Division of

CANCER CAUSE AND PREVENTION

Volume I
October 1, 1982-September 30, 1983

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ANNUAL REPORT

DIVISION OF CANCER CAUSE AND PREVENTION

NATIONAL CANCER INSTITUTE (U.S.)

October 1, 1982 through September 30, 1983

TABLE OF CONTENTS

ANNUAL REPORT

DIVISION OF CANCER CAUSE AND PREVENTION

Richard H. Adamson, Ph.D., Director

October 1, 1982 through September 30, 1983

OVERVIEW

This year plans for the reorganization of the Division have been formalized. It is proposed to change the name of the Division to the Division of Cancer Etiology, concomitant with changing the name of the Division of Resources, Centers and Community Activities to the Division of Cancer Prevention and Control. Also proposed is to appoint Associate Directors for a Biological Carcinogenesis Program and for a Chemical and Physical Carcinogenesis Program. Incorporated into the new Biological Carcinogenesis Program would be the biological carcinogenesis components of the existing Carcinogenesis Intramural and Carcinogenesis Extramural Programs. Similarly, the Chemical and Physical Carcinogenesis Program would include both intramural and extramural components of the chemical carcinogenesis research. This reorganization is proposed because it is anticipated that it will enhance interaction between intramural scientists and the extramural community, facilitate the sharing of scarce resources and generally improve the management of research in the areas of biological carcinogenesis and of chemical and physical carcinogenesis.

During the last two years the intramural research program has been undergoing changes and in July 1981 four new laboratories were created in order to strengthen the areas of chemical carcinogenesis, tumor promotion and studies of transformation at the molecular level. An Associate Director for the Field Studies and Statistics (Epidemiology and Biometry) Program was appointed last year; the organizational components of this program are now the Biometry Branch, the Clinical Epidemiology Branch and the Environmental Epidemiology Branch. The Chief of the Laboratory of Viral Carcinogenesis resigned in March 1983 and it is anticipated that the laboratory will be reorganized under the leadership of a new laboratory chief(s) according to the advice of the National Cancer Institute (NCI) Executive Committee and the Division's Board of Scientific Counselors. Subsequent to the departure of the Chief of the Laboratory of Tumor Virus Genetics, sections of that laboratory have been incorporated into appropriate existing laboratories and the laboratory will be abolished. The Experimental Oncology Section of the Laboratory of Cellular and Molecular Biology has been transferred to the Division of Cancer Biology and Diagnosis, NCI. Efforts continue to bring about better interaction between the biological carcinogenesis and chemical carcinogenesis laboratories and to have these laboratories interact with the three branches in the Field Studies and Statistics Program. The distribution of funds for the intramural laboratories was 16.8% and that for Field Studies and Statistics was 17.3% of the total DCCP budget. The current organizational chart for DCCP is shown in Figure 1.

The extramural component, at present known collectively as the Carcinogenesis Extramural Program, comprises several major activities: Biological Carcinogenesis, Chemical and Physical Carcinogenesis, and Special Programs, including major aspects of the Smoking and Health and the Diet and Nutrition Programs. The overall budget for research in these areas is given in Table 1 and Figure 2.

Last year renovations were completed for the Laboratory of Experimental Pathology and for a new laboratory, the Laboratory of Comparative Carcinogenesis, at the Government-owned facilities at the Frederick Cancer Research Facility (FCRF). This year the consolidation of the Laboratory of Cellular and Molecular Biology in Building 37 and the move of the Laboratory of Chemoprevention to Building 41 will be completed. These moves and the creation of the new laboratories will return all remaining off-reservation DCCP laboratories to Government facilities, provide geographic unity of individual laboratories, strengthen the area of chemical carcinogenesis, help integrate biological, chemical and physical carcinogenesis programs and bring about interaction with the Field Studies and Statistics Program, and reduce funds needed for resource support laboratories.

The past year has seen a continued reduction in overall contract support. This has been realized by gradual phase-out of contract-supported, investigator-initiated research in areas where grants provide adequate coverage, by reducing activities which provide materials and services, and by initiating various cost-recovery mechanisms. By the same token, as support for research contracts has dropped, support for investigator-initiated research grants has continued to increase. In addition, the Cooperative Agreement is now being utilized as an additional instrument of support.

In addition to its intramural and extramural research, the Division has been involved in other activities during the past year which merit attention. These are:

1. Frederick Cancer Research Facility (FCRF)

   Research conducted by the contractor began in the early 1970s as a series of unrelated tasks or projects. Today, the carcinogenesis program at the FCRF has gradually evolved into a coordinated effort on the mechanisms of action of viral and chemical carcinogens and has been transferred to the Office of the Associate Director, NCI (Dr. Peter Fischinger), which coordinates all FCRF activities. As a separate effort, the contractor provides support services and materials to intramural investigators who work in NCI laboratories at the Frederick facility. Government and contractor scientists have combined their efforts to create a center of excellence for cancer research.

2. The DCCP Board of Scientific Counselors

   Chartered in 1978, the Board is an advisory body whose members are drawn from the scientific community outside NIH. Chosen for their recognized expertise in chemical carcinogenesis, molecular biology, viral oncology, epidemiology, immunology, pathology, genetics, and cell biology, the members of the Board advise the Division Director on a wide variety of matters which concern the progress of the Division's programs.

   One particularly important responsibility of the Board has been the examination of the productivity and performance of staff scientists by site visit review of the intramural laboratories. These visits have been conducted by teams which, as a rule, comprise two to four members of the Board and four or more investigators from the scientific community outside NIH whose special fields of expertise match those of the scientists in the Laboratory or Branch being reviewed. The site visit

reports, which reflect a consensus of the members of the team, are critically examined by the entire Board of Scientific Counselors and, after discussion, recommendations based on the reports are submitted to the Division Director.

The first cycle of site visits to the Division's entire intramural operation was completed two years ago with the site visits to the Laboratory of Viral Carcinogenesis, which took place on September 14-15, 1981 and to the Experimental Oncology Section of the Laboratory of Molecular and Cellular Biology on May 19, 1982. The next cycle of site visits began in the Spring of 1983, with the site visit to the Environmental Epidemiology Branch. Site visits to the Biometry Branch and the Laboratory of Molecular Virology are scheduled for the Fall of 1983.

Another important function of the Board is that of concept review, in which the members pass judgment on concepts for grant- or contract-supported activities. The Board continued to examine new concepts this year as new directions in extramural research activities developed.

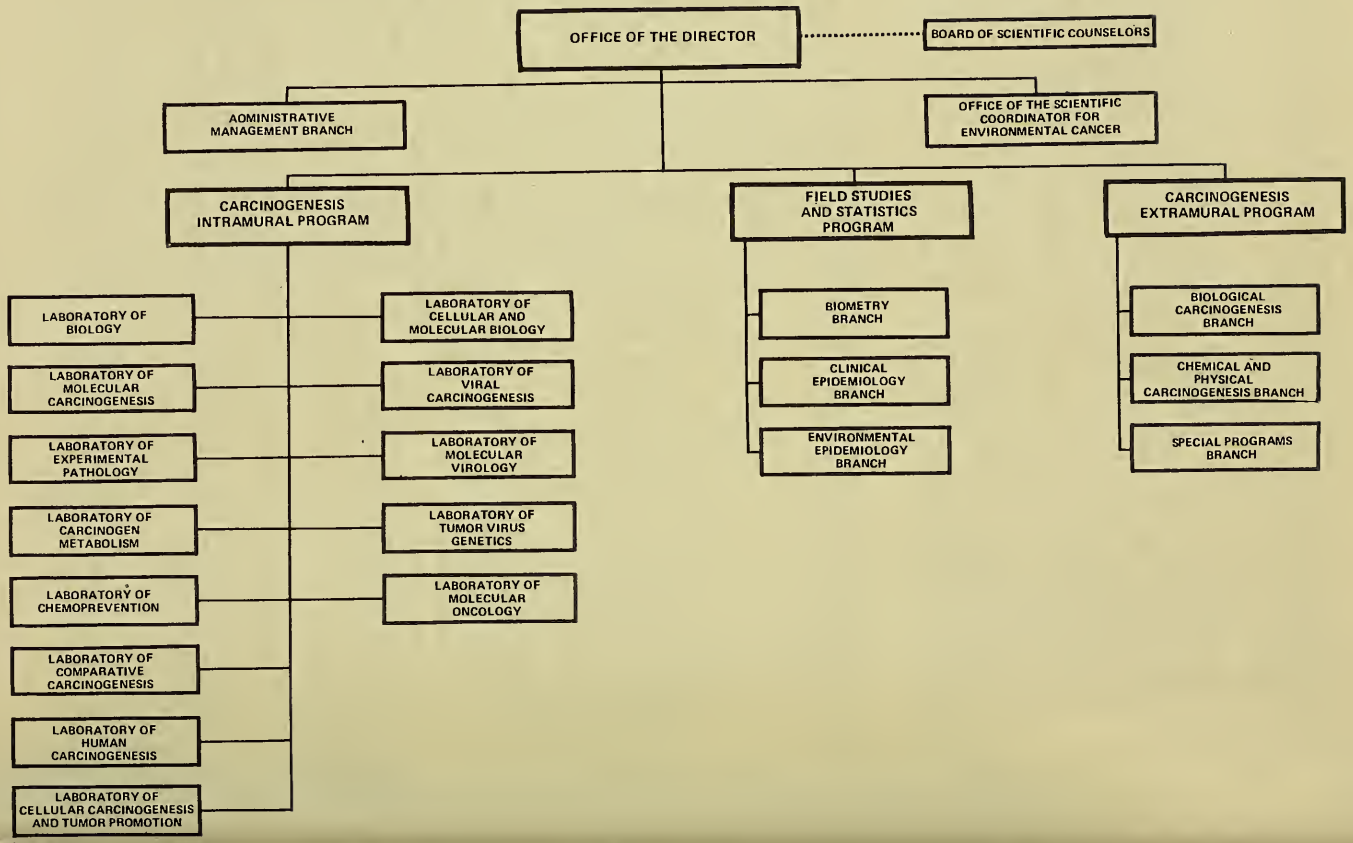
Several workshops involving Board members, as well as participants from the scientific community outside NIH, were held this year. As a consequence, new initiatives resulted using the mechanism of a Request for Grant Application (RFA) in the areas of Biochemical Epidemiology, the Epidemiology of Rare Cancers, the Accuracy of Questionnaire-Derived Historic Dietary Information, and the Pharmacological Role of Nicotine in Disease. In addition, in response to the emerging epidemic of Acquired Immune Deficiency Syndrome (AIDS) and because of the potential public health problem that it poses, this Division in collaboration with the Division of Cancer Treatment, NCI, issued an RFP entitled "Epidemiology, Diagnosis, Treatment and Immunology of AIDS." Subsequently, DCCP and NIAID jointly issued one additional RFA and an RFP addressing the AIDS problem: "The Infectious Etiology of AIDS and Kaposi's Sarcoma," and "The Natural History of AIDS in Homosexual Men," respectively.

Continuing modifications of funding mechanisms approved by the Board include a gradual transfer of current resources to a cost-reimbursement system (payback system), increased availability of funding to support new resource activities, the phasing out of contract-supported research in areas adequately covered by grant applications, an increased use of RFAs to stimulate research activity in high priority areas, and the use of the Cooperative Agreement as an additional mechanism of support for initiator-initiated research involving significant involvement of NCI staff.

The Division is most grateful to the members of the Board for giving so generously of their time, effort, and expertise in helping to guide the future of the Division's programs. There is every expectation that the Board will continue to play a vital role as the Division's foremost advisory group.

FIGURE 1

DIVISION OF CANCER CAUSE AND PREVENTION

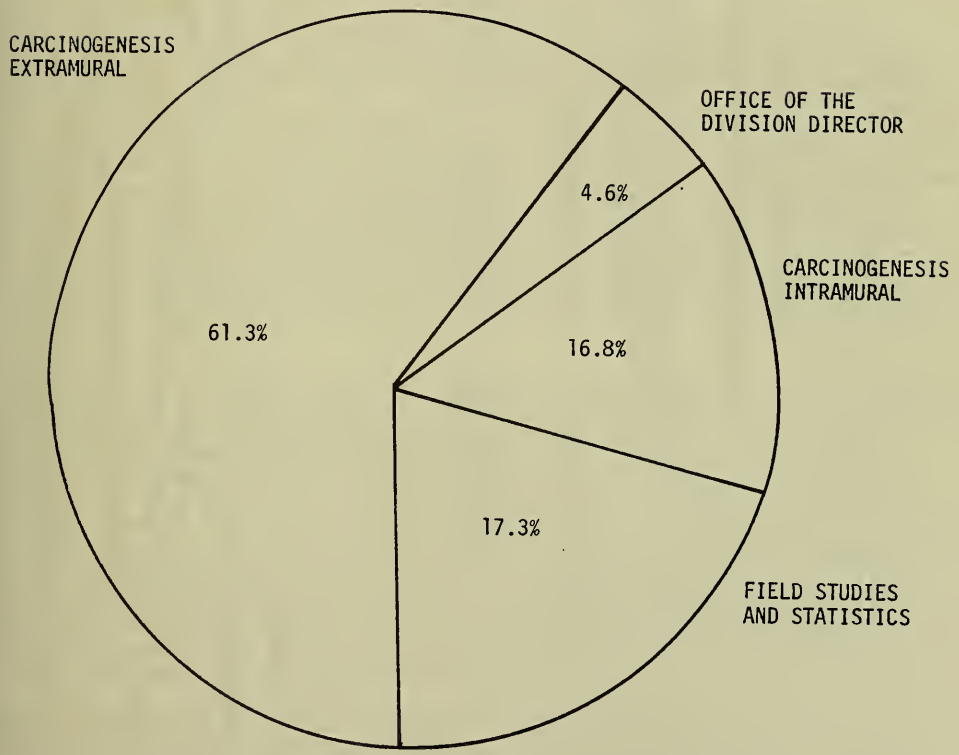


NATIONAL CANCER INSTITUTE

DIVISION OF CANCER CAUSE AND PREVENTION

Current Distribution of Funds

FY 1983 Estimate



NATIONAL CANCER INSTITUTE

DIVISION OF CANCER CAUSE AND PREVENTION

Table of Mechanisms by Organizational Unit Based on
Estimated Current Level of Expenditures
(Dollars in Thousands)

FY 1983 Estimate

	Office of the Division Director	Carcinogenesis Intramural	Carcinogenesis Extramural	Field Studies and Statistics	Total
Intramural	3,643	29,326	1,770	9,112	43,851
Contracts	5,163	4,306	7,656	25,522	42,647
CREG/RFA			8,718		8,718
Cooperative Agreements	530		1,460		1,990
Research Project Grants			103,378		103,378
Total	9,336	33,632	122,982	34,634	200,584

SCIENTIFIC HIGHLIGHTS

Introduction: The Division of Cancer Cause and Prevention is responsible for planning and conducting the Institute's program of coordinated research on cancer causation and basic research on prevention. The Division supports both intramural laboratories and branches and extramural programs which seek to elucidate the mechanisms of cancer induction at each step of the cellular process from initiation to tranformation of normal cells to malignant cells. The overall purpose of these studies is to provide information for reversing, interrupting or preventing this process before the development of clinical disease.

To accomplish these goals, investigators pursue fundamental studies on normal and malignant cells and on carcinogens or procarcinogens, such as viruses and chemicals, using the disciplines of cellular and molecular biology, immunology, biochemistry, and microbiology; epidemiologic studies of human populations are performed to identify risk factors predisposing to various cancers using the disciplines of clinical medicine, genetics, mathematics, and biometrics.

Excellent model systems are available to scientists studying the effects of exposure to a diversity of potentially carcinogenic factors in the environment. Much fundamental information has been obtained by studying tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Investigations in the area of biological carcinogenesis have shown that viral information present as nucleic acid sequences (oncogenes) in normal and malignant cells replicating with them may be intimately involved in the development of cancer. Triggered by chemical carcinogens, radiation, hormones, aging factors and other influences, these highly conserved viral sequences may direct the synthesis of proteins responsible for malignant transformation of the cell. The work is conducted on animal and human cancers under several general categories, including virus-cell interactions, virus-host interactions, and molecular pathways of viral replication.

Similarly, chemical carcinogens, both synthetic and naturally occurring, provide a diverse group of chemicals with cellular and tissue selectivities that influence transformation and progression from the normal to the malignant state. Studies in the area of chemical/physical carcinogenesis cover a broad range of approaches with emphasis on the mechanisms of action of chemical and physical agents. Investigators have focused on the effects of carcinogens on cell structure and function, the relationships between molecular structure and carcinogenic activity, enzyme systems associated with the metabolic activation of procarcinogens to carcinogenic forms, the effects of the binding of carcinogens to DNA, and systems that repair DNA damage. Other relevant efforts include investigation of the role of various factors in the environment of the cell; for example, promoters, hormones and growth factors which may be required for the progression of the "initiated" cell to the malignant state.

Finally, studies on the natural history of cancer in humans and on the incidence of cancers in different geographic locations help to identify causal associations of various intrinsic and extrinsic risk factors with various cancers. In view of the increasing importance of nutrition and lifestyle in the causation or prevention of cancer, in particular the role of micronutrients in the diet, alcohol consumption and smoking, special emphasis has been given to projects

that may have more immediate health implications. Many studies deal with determining mutagens/carcinogens in foods, natural inhibitors in foods, in assessing the carcinogenic components in cigarettes, and the influences of the total smoking experience. In addition, major studies on cancer incidence, mortality and survival in the U.S., cancer incidence in the workplace, effects of low-level radiation, and environmental pollutants in air, water, and soil are under investigation.

MAJOR FINDINGS:

Biological Carcinogenesis:

Oncogenes

The past year has been an exciting one in the area of biological carcinogenesis research in general and in research on oncogenes in particular. The structural and biological properties of viral onc genes present in both avian and mammalian acute transforming viruses have been analyzed, and the normal cellular homologs of these viral onc genes have been isolated, identified and characterized from their species of origin as well as from the human genome. A prokaryotic vector, developed for studying the expression of high levels of onc genes in E. coli, has provided a means for studying the chemistry of the protein and for preparing appropriate immunologic reagents for studying the expression of the oncogene product in transformed cells and tumor cell lines. In addition, it has been possible to assign several human oncogenes to specific chromosomes.

A screening method in nude mice has been developed for detecting dominant transgenes. Five such genes have been identified by this technique. One gene has been partially cloned from a human pancreatic carcinoma cell line which has been shown to be a member of the family of human sequences related to the Kirsten ras oncogene. A second dominant transforming gene has been isolated from a human teratocarcinoma cell line. This oncogene also is a member of the human ras oncogene family but is the n-ras oncogene. Several other genes are in the process of being cloned. One is clearly not a member of the ras family and is derived from a chemically transformed human tumor cell line. Attempts are also underway to develop human cells as recipients for transforming genes in DNA transfection assays since the use of the single recipient cell line (NIH/3T3) raises questions regarding the range of oncogenes detectable and the role of genes isolated in rodent cells. Diploid human and rodent primary cells are being evaluated for their ability to be transfected and transformed and the multiple factors required to render these cells tumorigenic following DNA transfection are under investigation. These approaches should enable detection of a wider range of human oncogenic sequences in a system more closely analogous to the one in which tumors are naturally induced in animals and man.

BALB and Harvey murine sarcoma viruses (MSV) comprise a family of retroviruses whose mouse- and rat-derived onc genes are closely related. These viruses induce sarcomas and erythroleukemias in susceptible animals. An in vitro colony assay that detects transformation of lymphoid cells by A-MuLV was used to demonstrate that BALB- and Harvey-MSV transform a novel hematopoietic cell both in culture and in vivo. Bone marrow colony formation was sarcoma virus-dependent, followed single-hit kinetics, and required the presence of mercaptoethanol in the agar medium. BALB- and Harvey-MSV-induced colonies could be established in culture as continuous cell lines that demonstrated unrestricted

self-renewal capacity and leukemogenicity in vivo. The cells had a blast cell morphology and lacked detectable markers of mature cells within the myeloid or erythroid series. They also lacked detectable immunoglobulin μ chain or Thy-1 antigen, markers normally associated with committed cells of the B- and T-lymphoid lineages, respectively. However, the transformants contained very high levels of terminal deoxynucleotidyl transferase (TdT), an enzyme believed to be specific to early stages within the lymphoid differentiation pathway. This phenotype distinguishes these BALB- and Harvey-MSV transformants from any previously reported hematopoietic targets of transforming retroviruses, including the pre-B lymphoid cell transformed by A-MuLV under identical assay conditions. These newly identified lymphoid progenitor cell transformants may provide an important means of studying early stages of lymphoid ontogeny and the possible role of TdT in lymphoid development.

A cloned transforming virus of Moloney sarcoma virus (MSV) containing the transforming sequence v-mos and its cloned normal cellular homolog (c-mos) from both mouse and human has been analyzed. The provirus of the MSV contains both the MSV-specific transforming sequence v-mos as well as sequences which enhance or activate RNA synthesis. These latter sequences are found in the MSV long terminal repeat (LTR) which has been shown to be necessary for the efficient expression of the transformation potential of v-mos and its murine cellular homolog c-mos. The LTR enhances v-mos transformation when linked either 5' or 3' relative to v-mos. RNA synthesis either initiates or terminates in the LTR depending on its location relative to mos. A cis-acting sequence has been identified within the 5' normal mouse DNA flanking sequence upstream from the murine cellular mos locus that inhibits activation of transformation by a 3' LTR. Removal of this normal mouse sequence permits activation of the transforming potential of c-mos. This is the first characterization of a negative acting cis control element.

The retroviral DNA sequences upstream from the v-mos gene in two molecularly cloned MSV isolates were determined in order to clarify the origin of the viral subgenomic mos mRNA. A number of sequence alterations have been identified which may be responsible for the production of the mos gene mRNA, and recombinants between the two MSV isolates are being prepared in order to test this hypothesis. Recent analyses indicate that the viral mos gene in cellular transformation is effected with low levels of expression (i.e., approximately 1 to 10 copies of RNA per cell). This is consistent with the amount of protein believed to be expressed in transformed cells (i.e., approximately one thousand molecules per cell). This identifies mos as one of the most potent transforming genes yet to be described.

A human DNA fragment containing a region homologous to the v-mos transforming gene of MSV was sequenced; comparison of this fragment with the sequence of the mouse cellular homologue of v-mos revealed extensive homology. Attempts to activate the human mos gene with LTRs and hybrid recombinants between human mos and the first 50 codons from the v-mos of MSV were unsuccessful. A technique was developed for isolating unique hybrid DNA recombinants between human mos and the viral mos region of Moloney sarcoma virus in E. coli. These recombinants were characterized both by identifying the region in mos where the hybrid molecules have been formed and testing their biological transforming activity in order to identify the region of the mos gene that is essential for focus formation. These analyses have provided the first application of recombinant DNA to make hybrid protein molecules and more importantly they have allowed the

identification of important domains in the mos gene that are essential for transforming activity. It is now possible by conventional recombinant DNA techniques to specifically identify the amino acids contributing to the structure of a transforming protein.

The complete nucleotide sequence of human cellular c-myc, which is homologous to the transforming gene, v-myc, of myelocytomatosis virus MC29 has been determined. Analysis of the genetic information and alignment with the known sequence of chicken c-myc and v-myc indicates: (1) an intervening sequence can be identified by consensus splice signals. The unique 5' sequence of c-myc and its junction with the v-myc region may be a canonical 3' splice acceptor; (2) the c-myc locus can generate a mRNA whose termination signals are downsteam from the translational termination signal; (3) the three myc genes share the same reading frame, including translational termination signals; (4) the homology is conserved only in the coding region; (5) most changes at nucleotide level result in no change in amino acid; and (6) there are two distinct domains--the 5' unique domain, which is different from the viral, and the 3' coding domain, which contains amino acids coded by the two exons whose sequences have been determined here. In the latter domain, the amino acid variation between v-myc and chicken c-myc is less than 2%, whereas that between the chicken v-myc and the human is 27%, with the variation concentrated in the region that flanks the splicing points.

Simian sarcoma virus (SSV) deletion mutants were constructed from a molecular clone containing the entire infectious provirus. Transfection analysis of these mutants localized the SSV-transforming gene to a small region of the viral genome encompassing its cell-derived sequence (v-sis). Antiserum to a peptide synthesized on the basis of the predicted amino acid sequence of the SSV-transforming gene detected a 28,000-dalton protein that was specifically expressed in SSV-transformed cells and that corresponded in size to that predicted from the v-sis coding sequence. The v-sis gene product, designated p28^{sis}, was not a phosphoprotein, nor did it possess detectable protein kinase activity. These findings distinguish p28^{sis} from a number of other retroviral onc proteins.

The complete nucleotide sequence of the proviral genome of SSV has been determined. Like other transforming viruses, SSV contains sequences derived from its helper virus, simian sarcoma-associated virus (SSAV), and a cell-derived v-sis insertion sequence. By comparison with the sequence of Moloney murine leukemia virus, it was possible to precisely localize and define sequences contributed by SSAV during the generation of SSV. Comparative sequence analysis of SSV and SSAV showed that SSAV provides regulatory sequences for initiation and termination of transcription of the SSV-transforming gene. Moreover, coding sequences for the putative protein product of this gene appear to initiate from the amino terminus of the SSAV env gene. Antibodies to synthetic peptides derived from the carboxy and amino termini of the putative protein predicted by the open reading frame identified within v-sis specifically detect a 28,000 dalton protein, p28^{sis}, in SSV-transformed cells. These and other findings confirm the predicted amino acid sequence of this protein and localize it to the coding region of the SSV-transforming gene.

Very recent findings have provided the first direct link between an oncogene and a known biologic function. Studies performed elsewhere on human platelet-derived growth factor (PDGF), a potent mitogen for cells of connective tissue origin, led

to the elucidation of its amino terminal amino acid sequence. Computer comparison of this protein sequence with the predicted amino acid sequence of p28^{sis} revealed a very high degree of homology. In earlier studies, SSV onc gene transcripts were detected in specific human malignancies, such as fibrosarcomas, osteosarcomas, and glioblastomas. These findings corroborate the present finding that the SSV onc gene may encode for a protein with mitogenic activity similar to PDGF which exerts its growth-promoting activity on fibroblasts, tumor muscle cells, and glial cells. These results suggest that malignant transformation by the SSV onc gene or its cellular homologues, in certain instances, may involve constitutive expression of a protein which could act as a potent mitogen similar to PDGF.

A contiguous region of a cellular DNA sequence, 64 kb in length and representing overlapping inserts from three independent cosmid clones, was isolated from a representative library of human lung carcinoma DNA. Within this region of the cellular genome, v-abl homologus sequences are dispersed over a total region of approximately 32 kb. These sequences represent the entire v-abl human cellular homolog, are colinear with the viral v-abl transforming gene, and contain a minimum of seven intervening sequences. At least eight regions of highly repetitive DNA sequences have been shown to map in close proximity to c-abl coding sequences. In addition to the major c-abl human locus, three regions of human DNA sequence, corresponding to only portions of the v-abl gene, have been identified. Two of these have been molecularly cloned and shown to be distinct from the primary human c-abl locus. Upon transfection to rat embryo fibroblasts in culture, none of the cosmid DNAs containing v-abl homologous sequences exhibited transforming activity. These findings identify and map a single genetic locus of human DNA, c-abl, representing the complete v-abl homolog, and demonstrate the existence of additional human DNA sequences corresponding to more limited, subgenomic regions of v-abl.

Sequences encoding the tyrosine phosphorylation acceptor region of the human c-abl oncogene have been identified and their nucleic acid sequence determined. Extensive sequence homology between this region of c-abl and the acceptor regions of the v-src, v-yes and v-fes/fps family of viral oncogenes was established, as well as more distant relatedness to the catalytic chain of the mammalian cAMP-dependent protein kinase. These findings suggest that, of the homologs of retroviral oncogenes with tyrosine protein kinase activity examined to date, all were probably derived from a common progenitor and may represent members of a diverse family of cellular protein kinases.

Methylcholanthrene-transformed C3H/10T1/2 mouse cells were treated with iododeoxyuridine and a new, acute transforming, replication-defective, mouse type C retrovirus, designated 3611-MSV, was induced. It transforms embryo fibroblasts and epithelial cells in culture and induces fibrosarcomas in mice. Analysis of viral protein expression led to the demonstration of 90,000 and 75,000 molecular weight polyproteins (P90 and P75), both containing amino-terminal murine leukemia virus (MuLV) gag gene proteins, p15 and p12. The oncogene v-raf was isolated from this virus, molecularly cloned and compared with previously isolated retrovirus oncogenes. It was shown to be unique. Transfection of NIH/3T3 and RAT-2 cells with cloned v-raf DNA led to efficient transformation and expression of P75 and P90, with NH_2-terminal gag gene-encoded components linked to the acquired sequence (v-raf) translational product. The v-raf-encoded polyproteins lack detectable protein kinase activity, and 3611-MSV-transformed cells do not exhibit elevated levels of phosphotyrosine.

DNA sequence determination of v-raf and its flanking viral elements has provided essential information pertaining to its acquisition, composition, and mechanism of action. The parent of 3611-MSV was determined to be an ecotropic murine type C virus; that is to say, no prerequisite recombinational events need to occur prior to the transduction of this cellular oncogene. 3611-MSV acquired its transforming sequences by the substitution of 2.39 kilobases (kb) of viral structural information with 1.49 nucleotides of cellular oncogene. Interestingly, this oncogene adoption occurred in a way that precluded its expression (it entered into a non-translatable reading frame). Thus, the oncogenic potential of this recombinant was carried cryptically in the virus stock until a nucleotide deletion shifted the transforming sequences into the proper reading frame. The two polyproteins detected in transformed cells represented the translation products of the molecularly fused viral gag gene and the raf oncogene.

The amino acid sequence, predicted from the nucleic acid sequence of v-raf, revealed domains homologous to v-src and v-mos and, therefore, a close relationship between the members of the tyrosine kinase-oncogene superfamily and v-raf. The fact that the latter enzymatic activity is not demonstrable in 3611-MSV-transformed cells suggests that tyrosine phosphorylation may not be essential for oncogenicity. Family comparisons now allow for the prediction of which sequences are related to the kinase activity and which are necessary for transformation. Of a unifying interest is the observation that one of these latter sequences is nearly identical to the presumed active site of the ras family of oncogenes. As with many oncogenic sequences, raf-related RNA is found in uninfected cells. The specific role of these sequences in human malignancy is not yet understood. Preliminary experiments indicate that cells derived from human lung carcinomas express large amounts of raf-related RNA (>1000 copies per cell). The qualitative analysis of these RNAs is in progress.

Localization of Onc Genes to Specific Human Chromosomes

A survey of human genomes showed the presence of cellular homologs of v-raf in two related foci, designated c-raf-1 and c-raf-2. A DNA was constructed which contained the 5' portion of the transforming virus including the 5' one-third of the v-raf gene spliced to the 3' two-thirds of the human c-raf-1 gene; upon transfection, this construct transformed NIH/3T3 cells. Since the 5' one-third of the v-raf gene will not transform by itself, the transforming ability of the construct implies the expression of a polyprotein containing Moloney MuLV gag, mouse v-raf and human c-raf components. Somatic cell hyrids were used to map c-raf-1 to human chromosome 3 and c-raf-2 to chromosome 4. The presence of c-raf-1 on chromosome 3 suggests that it might be involved in small cell lung carcinomas which characteristically have deletions on the short arm of chromosome 3, usually beginning at band p14 and extending to p23 or beyond. The possibility of rearrangements in the vicinity of c-raf-1 in these tumors is under intense study.

Cell derived onc genes from acute transforming retroviruses have been used as probes for identifying the chromosomal locations of additional related human cellular genes. The primate cell-derived transforming gene (v-sis) of SSV is represented as a single copy marker within cellular DNAs of mammalian species, including human. By testing for the presence of the human v-sis-related fragment, c-sis (human) in somatic cell hybrids possessing varying numbers of human

chromosomes, as well as in segregants of such hybrids, it was possible to assign c-sis to human chromosome 22. Sequences homologus to the transforming gene (v-mos) of Moloney MSV have been identified in human DNA and cloned in a bacteriophage vector. This sequence (c-mos) has been assigned to human chromosome 8. A transforming gene has been isolated by molecular cloning techniques from the T24 and EJ human bladder carcinoma cell lines. This bladder carcinoma oncogene has been shown to be closely related to the onc genes (v-bas and v-ras) of BALB and Harvey MSVs and to be carried on chromosome 11 in normal cells. Further, the normal human homolog of v-bas was found to be indistinguishable from the T24 oncogene, suggesting that rather subtle genetic alterations have led to the activation of the normal human homolog of v-bas as a human transforming gene.

The genetic change that leads to the activation of the oncogene in T24 human bladder carcinoma cells was shown to be a single point mutation of guanosine into thymidine. This substitution results in the incorporation of valine instead of glycine as the 12th amino acid residue of the T24 oncogene-encoded p21 protein. Thus, a single amino acid substitution appears to be sufficient to confer transforming properties on the gene product of the T24 human bladder carcinoma oncogene.

A transforming gene related to c-bas/has (human) was cloned in biologically active form from a human lung carcinoma-derived cell line. Studies on recombinants constructed between the oncogene and its normal allele revealed that the genetic change that led to the activation of the oncogene is a point mutation in the second exon. This point mutation results in the incorporation of leucine instead of glutamine in the 61st amino acid of the predicted protein. No changes were observed in the first exon, the region of c-bas/has in which a point mutation is responsible for activation of the T24 and EJ bladder carcinoma oncogenes. Thus, single amino acid substitutions within different structural domains of the same protooncogene can be independently responsible for its malignant conversion under natural conditions.

The human cellular homolog (c-fms) of the transforming sequence of FeSV was localized to human chromosome 5. Regional localization of c-fms to band q34 on chromosome 5 was accomplished by analysis of Chinese hamster-human cell hybrids containing terminal and interstitial deleted forms of chromosome 5. The localization of c-fms to human chromosome 5(q34) is of interest in view of reports of a specific, apparently interstitial, deletion involving approximately two-thirds of the q arm of chromosome 5 in acute myelogenous leukemia.

Analysis of a large series of mouse x human somatic cell hybrids permitted the localization of the c-fes oncogene to human chromsome 15 and a more precise mapping of several genetic markers on chromosomes 15 and 17. Somatic cell hybrids were prepared between a thymidine kinase-deficient mouse cell line and blood leukocytes from a patient with acute promyelocytic leukemia (APL) showing the $15q^{+};17q^{-}$ chromosome translocation frequently associated with that disease. One hybrid contained the $15q^{+}$ translocation chromosome and very little other human material. The c-fes oncogene was not present in this hybrid and was, therefore, probably translocated to the $17q^{-}$ chromosome. Analysis of the genetic markers present in this hybrid allowed a more precise localization of the translocation breakpoints on chromosomes 15 and 17.

The human cellular homolog (c-abl) of the transforming sequence of Abelson MuLV was localized to human chromosome 9 by analysis of a series of somatic cell hybrids. The long arm of this chromosome is involved in a specific translocation with chromosome 22, the Philadelphia translocation, t(9;22) (q34, q11), occurring in patients with chronic myelocytic leukemia (CML). Investigations were made on whether the c-abl gene was included in such translocations. Using c-abl and v-abl hybridization probes on blots of somatic cell hybrids, positive hybridization was found when the $22q^-$ (the Philadelphia chromosome), and not the $9q^+$ derivative of the translocation, was present in the cell hybrids. From this it was concluded that in CML, c-abl sequences are indeed translocated from chromosome 9 to chromosome $22q^-$. Moreover, it has been demonstrated, for the first time, that this translocation is reciprocal, since c-sis, another oncogene, was found also to be simultaneously translocated from chromosome 22 to 9, also in CML.

The consistent appearance of specific chromosomal translocations in human Burkitt's lymphomas and murine plasmacytomas has suggested that these translocations might play a role in malignant transformation. Transformation of these cells is frequently accompanied by the somatic rearrangement of a cellular analogue of an avian retrovirus transforming gene, c-myc. Moreover, c-myc was mapped to chromosome 8 band q24. This chromosomal segment is involved in the reciprocal Burkitt's translocations [t(8;14), t(8;22) and t(2;8)]. In two t(8;14) human Burkitt's cell lines, c-myc appears to have been translocated directly into a DNA restriction fragment that also encodes the immunoglobulin { chain gene. In the case of a specific cloned fragment of DNA derived from a mouse plasmacytoma, we demonstrated directly that c-myc has been translocated into the immunoglobulin α switch region. These data provide a molecular basis for considering the role that specific translocations might play in malignant transformation.

Control of Gene Expression

Considerable progress has been made during the past year in elucidating the signals associated with gene expression. In particular, interest has been directed toward regulatory events which take place at the level of transcription and processing of RNA. The elucidation and analysis of novel genetic elements, enhancer sequences, which appear to be responsible for controlling the rate at which particular genes are transcribed have been undertaken. The existence of these enhancer sequences has been demonstrated, not only in the genomes of DNA viruses such as SV40 and BKV, but also in the long terminal repeats (LTRs) of retroviruses. Using a combination of in vivo and in vitro assays, it has been shown that enhancer sequences show host-cell specificity, and thus may be among the elements involved in controlling the host range of certain viruses as well as the tissue-specific expression of certain eukaryotic genes. Several lines of research have suggested that enhancer sequences are not unique to viruses but are also present within the eukaryotic genome. A major effort will be directed at determining whether or not enhancer sequences play a role in the developmental and tissue-specific regulation of gene expression. In addition, mutagenizing regions of enhancer elements will be studied to elucidate those sets of nucleotides associated with the general activation phenomenon as well as the cellular specificity. Experiments have been designed in an attempt to elucidate the mechanism by which the activator/enhancer sequences function. A principal focus for the future will be the definition of biological macromolecules which interact with these regulatory elements.

Other efforts to characterize the control mechanisms for the expression of normal and transformed genes have involved studies of mink cells morphologically transformed by either Snyder-Theilen FeSV or Abelson MuLV. Such cells exhibit relatively high rates of reversion to the nontransformed phenotype. The proviral DNAs are conserved within the revertant lines and have not undergone changes in integration sites due to translocations or other genomic rearrangements. In contrast, expression of well-defined, viral encoded transforming proteins is blocked and elevated levels of phosphotyrosine, characteristic of the parental transformed cells, are reduced to control levels. Loss of the transformed phenotype is associated with increased cytosine methylation of proviral DNA sequences, while levels of methylation resume control values upon spontaneous retransformation of revertant clones. Following molecular cloning and transfection to Rat-2 cells, ST-FeSV proviral DNAs from revertant and transformed cells induced similar numbers of transformed foci. Cytosine methylation sites involved in regulation of expression of the major ST-FeSV-encoded transforming protein have been localized within the proviral DNA itself, rather than in adjacent cellular flanking sequences. In contrast to the v-fes proviral DNA, c-fes, the cellular homolog of the ST-FeSV-acquired transforming sequences, is highly methylated in cytosine residues in both transformed and revertant clones. These findings demonstrate regulation of viral oncogene-mediated transformation by cytosine methylation and suggest that expression of cellular homologs of viral oncogenes, such as c-fes, are also subject to regulation at this level.

Transforming Growth Factors

Another area of emphasis during the past year has been the identification and characterization of transforming growth factors (TGFs) which can confer reversible malignant characteristics to normal cells when added to appropriate soft agar cell cultures. TGFs were purified from serum-free medium conditioned by retrovirus-transformed Fischer rat embryo fibroblasts, mouse 3T3 cells and two human melanoma cell lines. The amino-terminal sequences of rat, mouse and human TGFs were determined. Extensive (>90%) sequence homology was found among TGF polypeptides from different species and cell types. The complete amino acid sequence of rat TGF also was deduced from microsequence analysis data of reduced and S-carboxyamidomethylated TGF and Lys-c peptides, and from results of carboxypeptidase digestion of modified rat TGF. Rat TGF was found to be a single chain polypeptide with a calculated molecular weight of 5,600 which displays 33% sequence homology with murine EFG and 44% sequence homology with human urogastrone.

Sarcoma growth factor (SGF) from serum-free media conditioned by murine sarcoma virus (MSV)-transformed 3T3 cells was purified to homogeniety using rpHPLC. The amino acid sequence analysis indicated that SGF is distinctly different from EGF. Comparison of the N-terminal amino acid sequence of SGF with a TGF from a rat cell transformed by a feline sarcoma virus or the TGF from a human melanoma cell line showed greater homology between the members of the TGF class than with either the TGFs and EFGs or even between mouse EGF and human EGF (urogastrone).

Urine specimens from patients with a variety of pediatric malignancies including small cell carcinoma, osteogenic sarcoma and rhabdomyosarcoma contained acid- and heat-stable, high molecular weight factors which competed for binding to EGF and promoted anchorage-independent growth of nontransformed cells in semi-solid media. High levels of this activity were not found in the patients with acute or chronic lymphocytic leukemia or in the majority of healthy control children. This transforming activity was enhanced by a transformation potentiating factor (TPF) which

is distinct from and functionally unrelated to EGF. Highest levels of activity were found in lymphoma patients and a patient with undifferentiated small cell carcinoma.

Fischer rat embryo cells transformed by v-abl, v-fes and v-fms release TGFs into the cell culture medium. These peptides stimulate phosphorylation of the EGF membrane receptors and promote anchorage-independent cell growth. Cells transformed by v-abl and v-fes produce high titers of TGF (60-200 ng eq EGF/liter) while cells transformed by v-fms produce TGF at only low levels (<10 ng eq EGF/liter). Upon purification, TGF progressively loses transforming activity. A second potentiating factor, when added to purified TGF preparations, restores transforming function. This factor has been partially purified and shown to be distinct from previously described growth factors.

Serum-free conditioned media from several human tumor cell lines (e.g., epidermoid carcinomas, melanomas, bronchogenic carcinomas, and rhabdomyosarcomas) and from NIH/3T3 cells transfected with DNA from a human lung carcinoma were observed to produce a class of factors which inhibits the growth of human melanoma and carcinoma cells in soft agar and in monolayer cultures. These inhibitors of tumor cell growth have been designated tumor inhibiting factors (TIFs). Normal human fibroblasts and epithelial cells, however, are stimulated to proliferate by these same factors. A rich source of TIFs (certain normal tissues) has been identified which should facilitate their purification and characterization. TIFs produce gross cell surface and internal cytoskeletal changes in tumor cells. Scanning electron microscopy of human lung carcinoma, melanoma, and normal human fibroblasts treated with TIF showed considerable cell surface changes only in the transformed cells. Lung carcinoma cells showed dramatic changes in cell-cell contact and in the distribution of microvilli. Normal human fibroblasts treated with TIF were indistinguishable from the untreated controls. Fluorescent microscopic studies using antisera against actin showd lung carcinoma cells treated with TIF to have microfilaments which were much thicker and more directionally oriented than the untreated control cells. Additional studies performed during the past year on growth factors are described under Chemical Carcinogenesis.

Chemical Carcinogenesis

In Vitro Models

Remarkable progress has been made during the last few years in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. Chemically defined media for long-term culture of human bronchus, colon, esophagus, and pancreatic duct have been developed. Primary cell cultures of human epithelial outgrowths have been obtained from many different types of human tissues. Isolated epithelial cells from human bronchus and esophagus can be transferred three or more times and can undergo more than 30 cell divisions. Human bronchial epithelial cells can also be grown in serum-free culture medium.

One important use of cultured human tissues has been in the investigation of the metabolism of chemical carcinogens. The studies of activation and deactivation of representative procarcinogens have revealed that the metabolic pathways and the predominant adducts formed with DNA are generally similar between humans and experimental animals. Wide quantitative interindividual

differences (50- to 150-fold) are found in humans and other outbred animal species. When the metabolic capabilities of specimens from different levels of biological organization are compared, the profile of benzo[a]pyrene metabolites between the explant culture and the epithelial cell cultures were observed. Cultured human colon is being used in systematic studies of the metabolic activation of several chemical classes of carcinogens. The following carcinogens are converted by cultured human colon to metabolites that bind to DNA: benzo[a]-pyrene; 6-nitrobenzo[a]pyrene; aflatoxin B_1; N-nitrosodimethylamine; 1,2-dimethylhydrazine; and 3-amino-1,4-dimethyl-5H-pyride(4,3-b) indole. The latter compound is formed by pyrolysis of tryptophan.

Cultured human bronchial epithelial and fibroblastic cells are also being used to investigate DNA damage and repair caused by chemical and physical carcinogens as examined by the formation and removal of carcinogen-DNA adducts. Human bronchial epithelial cells repair single-strand breaks in DNA induced by X-irradiation, UV-irradiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines at rates similar to bronchial fibroblasts. The presence of formaldehyde significantly inhibited the repair of the X-ray-induced single-strand breaks in human bronchial cells, correlating with the potentiation of cytotoxicity in human cells and mutation frequency in Chinese hamster V79 cells by combinations of the agents. Formaldehyde, a common environmental pollutant and metabolite of carcinogenic N-nitrosamines, also inhibits repair of O^6-methylguanine, decreases O^6-alkylguanine transalkylase activity, is mutagenic at high concentrations and potentiates the cytotoxicity and mutagenicity of N-methyl-N-nitrosourea in normal human cells. Exposure to formaldehyde may lead to the dual genotoxic mechanism of both directly damaging DNA, i.e., formation of DNA-protein cross-links and single-strand DNA breaks, and inhibiting repair of mutagenic and carcinogenic DNA lesions caused by alkylating agents and physical carcinogens.

Cultured human cells are also being used to characterize chromatid DNA repair lesions associated with malignant transformation and/or susceptibility to cancer. Cell lines from diverse human tumors, including sarcomas, carcinomas and glioblastomas have significantly more chromatid breaks and gaps than their normal counterparts when X-irradiated, suggesting deficiencies in DNA repair. Further, skin fibroblasts from cancer-prone individuals, including those with ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia, Gardner's syndrome, familial polyposis or xeroderma pigmentosum, show similar defects in chromatid DNA repair. It appears that acquisition of defects in chromatid DNA repair operative during G_2-prophase is a prerequisite, possibly the initiating step, for carcinogenesis in human cells. Additional studies on cell survival, mutagenesis, DNA synthesis and repair, histone synthesis and chromosome integrity in cultures of skin and blood from patients with xeroderma pigmentosum and ataxia telangiectasia are being performed after the cells are exposed to DNA damaging agents.

Factors controlling growth and differentiation of normal epithelial cells from bronchus and esophagus, and their normal counterparts, are under investigation. Comparative studies of normal and malignant cells have revealed differences in their response to inducers (blood-derived serum platelet lysates, >1mM calcium, or serum) of terminal differentiation, in their pattern of cytoskeletal proteins including keratins, and their production of polypeptide hormones. The effects of a variety of chemicals including phorbol, 12-O-tetradecanoylphorbol-13-acetate (TPA), teleocidin B, 2,7-dichlorodibenzo-p-dioxin (DCDD), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and for cigarette smoke condensate, catachol,

benzo[a]pyrene, benzo[e]pyrene, and pyrene have been assessed in relation to cellular morphology, clonal growth rate, and enzyme assays, i.e., plasminogen activator, ornithine decarboxylase, and aryl hydrocarbon hydroxylase.

Methods to culture replicative normal mesothelial cells from adult human donors have been developed in order to study the long-term effects of asbestos fibers. The mesothelial cells were 10 and 100 times more sensitive to the cytotoxicity of asbestos fibers than were bronchial epithelial or fibroblastic cells, respectively, from normal adult humans. Exposure of the mesothelial cells to amosite asbestos caused chromosomal rearrangements, including dicentrics. These aneuploid mesothelial cells had an extended population doubling potential of more than 35 divisions beyond the culture life span (30 doublings) of the control cells. Mesothelial cells have distinct keratin proteins and an ability to regulate their cytoskeletal composition; the content of keratin or vimentin in the cytoskeleton reflects the growth conditions. This uniquely fluid cytoskeleton may be more easily perturbed by penetrating asbestos fibers than are the cytoskeletons of other cell types and this may lead to an increased risk for chromosomal instability and transformation.

The effects of asbestos were also investigated in human bronchial epithelial cells. The differential cytotoxic activity of various asbestos and glass fibers was estimated by measuring the inhibition of epithelial cell growth as a function of fiber concentration. The data show that various fiber types have different effects on human bronchial epithelial cells. Chrysotile was extremely toxic; amosite and crocidolite were less toxic; glass fibers were only mildly toxic. For comparison, human bronchial fibroblastic cells were also exposed to fibers and were found to be markedly more resistant (more than 10-fold) than the epithelial cells to all the types of asbestos tested. Asbestos fibers also induce abnormal cell growth. Addition of amosite asbestos to human respiratory mucosa in explant culture caused numerous focal lesions including squamous metaplasia and dysplasia. When examined by scanning electron microscopy, the epithelial lesions appeared as focal elevations of nonciliated cells. Cytopathological aberrations of the bronchial epithelial cells were manifested by cellular polymorphism and variation in nuclear size. These studies are being extended to determine the progression of these lesions and eventually their malignant potential.

The role of arsenic in epithelial carcinogenesis is under investigation in studies of combined exposures with other carcinogens; it was selected for studies on the interaction of organic and inorganic carcinogens as a unique example of discrepancy between positive human findings and negative animal tests. Metabolic species differences and possible requirements for a cofactor role in carcinogenesis are under investigation. Preliminary results indicate that inorganic arsenic is methylated in vivo by the mouse much more effectively than by the rat. Arsenic was found to bind to human keratins in vitro, and the role of arsenic binding in the induction of hyperkeratosis and keratinizing cell tumors is under investigation. Trivalent arsenic was more cytotoxic than pentavalent arsenic for cultures of primary BALB/c mouse epidermal cells and of the BALB 3T3 clone A31-1-1 cell line; in the latter system, trivalent arsenic was found to induce neoplastic transformation, while pentavalent arsenic appeared negative.

Other studies have focused on the regulation of epidermal growth and differentiation, and in particular on the importance of extracellular calcium

concentrations to these processes. Culture medium of 0.02 - 0.09 mM calcium concentration selects for proliferating cells which have morphological, immunological and biochemical characteristics of basal cells. Culture medium of >0.1 mM induces epidermal differentiation resulting in cessation of proliferation, vertical stratification, cornification and sloughing of mature squames. The regulation of differentiation by calcium is not associated with changes in cyclic nucleotide levels but appears dependent on a functioning Na^+-K^+ ATPase pump, as it is inhibited by ouabain. A number of other inhibitors of Ca^{++} induced terminal differentiation have been identified, and their common actions together with studies of intracellular ion concentrations indicate that an increase in intracellular K^+ is required for the differentiation program to proceed. Epidermal proliferation and differentiation may also be modified via a specific cytosolic skin calcium binding protein which is synthesized only under conditions of high proliferation in normal cells. Retinoids alter epidermal differentiation and this may be mediated through an action on the cornification process by altering the function or subcellular distribution of the key enzyme in this pathway, epidermal transglutaminase.

Exposures of primary cultures of mouse keratinocytes to chemical carcinogens results in foci which resist the Ca^{++} signal to differentiate and continue to proliferate under high Ca^{++} conditions, producing countable colonies which stain red with rhodamine B. Cells obtained from mouse skin initiated in vivo show the same characteristics. A series of mouse skin initiators show similar potency for the production of altered foci in vitro and initiation of tumorigenesis in vivo. Cell lines derived from these experiments are initially non-tumorigenic, retain epidermal characteristics and all have in common the ability to proliferate in 1.2 mM Ca^{++}. They share these properties with cells isolated from chemically induced papillomas. Cells derived from altered foci are resistant to induced differentiation by phorbol ester tumor promoters but are stimulated to proliferate by these agents. Infection of epidermal cells with oncogenic retroviruses containing an activated ras gene indicate that expression of ras and subsequent synthesis of p21 provides a marked proliferative stimulus to basal cells. However such cells respond to high Ca^{++} by cessation of proliferation. These cells do not terminally differentiate but appear to be blocked in some nonterminal but advanced state of differentiation.

In an attempt to probe for functional changes in gene expression which may occur in carcinogen- or promoter-treated normal cells or in malignant cells, cloning of the genes for keratin peptides, the major differentiation proteins of the epidermis, has been accomplished. Clones corresponding to the 55, 59 and 67 kilodalton keratins synthesized by mouse epidermis in vivo and the 50, 54 and 60 kilodalton keratins synthesized by cultured mouse keratinocytes have been isolated and in some cases sequenced. Specific mRNA has been identified for each probe in embryonic, newborn, and adult skin with the in vivo probes and in normal cultured epidermal cells for the in vitro probes. The expression of each set of keratin genes may represent a program characteristic of predominantly differentiating cells (in vivo) or predominantly proliferating cells (in vitro). Malignantly transformed cells and preneoplastic cells show alterations in their programs of keratin gene expression suggesting that regulation of keratin genes in these cells differs from normal cells. It is anticipated that the identification of keratin profiles of individual cells or cell clones by in situ methods will be useful in characterizing their particular stage of differentiation.

Tumor promotion by phorbol esters has been an area of intense study during the past year. Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Basal cells appear to be heterogenous in response to phorbol esters in that some cells are induced to differentiate while others are stimulated to proliferate. This could form the cellular basis for selection. The induction of terminal differentiation by phorbol esters appears to be mediated by the phorbol ester receptor, and this action of phorbol esters is enhanced by Ca^{++}. The molecular basis for the pharmacological heterogeneity is suggested by studies of the phorbol ester receptor in cultured keratinocytes. Multiple receptor classes are found in differentiating cultures indicating that maturation state may modify receptor affinity. Putative initiated cells have been isolated which cannot be stimulated to differentiate by phorbol esters and have only a single receptor class. The altered response pattern of initiated cells suggests a mechanism for their selective clonal expansion from a larger population of normal cells undergoing terminal differentiation induced by phorbol esters, and this is mediated by opposing actions on transglutaminase and cornification. This mechanism may be related to the anti-promoting activity of retinoids. Studies on the progression of benign to malignant tumors in vivo indicate that promoters are incapable of accelerating the conversion process while genotoxic carcinogens have a marked enhancing and accelerating effect on malignant conversion. These results suggest a mechanism of multistage carcinogenesis involving three steps. A genetic change in the program of terminal differentiation characterizes the initiation step. This is a preneoplastic change. Tumor promotion involves cell selection and clonal expansion of initiated cells but does not alter their preneoplastic character. A second genetic change is required in the third step to convert benign to malignant lesions.

In the course of studies aimed at the identification of phorbol ester receptors and endogenous ligands, a specific phorbol ester apo-receptor present in brain cytosol was characterized which requires addition of phospholipids to reconstitute activity. Structure-activity relations for the reconstituted apo-receptor closely resembled those for the membrane receptor, suggesting that they might be the same. The phorbol ester apo-receptor co-fractionated with protein kinase C activity, indicating a biochemical function for the receptor. Diacylglycerol is an endogenous activator for the protein kinase C, and the phorbol esters can substitute for diacylglycerol in this stimulation. Diacylglycerol therefore appeared to be a possible candidate for an endogenous phorbol ester analog. In confirmation of this prediction, diacylglycerol was shown to competitively inhibit phorbol ester binding to the apo-receptor with a K_I of 0.4% (w/w), the concentration of the phospholipid. Structure-activity analysis of various diacylglycerol derivatives indicated that short or unsaturated side chains were required for activity.

The phorbol ester receptor could recognize either free phorbol ester or that dissolved in the membranes. The ability of insoluble diacylglycerols to mimic the phorbol esters strongly supports the latter possibility. A phorbol ester which was relatively insoluble in aqueous solution was synthesized. It was shown to be a potent inhibitor of phorbol ester binding if introduced into the phospholipids used for apo-receptor reconstitution, whereas it was substantially less potent if added to the aqueous incubation mixture instead. These findings support the interpretation of the structure-activity relations for highly lipophilic phorbol esters. They suggest, additionally, that relatively insoluble phorbol esters may provide a convenient reservoir for maintaining constant phorbol ester concentrations during systemic promotion experiments.

Another model, the JB6 mouse epidermal cell system, has been developed to elucidate the critical rate-limiting steps of the long latent period of premalignant progression, in particular when mediated by tumor promoters. JB6 cells are stably nontumorigenic and anchorage-dependent, and, in response to a variety of tumor promoters, become irreversibly tumorigenic and anchorage-independent. All promotable cells tested have shown specific cell surface receptors for phorbol esters. This is, however, not sufficient for either promotion or mitogenesis, since both promotion-resistant and mitogen-resistant variants of JB6 cells have receptors comparable in number and affinity to their sensitive counterparts. Fusion of promotable with nonpromotable JB6 cells yielded promotable hybrids indicating dominance of promotability. DNA from promotable cells, when transfected into nonpromotable cells yielded a 5- to 10-fold increase in promotion of anchorage-independence in response to TPA. Genes for promotability, and which specify sensitivity to induction of neoplastic transformation, are expected to be different from oncogenes.

Another in vitro model, used in studies of chemical hepatogenesis, is cultured hepatocytes; these studies have focused on early events in the production of liver neoplasia. Cultured hepatocytes were treated with non-toxic concentrations of the hepatocarcinogen N-hydroxy-2-acetylaminofluorene (N-OH-AAF) in order to obtain only "initiated" cell populations. Two-dimensional electrophoretic analysis of total cellular proteins from treated and untreated cells revealed few (6-8) qualitative protein changes between the groups. In contrast 10-15% of the 600-800 readily detectable proteins were undergoing at least a 50% quantitative change following treatment with N-OH-AAF. In order to examine the relationship between the covalent binding of carcinogens (aromatic amines) to cellular DNA with gene expression in early initiated cells, the major known or proposed deoxyguanosine and deoxyadenosine DNA adducts of N-OH-AAF have been synthesized and will be tested.

In Vivo Models

A number of in vivo models have been used for studies of chemical carcinogenesis during the past year. The development through selective breeding of animal strains with high susceptibility to tumor induction at a particular organ site provides an excellent model for the study of susceptibility determinants. One such strain is the SENCAR mouse strain, which is especially sensitive to chemically induced skin carcinogenesis. SENCAR mice are markedly susceptible to two-stage skin carcinogenesis compared to BALB/c mice. Grafting studies have shown that susceptibility is a property of the skin itself and other studies indicate that sensitivity is not due to differences in metabolism of polycyclic aromatic hydrocarbons. Yet by a variety of biological and biochemical parameters SENCAR epidermal cells behave identically to epidermal cells from less sensitive strains. SENCAR epidermis, however, appears to have a population of initiated cells which can be isolated from untreated cultures because they resist the terminal differentiation signal of 1.2 mM Ca^{++}. Thus SENCAR may be constitutive for an alteration in gene expression associated with the earliest changes in transformation.

The tumor-promoting properties of the commonly used plasticiser, di(2-ethylhexyl) phthalate (DEHP) has been demonstrated in mice. This chemical promotes hepatocellular tumors, but at extremely high dosage levels. The dose/effect curve

is extremely steep, and encourages the speculation that there may be a true no-effect level of exposure to non-genotoxic agents such as DEHP, thus providing a strategy for effective prevention by reducing exposure within measurable limits. Promotion of hepatocellular neoplasia by DEHP differs significantly from that by phenobarbital in regard to the biologic and histochemical properties of the promoted neoplastic cells. Intrinsically higher malignant potential is characteristic of neoplastic hepatocytes promoted by DEHP, illustrating that more than one path exists for tumor promotion in this, and presumably other, cell types. Phenobarbital, the classic promoter for liver cells, has now been shown to promote carcinogenesis in the rat thyroid as well, and becomes the first well-defined promoter clearly to act on different unrelated tissues in vivo.

The importance of dietary deficiencies in carcinogenesis has been shown clearly to relate to tumor promotion. Many carcinogens of the non-genotoxic type are known to affect the thyroid, and those that do so are frequently goitrogenic. It has been shown that simple iodine deficiency, the classic cause of diffuse goitrous thyroid hyperplasia, efficiently promotes thyroid carcinogenesis in rats. This not only identifies a promoting influence potentially significant in human populations, but provides an experimental approach to investigation of purely hyperplasiogenic stimuli in tumor promotion in the absence of any critical cell/agent interaction step corresponding to the "conversion" stage of promotion, and to investigating the comparative mechanisms of action of phenobarbital on the thyroid and on the liver. Diets devoid of the lipotropes methionine or choline have been shown to promote hepatocarcinogenesis in nitrosamine-initiated rats, and to elicit hepatocarcinoma development when given alone in the absence of pre-exposure to a genotoxic carcinogen. Conversely, high dietary levels of the physiological methyl donor methionine suppressed the carcinogenic activity of phenobarbital in mice. These dietary modifications have obvious, testable implications for man. Further, since normal bioregulatory methylation of cytosine in nucleic acids in the rat liver is disturbed by dietary methyl deprivation, the possible relation of nucleic acid methylation to mechanisms of tumorigenesis and tumor promotion can be investigated by dietary manipulation.

Transplacental carcinogenesis studies in nonhuman primates have shown that phenobarbital, at dosage levels comparable to pediatric anticonvulsive therapy in man, promotes liver tumors initiated by direct or transplacental exposure to diethylnitrosamine. This suggests that long-term exposure to barbiturates may be a significant carcinogenic risk in man. The observation that risk of gestational choriocarcinoma in postpartum females is correlated with risk of neoplasia in the offspring in patas monkeys given ethylnitrosourea during pregnancy suggests an epidemiologically testable hypothesis that in regions of the world where gestational choriocarcinoma is reported to be great, an environmental carcinogen may be involved that may also affect children born in these geographic regions. The possible role of aflatoxins as candidate agents will be investigated.

Carcinogen Metabolism

Studies on the metabolism of chemical carcinogens continued to receive emphasis during the past year. The primary interface between environmental chemicals and higher organisms is the mixed function oxidase system. The various forms of cytochrome P-450 constitute a major part of this system and are the major receptors for a wide variety of drugs, carcinogens, and other environmental

chemicals. Efforts to identify and characterize these isozymes in different tissues, species, populations, and individuals have involved developing a library of monoclonal antibodies (MAbs) which are highly specific for individual cytochromes P-450.

Inhibition of enzyme activity by a MAb prepared to 3MC-induced rat liver cytochrome P-450 was used to detect the cytochrome P-450 recognized by this MAb. The MAb not only inhibited the aryl hydrocarbon hydroxylase (AHH) activity of MC-induced rat liver microsomes, but also the AHH activity of human placenta. Among placentas from different individuals, the extent of inhibition of AHH by this MAb was virtually complete. Inhibition of 7-ethoxycoumarin deethylase (ECD), however, exhibited a large degree of individual variation. Placentas from both dizygotic and dichorionic monozygotic twins were examined both for absolute amounts of AHH and ECD, and for their degree of inhibition by the MAb; high intra-pair concordances were observed, relative to that found in unrelated individuals. The MAb-sensitive activities were also found in human lymphocytes, but not in liver and monocytes. The cytochromes P-450 responsible for these activities in liver and monocytes are therefore antigenically distinct from the enzymes in placenta and lymphocytes.

The results demonstrate the value of MAbs for defining antigenic site relatedness for different enzymatic functions of P-450s, and for identifying and quantifying the amount of a particular enzyme activity in a tissue that is dependent on specific cytochromes P-450. This study may be a prototype for the use of MAbs in phenotyping and mapping of the P-450s responsible for specific metabolic reactions and thus be useful in determining the relationship of P-450 phenotype to individual differences in drug metabolism and carcinogen susceptibility.

The molecular mechanisms of regulation of P-450 gene expression, the multiplicity of P-450s, and the structural-function relationships among the P-450s is also being assessed. The structure of the P-450 gene isolated last year has been characterized in greater detail using subclones of the different domains of this gene and of adjacent sequences as hybridization probes, repetitive DNA in the intervening sequences nearest the 5' and 3' ends of the gene have been discovered. The repetitive sequences in these two locations are different. Preliminary evidence has been obtained that the large intervening sequence nearest the 5' end of the gene contains sequences that code for a second mRNA that encodes a peptide of about 70 kilodaltons.

Regulation of Gene Expression

The nature of the genes and proteins responsible for the expression of the neoplastic phenotype of chemically transformed human cells continues to be the focus of research. A new polypeptide recognized in a chemically transformed human cell line has been identified as a mutant of β-actin. The presence of the mutated β-actin was correlated with the expression of the transformed phenotype in the variants of the transformed line and its hybrids with normal human fibroblasts. A mutation in the β-actin molecule resulted in several defects in the function of β-actin which were associated with the disruption and loss of the structural organization of the cytoskeleton. The cytoskeletal structure is responsible for cell shape and the motility and fluidity of the cytoplasmic membrane. Alteration of this structure is one of the most striking and universal characteristics of neoplastic cells. The results suggest that a mutation in β-actin leads the cells to express transformation by disrupting the cytoskeletal structure and its function.

As one of the gene sources for studying the regulation of gene expression, families of actin genes were isolated from human fibroblasts. Actin families are well-conserved through evolution and their expression is specific to the type of tissues in spite of the high similarity of the encoded amino acid sequences. One cardiac muscle actin gene and two smooth muscle actin genes have been isolated and shown to contain DNA sequences which completely match the amino acid sequences of the corresponding actin proteins. They also have the necessary consensus sequences at the boundary between exon and intron sequences, as well as other sequences known to be required for transcription and translation. A cysteine codon was found in all of the muscle actin genes at the position of the first amino acid following the methionine codon; this codon was not found in nonmuscle actin genes. Since no known actin protein starts with a cysteine, it is likely that post-translational removal of both cysteine and methionine accompanies muscle actin synthesis. This observation has interesting implications for actin gene functions, regulation, and evolution. A new intron site was found at the position of amino acid 84 in both types of smooth muscle actin genes. This intron location has never been found in any actin gene family of any species. Comparison of the intron location in all actin genes in all species examined indicates that both deletion and insertion of the intron have been involved in the evolution of the actin gene family.

It is likely that the conformational state of DNA is involved in the regulation of expression of the eukaryotic genome. A large copy number of a dT-dG alternating sequence, a sequence with potential to adopt Z-DNA conformation has been found in a variety of eukaryotic genomes. Although most studies on Z-DNA in the past have focused on dG-dC alternating sequences, the importance of the dT-dG alternating sequence based on its ubiquitousness in the genomes of various species is now being emphasized. Recently, the conversion of dT-dG alternating sequences from B-form to Z-form was found to be dependent on the degree of negative supercoiling of the flanking DNA. Because isomerization between the topological forms of DNA has been implicated in DNA replication, transcription, repair, and recombination, dT-dG alternating sequences may play an important role in these fundamental molecular processes. Experiments are now underway to develop an assay system to detect Z-DNA in biological systems and to test the hypothesis that such a Z-DNA sequence may be involved in the regulation of gene expression, mutation, and in carcinogenesis.

Another research area involves the identification and characterization of endogenous and exogenous factors that may control initiation, promotion and progression of chemically induced murine hepatomas, and includes both in vivo and in vitro studies. The in vivo studies have focused on isolation and characterization of preneoplastic cell populations. A centrifugal elutriation method for isolation of different populations (based on size) of parenchymal liver cells from untreated and carcinogen treated rats has been developed and combined with a cell surface receptor-based separation. Since an early event in hepatocarcinogenesis is a highly significant reduction in the surface receptors for asialoglycoproteins, asialofetuin (coated on tissue culture plates) was used as a ligand to selectively bind normal hepatocytes and thereby partially purify the preneoplastic cell population. In addition, an in vivo "bioassay" system for testing the growth potential, as well as the malignancy potential (invasiveness, metastasis, etc.) of the preneoplastic cells has been established. The biochemical characterization of the preneoplastic hepatocytes

includes computer assisted analysis of total cellular proteins measured by two-dimensional gel electrophoresis, and comparison to normal hepatocytes. To date, relatively few (5-8) qualitative protein differences between the preneoplastic and normal hepatocytes have been found, whereas 10-15% of the cellular proteins in the preneoplastic cells are undergoing at least a 50% quantitative change during the initiation stage when compared to normal hepatocytes.

Transforming Growth Factors

The problem of the isolation and characterization of transforming polypeptide growth factors (TGFs) continued to receive emphasis during the past year. TGFs can be isolated from a variety of epithelial and mesenchymal tumors of murine, chicken, and human origin, caused by chemicals or viruses, or of spontaneous origin. All of these TGFs are acid-stable, low molecular weight materials, and amino acid sequencing will commence when they are purified to homogeneity. New methods to achieve this desired purification have been developed during the past year, and the total purification of TGF-β from 3 non-neoplastic tissues (human placenta, human platelets, and bovine kidney) has been accomplished within the past 12 months. The experimental use of TGF-β in wound healing has been a finding of major importance and has provided a great deal of encouragement to proceed further with the entire problem of the molecular biology and molecular genetics of these growth factors.

TGFs able to confer a transformed phenotype on untransformed indicator cells have been isolated from all neoplastic and non-neoplastic tissues examined thus far. Two major subsets of the TGF family have been separated and characterized in terms of their interactions with epidermal growth factor (EGF). TGF-α competes with EGF for binding to membrane receptors while TGF-β binds to an as yet uncharacterized membrane receptor distinct from the EGF receptor. The presence of both TGF-α (or EGF) and TGF-β is required to induce transformation of normal rat kidney (NRK) cells; this concerted action of these two distinct subclasses of the TGF family has been demonstrated both for TGFs extracted directly from cells, and for TGFs isolated from conditoned medium of murine sarcoma virus-transformed mouse cells (often referred to as sarcoma growth factor). In addition, the effect is unchanged whether TGF-α or TGF-β is derived from a neoplastic or non-neoplastic source.

TGF-β has been purified over 200,000-fold from bovine kidney and is characterized by its ability to induce anchorage-dependent NRK cells to grow in soft agar in the presence of EGF; TGF-β is not mitogenic for cells grown in monolayer culture. Purified TGF-β does not compete with EGF for binding to membrane receptors. Analysis of the structure of TGFs revealed 16 half-cystine residues per mole and indicates that it is composed of two closely related polypeptide chains cross-linked by disulfide bonds. Sequence analysis indicated that at least the first 15 N-terminal amino acids of the two TGFs subunits are identical. Amino acid sequencing has also begun on TGFs from human platelets and human placenta. It is of major significance that the sequences that have been determined thus far for all 3 peptides (bovine kidney, human placenta, human platelet) are identical, indicating that TGF-β is a highly conserved structure.

Another major area of TGF research that has been explored for the first time within the past year is the development of a receptor binding assay for TGF-β. Methods have been developed for the successful iodination of TGF-β, and results of recent experiments show that there are membrane-bound, high affinity, saturable receptors for TGF-β in NRK cells. Efforts are currently underway to characterize these receptors and to determine the physiological response that occurs after interaction of TGF-β with its receptors. The development of a routine receptor assay for TGF-β should be of use in diagnostic screening for the possibility of abnormal expression of high levels of this peptide in a variety of disease states characterized by abnormal cell proliferation.

Finally, a long-term goal in studies of transforming growth factors is the development of peptide antagonists of the TGFs. Such agents should have use both as chemopreventive and chemotherapeutic agents. Recent reports regarding the chemical synthesis of the EGF gene and its incorporation into bacteria suggest that it will also be possible to make anti-EGFs by such recombinant DNA techniques. With the discovery that EGF and EGF-like peptides are required for the expression of TGF activity, the possibility of using EGF antagonists to block malignant transformation now becomes more a reality.

Studies in a related area, the lymphokines, have continued during the past year. Lymphotoxin, a lymphokine produced by mitogen- or antigen-stimulated lymphocytes, inhibits tumor cell growth and is anticarcinogenic. Alteration of HEC surface by lymphotoxin causes a persistent resistance to neoplastic transformation induced by carcinogens. Effectiveness of lymphotoxin prior to carcinogen treatment is not due to inhibition of hamster embryo cell (HEC) growth because there is no evidence of cytostatis of HEC with the lymphotoxin concentrations used. Lymphotoxin can be anticarcinogenic if given before or after carcinogenic insult or after TPA addition. Recent results indicate that lymphotoxin induces an anticarcinogenic physiological state that is transient and that the temporal relationship between lymphotoxin and carcinogen exposure is important for preventing initiated or promoted transformation by lymphotoxin. A major observation involving the anticancer activities of hamster and human lymphotoxin is that each consists of two distinct activities. The two proteins are separable by isoelectric focusing; the more acidic one contains nearly half the anticarcinogenic as well as other anticancer activities. These activities include the cytostatic effects as well as sensitization of target cells to increased destruction by naturally immune lymphocytes. The other protein, in addition to having anticarcinogenic, tumor cell cytostatic, and natural killer cell sensitizing activities, also has cytolytic activity.

Two observations concerning the specificity of interaction of lymphotoxin with the cell surface are that insulin susceptible rat mammary carcinoma cells become resistant to the growth inhibitory activity of lymphotoxin and that lymphotoxin reverses the results of interferon treatment of target cells which ordinarily decreases their sensitivity to naturally immune lymphocyte destruction. It will be important to establish whether the insulin and interferon effects are surface related and operative when considering anticarcinogenic lymphotoxin activity because insulin and interferon may normally be present.

In another area, the fluorescence-activated cell sorter was used to identify the normal lymphocyte subpopulations capable of producing lymphotoxin and responding with enhanced cytolytic activity to target cells previously treated with lymphotoxin. Fluorescein-labeled monoclonal antibodies were used to identify the

leu-7 positive subpopulation of natural killer cells that produces lympohotoxin and that can also respond with enhanced cytolytic activity after lymphotoxin treatment of the target cells. Therefore, the cell sorter can be used to isolate functionally active effector populations and to study changes occurring in the target cells. The data also provide further evidence that a lymphotoxin-like soluble mediator is secreted by natural killer cells and is involved in the destruction of the target cells. Identification of antigenic and structural membrane changes will permit sorting of cells for biochemical analysis of the lymphotoxin-induced changes. The latter will permit correlation of biochemical properties of sorted cells with their susceptibility to carcinogenesis.

Biochemical Epidemiology

The new field of biochemical epidemiology has received emphasis during the past year. Biochemical epidemiology is a multidisciplinary field whose primary goal is to identify individuals at high cancer risk by obtaining pathobiological evidence of high exposure of target cells to carcinogens and/or increased host susceptibility due to inherited or acquired factors. Laboratory methods have been recently developed for use in combination with analytical epidemiology to identify individuals at high cancer risk. These methods include: (1) techniques to assess specific host susceptibility factors; (2) assays that detect carcinogens in human tissues, cells, and fluids; (3) cellular assays to measure pathobiological evidence of exposure to carcinogens; and (4) methods to measure early biochemical and molecular responses to carcinogens.

The ability to detect carcinogens in human material is based on a unique and recently developed methodology. Antibodies were developed in rabbits against guanosin-(8-yl)-2-acetylaminofluorene (G-8-AAF) and guanosin-(8-yl)2-amino-fluorene (G-8-AF), the major guanosine adducts formed in vivo and in vitro by the interaction of nucleic acids with the aromatic amine carcinogen 2-acetylamino-fluorene (AAF). Antisera were also developed against DNA substituted with the 7,8-diol 9,10 epoxide of benz[a]pyrene (BPDE) yielding the trans (7R)-benzo[a]-pyrene-N^2-deoxyguanosine (BPdG) as the major antigen. A third antiserum developed is to cis-dichlorodiammine platinum DNA (cis-DDP-DNA). These antisera, which are highly specific for adducts, have been used to develop sensitive quantitative immunoassays for monitoring carcinogen binding and for morphological localization of binding sites. Currently these assays are able to detect one adduct per 10^7-10^8 nucleosides. Using immunological assays the persistence and removal of AAF adducts has been monitored during liver carcinogenesis by AAF. These studies have shown that binding to liver DNA is saturable and that adduct removal reaches a steady state after several weeks. In these studies, carcinogen substitution of liver DNA does not occur uniformly, and both inter-lobular and intralobular differences are observed.

Using the same technique, specimens from populations known to be exposed to carcinogens, such as benzo[a]pyrene and aflatoxin B_1 are being examined. For example, roofers who work with hot asphalt are exposed to high levels of polynuclear aromatic hydrocarbons including benzo[a]pyrene. Antibodies detecting benzo[a]pyrene-DNA antigens have been used to demonstrate this adduct in peripheral blood cells from many, but not all workers. The significance of this inter-individual difference is as yet unknown. In addition, the formation of carcinogen-DNA adducts may represent only the initial stages of carcinogenesis and measures of later stages, i.e., tumor promotion and progression, are also needed to predict an individual's cancer risk. The antibody has also been used

in immunoassays to screen DNA obtained from lung cancer patients and controls. Several positive samples were obtained in lung tissue from cancer patients and in DNA obtained from circulating lymphocytes. The production of a cis-DDP-DNA antibody has provided the first evidence that the cis-DDP-DNA adducts produced synthetically are structurally similar to those produced in vivo. DNA from buffy coats of patients receiving cis-DDP chemotherapy indicates that substitution by cis-DDP is readily measurable. Current studies involve a prospective analysis comparing clinical course and DNA binding levels of patients receiving cis-DDP chemotherapy.

Field Studies and Statistics

A major objective of the Field Studies and Statistics program is to generate national statistics on cancer incidence, mortality, and survival. This provides valuable signals for further epidemiologic study and for monitoring the progress of the National Cancer Program. During the early years of operation of the SEER Program, a network of population-based cancer registries which cover about 10% of the U.S. population, emphasis was placed on the analysis of incidence and mortality statistics. This year, with accumulation of sufficient follow-up information, the first report on cancer patient survival was published from the SEER Program for patients diagnosed from 1973 to 1979 and followed through 1980. For various sites of cancer the observed and relative survival rates were summarized by race, sex, age and time period. The overall five-year relative survival rates for all sites combined were 47% for whites and 35% for blacks. For certain sites the black-white differences in survival were substantial and have stimulated collaborative studies to identify factors responsible for ethnic differences in natural history and prognosis. Because of insufficient coverage of black and Hispanic populations in the SEER program, another registry will soon be added with appropriate ethnic representation. Data from the SEER Program were used extensively this year by the NCI Director to indicate progress as well as problems requiring special attention in cancer research and control. Toward this end, an NCI-wide advisory committee was formed to more fully utilize the SEER resource for various purposes, such as identifying national objectives and target areas for intervention as well as monitoring the impact of such programs.

With the continuing maturation of the SEER Program, systematic and comprehensive analyses of collected data are planned, including detailed survival tables by cancer site, cell type, extent of disease, initial treatment, race, sex, and age. In addition, an analysis of time trends through 1980 in cancer incidence and mortality will be completed for the five areas common to the Second National Cancer Survey (1947-48), the Third Survey (1969-71), and three points in the SEER Program (1975-76, 1977-78, 1979-80). Surveillance of the SEER data for occurrence of multiple primary cancers is also being actively pursued, with emphasis on treatment-induced second cancers and multiple primaries that may share etiologic factors. Since non-melanoma skin cancer is not covered routinely by cancer registries, a special incidence survey was carried out in 1977-78 in various areas of the country, and the results published this year in a monograph. In the past a county-by-county survey of cancer mortality in the United States identified geographic patterns that have provided etiologic clues for more definitive study, and updated maps of cancer mortality are now being completed for the more common tumors.

Continued emphasis was given this year to case-control and cohort studies aimed at evaluating key hypotheses in cancer etiology. Case-control studies of selected cancers have been undertaken when high-risk communities are identified on the cancer maps, or when major testable hypotheses and special resources become available. Based on leads provided by the U.S. cancer atlases, field studies have implicated shipyard work and asbestos exposures during World War II as the explanation for the high rates of lung cancer in several areas along the Atlantic coast. In southern Louisiana, the high rates of lung cancer appeared at least partly due to smoking habits, particularly the heavy use of hand-rolled cigarettes. The use of smokeless tobacco accounted for the elevated rates of oral cancer among women in southern rural areas, and the study also suggested relationships to the use of mouthwash, work in the electronics industry, and dietary deficiencies. A cluster of colorectal cancer in rural Nebraska was linked to a concentration of Czechoslovakian migrants, especially those with high fat diets and familial occurrence of digestive tract diseases. The high rates of renal adenocarcinoma in the north central region appeared related to ethnic factors (especially German ancestry), cigarette smoking, and obesity and dietary habits, particularly in females. A parallel study of renal pelvis cancer in this area indicated a substantial influence of cigarette smoking, plus an assocation with phenacetin-containing analgesic drugs and occupational exposures previously implicated in bladder cancer. A case-control study of nasal cancer in North Carolina and Virginia pointed to the role of smoking, chronic nasal disease, and occupational exposure to wood dusts, chromates, chemicals and textiles. A large case-control study of bladder cancer which previously evaluated the risk of artificial sweeteners is also being analyzed with respect to a number of other potential risk factors. Of special interest is an elevated risk of bladder cancer found among truck drivers, especially those using diesel engines.

Occupational studies, a time-tested means of identifying physical and chemical carcinogens, were pursued to assess hazards suspected on the basis of experimental, clinical, and field observations. This year surveys revealed excesses of brain and other cancers in petroleum workers; leukemia and bladder cancer in professional artists; stomach cancer in iron ore miners; lung cancer and leukemia among pesticide applicators; leukemia and non-Hodgkin's lymphoma in agricultural workers; pancreas cancer in workers involved in corn wet milling and in press photographers; lung cancer in various groups exposed to asbestos, in steel and foundry workers exposed to polycyclic hydrocarbons, in copper and zinc smelter workers exposed to inorganic arsenic, and in workers exposed to talc during ceramic plumbing manufacturing. A large follow-up study of workers in contact with formaldehyde during manufacturing and usage is underway. Preliminary surveys of morticians have revealed no excess of nasal cancers as observed in animal bioassay studies of formaldehyde, but have suggested increased mortality from brain tumors, colon cancer, and leukemia. A systematic evaluation of occupational risks of cancer, with adjustment for smoking habits, is being carried out among participants of the Veteran's Followup Study initiated many years ago by Harold Dorn.

Radiation studies received further emphasis in efforts to clarify the effects of low-level exposure and the shape of the dose-response curve. In an international survey of cervical cancer, the radiation regimens were less effective in inducing leukemia than other radiation exposures that have been studied, perhaps related to the cell-killing potential of high-dose radiation to the pelvis. However, a slight risk was found that may be associated with low-dose radiation received by marrow outside the pelvis. A survey of breast cancer

among atomic bomb survivors revealed elevated risks among women exposed at younger ages, and for the first time women exposed under the age of 10 showed a dose-related excess risk. A follow-up of patients with non-Hodgkin's lymphoma revealed an elevated risk of acute non-lymphocytic leukemia, strongly correlated with radiation dose to the bone marrow. In a follow-up of children irradiated for enlarged tonsils, preliminary findings suggest a two-fold risk of thyroid nodules and persistent chromosome aberrations in circulating lymphocytes. A case-control study of thyroid cancer has revealed an elevated risk associated with radiotherapy for benign head and neck diseases in childhood, and a study of childhood cancer in twins indicated a two-fold excess risk associated with prenatal x-ray, suggesting that the association is due to radiation rather than the indications for pelvimetry. A survey of children who developed multiple primary cancers revealed dose-response relationships between radiation exposure and the risk of developing second cancers. Among children irradiated for ringworm of the scalp in Israel, preliminary data suggest elevated risks of thyroid cancer, brain tumor and leukemia. In collaborative studies with the Radiation Effects Research Foundation in Japan, current emphasis is being placed on the analysis of case-control interview studies of breast and lung cancers in efforts to evaluate interactions of radiation with other risk factors.

Drug studies were continued to evaluate the effects of estrogenic compounds, which appeared related to the risk of breast cancer in certain high-risk groups, including women with familial predisposition or benign breast disease. No excess risk of breast cancer was associated with thyroid medications or diazepam (Valium) as previously suggested, and patients with Hansen's disease showed no increased risk of cancer that could be linked to the use of dapsone, a carcinogen in animal studies. Among patients with cancer or rheumatologic diseases treated with various alkylating agents, there was a substantial elevated risk of acute non-lymphocytic leukemia. Most recently, methyl-CCNU, a nitrosourea used in cancer chemotherapy, was linked to acute leukemia and pre-leukemia.

Nutritional studies were intensified this year to clarify the role of dietary constituents in cancer etiology. Several studies have utilized geographic areas in the U.S. (e.g., north/south differentials for large bowel cancer) and migrant groups (e.g., Japanese- and Norwegian-Americans) whose cancer risks may be altered by changing dietary habits. In case-control studies the role of dietary fat was suggested for breast and colorectal cancers, a broad nutritional deficiency for esophageal cancer, and a deficiency of fruits and vegetables in oral cancer. Case-control studies of various cancers are underway to measure the intake of various micronutrients, both by interview about usual dietary patterns and by laboratory assays of serum samples. Efforts to develop and utilize national resources, including HANES I, the first Health and Nutrition Examination Study of the U.S., continue so that dietary habits can be correlated to the subsequent risk of cancer.

Family and genetic studies, enhanced by collaborative ties with laboratory investigators and a computer-based data resource, have resulted in delineation of familial cancer syndromes and several leads to mechanisms of host susceptibility. For example, the discovery of the dysplastic nevus syndrome has provided a marker of susceptibility to melanoma, enabling early detection and treatment of this potentially lethal cancer. Surveys of neurofibromatosis and other hereditary syndromes have helped clarify the risks of various cancers, and have explored the role of several genetic markers. Studies of a familial disorder featuring sarcomas and other neoplasms have led to the discovery of in

vitro cellular radioresistence in this syndrome. The Interinstitute Medical Genetics Clinic provides a multidisciplinary setting for studying families and individuals prone to cancer. The repository of cancer-prone families in the program is now of special interest to experimentalists involved in the identification of human oncogenes.

Environmental pollutants were evaluated through epidemiologic studies that have utilized relevant environmental and body measurements, whenever possible. To test the hypothesis that arsenical air pollution may be related to lung cancer, a case-control interview study was carried out in the vicinity of a large zinc smelter. An elevated risk was found among people living near the smelter and in areas with high soil levels of arsenic, even after controlling for the effects of smoking and occupation. Also underway is a case-control study of bladder cancer to investigate the role of halogenated hydrocarbons in drinking water, a relationship initially suggested by geographic correlation studies.

Infectious agents received substantial attention as the program became heavily involved in investigating the epidemic outbreaks of AIDS (Acquired Immune Deficiency Syndrome), which predisposes to Kaposi's sarcoma and opportunistic infections. Studies have focused on the epidemiologic, immunologic, and virologic characterization of certain high-risk groups, including male homosexuals and patients with hemophilia. In collaboration with the NCI Laboratory of Tumor Cell Biology, a series of investigations has been initiated to evaluate the role of a newly discovered human retrovirus in the origins of T-cell leukemia, which is endemic in certain parts of the world, including Japan and the West Indies. Preliminary evidence also suggests that this retrovirus may be involved in the development of AIDS. Also conducted this year were studies to clarify the role of the Epstein-Barr virus in Burkitt's lymphoma and nasopharyngeal cancer, and of herpes virus type II and papilloma virus in cervical cancer.

REPORTS ON INTERNATIONAL AGREEMENTS AND INFORMATION EXCHANGE ACTIVITIES

(Fiscal Year 1983)

The Division of Cancer Cause and Prevention (DCCP) participates in several of the major international agreements on cooperation in cancer research: U.S.-U.S.S.R. (1972); U.S.-France or NCI-INSERM (1972); U.S.-Japan (1974); U.S.-Italy (1979); U.S.-Germany (1979); and U.S.-Peoples Republic of China (1980). Collaborative efforts include studies in cancer epidemiology and chemical, physical and biological carcinogenesis, with emphasis on factors related to the etiology and prevention of cancer. Basic and applied research also is conducted in foreign institutions under grants, contracts and cooperative agreements administered through the Division's extramural programs. The activities supported under these funding mechanisms are described in the reports of the respective branches.

U.S.-U.S.S.R. Agreement. This year we have attempted to redefine and restructure the American-Soviet Cooperative Program in Carcinogenesis. Since this represents a new field of scientific endeavor in the U.S.-U.S.S.R. Cooperative Cancer Program, we deem it essential to obtain from the U.S.S.R. as much information as possible on the ongoing work in research areas that were identified as potentially benefiting both nations at our meeting in Bethesda, 14-15 September 1981. These include: (1) modifying effects of chemicals on gene expression of normal and neoplastic cells; (2) role of tumor promotors in biologic, chemical, and physical carcinogenesis; (3) genetic analysis of malignancy by means of somatic cell hybridization; (4) chemical induction of tumors in specific target organs; (5) gene regulation and gene amplification relating to viral and chemical carcinogenesis; (6) the role of viral-related transforming (onc, sarc) genes in the genesis of spontaneous and induced tumors of animals and man; (7) development and exchange of monoclonal antibodies (hybridomas) directed against different antigens in biologically and chemically induced tumors; (8) studies on the development of cancers resulting from transplacental and perinatal exposures to biological and chemical carcinogens; (9) chemical/viral co-carcinogenesis studies in primates; (10) genetic disorders which predispose to malignancy; (11) mutagenic action of anticancer drugs; (12) clinical aspects of somatic cell genetics; and (13) studies on new candidate oncogenic virus isolates from primates, including man. We have asked for the following information: (1) the number of research groups or institutions and their location in the Soviet Union where scientific effort is underway in the areas we listed; (2) the principal scientists that participate in these activities, their affiliation and a bibliographic listing of their contributions in areas of carcinogenesis; and (3) the availability of Soviet principal scientists in carcinogenesis for in-depth discussion of research interests and results for consideration of carcinogenesis problem areas amenable to joint investigation. Without this information we cannot, in any meaningful way, plan collaboration and/or selection of American scientists unique and appropriate to the research areas in which American and Soviet specialists will interact. We await the Soviet response to our requests.

The major contribution to the U.S. in this program is access to a large monkey colony in Sukhumi. Drs. Gallo and Saxinger (Division of Cancer Treatment, NCI) have found some of these primates to be seropositive for HTLV antibody, and further collaborative research in this area is planned.

No scientist exchange took place this year.

U.S.-France (NCI-Institut National de la Sante et de la Recherche Medicale-INSERM) Agreement. The NCI-INSERM Committee on Basic Cancer Research has the responsibility to support high quality nonclinical research relating to understanding the process of carcinogenesis. The exchange program is open to all qualified U.S. or French scientists who are presently engaged in basic research in carcinogenesis. Proposals submitted by candidates from each country are reviewed for scientific merit by both U.S. and French working groups. Information about this cooperation has been given wide circulation.

SCIENTIST EXCHANGES

U.S. to France:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. P. G. Satyaswaroop Pennsylvania State Univ. (Hershey, PA)	Dr. E. Baulieu Universite de Paris Sud (Bicetre)	6 months	Hormonal Response of Human Endometrial Carcinoma
Dr. J. A. Katzenellenbogen Harvard Univ. (Boston, MA)	Dr. Pierre Martin Faculte de Medicine de Marseille	2 weeks	Fluorescent Estrogens for Assay of Estrogen Receptors on Breast Cancer Cells
Dr. R. Weinmann Wistar Inst. (Philadelphia, PA)	Dr. Yaniv Pasteur Institute (Paris)	3 months	Adenovirus VA RNA Expression in Cells Transformed by a Papilloma Virus Vector
Dr. N. Salzmann NIAID, NIH (Bethesda, MD)	Drs. Pierre & Evelyn May CNRS (Villejuif)	2 months	Effect of T Antigens on Transcription of SV40 DNA

France to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. M. Leng CNRS (Orleans)	Dr. G. Felsenfeld NIADDK, NIH (Bethesda, MD)	1 month	Interactions of Carcinogens with Methylated DNA
Dr. J. Pavlovitch Hopital des Enfants-Malades (Paris)	Dr. S. Yuspa NCI, NIH (Bethesda, MD)	5 weeks	Biological Carcinogenesis

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. J. Saez INSERM (Lyon)	Dr. D. Gosporadowicz Univ. Calif. (San Francisco, CA)	4 months	Antagonist Effects of Retinoids and Growth Factors
Dr. C. Brechot Pasteur Institut (Paris)	Dr. J. Summers Fox Chase Cancer Center (Philadelphia, PA)	6 months	Chronic Hepatitis B Infection and Hepatocellular Carcinoma
Dr. J. C. Nicolas Hopital Trousseau (Paris)	Dr. A. Levine Stony Brook (NY)	4 months	Transfection of B Lymphocyes with Epstein-Barr Virus DNA

U.S.-Japan Agreement. This year marks the fourth in the second five-year program of this agreement, which consists of four broad program areas: Etiology, Cancer Biology and Diagnosis, Cancer Treatment, and Interdisciplinary Research. The cooperation between U.S. and Japanese scientists still remains one of the most active. This binational program is especially well suited to the study of malignancies that differ markedly in their occurrences within the two nations. Within the Etiology Area of the U.S.-Japan Agreement, seminars were held on "Multiple Primary Neoplasms," "New Etiology of Lung Cancer," and "Carcinogenesis and Environmental Factors."

Several members of this Division participated in exchange programs in Etiology Research which has as its mission to provide a fundamental basis for understanding cancer causation that, in turn, would identify effective means for preventing or modulating this process.

SCIENTIST EXCHANGES

U.S. to Japan:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. J. A. McCloskey University of Utah (Salt Lake City, UT)	Dr. S. Nishimura Natl. Cancer Res. Inst. (Tokyo)	2 months	Structure Elucidation of New Modified Nucleosides from Transfer RNA

Japan to U.S.:

Dr. K. Yoshiike Natl. Inst. of Health (Tokyo)	Dr. K. K. Takemoto NIAID (Bethesda, MD)	2 1/2 months	Molecular Biology of Papovaviruses

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. T. Kamatāki Keio University (Tokyo)	Dr. F. P. Guengerich Vanderbilt University (Nashville, TN)	1 month	Role of Cytochrome P-450 in the Metabolic Activation of Carcinogens
Dr. Y. Hayashi Nat. Inst. of Hygienic Sciences (Tokyo)	Dr. R. W. Moch Bureau of Foods, FDA (Washington, DC)	3 weeks	Chemical Carcinogenesis and Toxicological Pathology
Dr. H. Fujiki Natl. Cancer Center Research Institute (Tokyo)	Dr. R. E. Moore Univ. of Hawaii (Manoa)	3 weeks	Isolation of Tumor Promoter (Aplysiatoxin) from Seaweed
Dr. K. Oda Univ. of Tokyo (Tokyo)	Dr. W. Eckhart Salk Institute (La Jolla, CA) and Dr. H. Okayama NICHD (Bethesda, MD)	1 month	Analysis of Viral Transforming Genes

U.S.-Italy Agreement. Research pertinent to this Division is included in the Cancer Prevention Program. The program involves a variety of activities related to cancer etiology, with recent emphasis on epidemiology. This year a workshop on the Epidemiology of Gastric Cancer was held in Florence, Italy. The striking geographic variation in Italy in mortality from stomach cancer, the country's leading cause of cancer death, was described. Plans were then formulated to develop a collaborative case-control study to identify etiologic factors for this cancer in high-risk areas of northern Italy.

SCIENTIST EXCHANGES

Italy to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. M. Biocca Inst. Superiore di Sanita (Rome)	Dr. Jeanne Stellman Columbia Univ. (NY)	1 month	Computer Analysis of Epidemiologic Data
Dr. G. Costa Univ. Turin (Turin)	Mr. Todd Frazier	2 months	Collaborative Analyses of Occupational Data

U.S.-Federal Republic of Germany. This agreement primarily concerns cooperation in environmental carcinogenesis. The working groups have agreed to conduct joint investigations on mechanisms of carcinogenesis, including prevention and modulation of the process. In 1982, with the assistance of various German foundations, 15 German scientists visited the U.S as fellows. In addition, the Germans sent

approximately 500 scientists to a variety of congresses, symposiums, and workshops in the United States. In March 1983, the U.S. Chairman visited the Ministry of Health in Bonn, as well as some laboratories, as a guest of the German Government. The German Ministry feels that the Germans have much to gain by further increasing their involvement in the cancer research that is taking place in the U.S., particularly since so little collaboration has occurred in the area of carcinogenesis. As a result of the meeting in Bonn with the U.S. Chairman, the German side wishes to determine how they should focus additional collaboration with the U.S. They are prepared, if necessary, to obtain supplemental funds to support collaborative research. This area of research is of such high priority that a meeting is planned at the German Embassy in Washington for August 1983. In attendance will be at least 2 representatives of the German Government as well as a German scientist who is currently in the U.S. At that time, the extent of the involvement of German scientists in areas identified as promising research areas will be formalized.

U.S.-People's Republic of China. The newest initiative differs from other binational health programs in that it has no joint steering committee. Cancer epidemiology has been given the highest priority for collaborative research. Molecular biology and related disciplines will await the training of the younger Chinese scientists. Of particular interest to the DCCP are joint studies on the biochemical epidemiology of esophageal, nasopharyngeal and hepatocellular carcinomas; other epidemiologic studies are focused on lung cancer, stomach cancer, choriocarcinoma and T-cell lymphoma. Pilot studies of these cancers, supported by a contract from DCCP, involved case-control studies to identify environmental determinants, including diet and nutrition, occupational exposures, air pollution, and infectious agents. Members of this Division have already begun collaborative projects. This year, substantial progress was made in (1) establishing culture conditions for human liver and esophagus; (2) studying the metabolism of chemical carcinogens, including studies on DNA damage and repair; (3) initiating biochemical and immunochemical epidemiological studies of individuals at high risk of developing liver or esophageal cancer; and (4) investigating the in vitro transformation of human epithelial cells by microbial and chemical agents.

SCIENTIST EXCHANGES

U.S. to China:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. C. Harris NCI (Bethesda)	Dr. Hsia Chu-chieh Cancer Institute (Beijing)	3 weeks	Risk Factors in Esophageal and Hepatic Cancer
Dr. J. Lechner NCI (Bethesda)	Dr. Sun Tsung-tang Cancer Institute (Beijing)	1 month	In Vitro Transformation of Esophageal and Hepatic Epithelial Cells

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. Steven Fox NIOSH (Cincinnati)	Dr. Li Bing Cancer Institute (Beijing)	3 weeks	Epidemiologic Studies of Esophageal Cancer, Lung Cancer, and Choriocarcinoma

China to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. Hsia Chu-chieh Dr. Sun Tsung-tang Cancer Institute (Beijing)	Dr. Curtis Harris NCI (Bethesda)	1 month	Risk Factors in Esophageal and Hepatic Cancer
Dr. Xu-dong Dai Xarbin Med. Univ. (Xarbin)	Dr. Robert Miller NCI (Bethesda)	1 year	Methodology Training
Dr. Song-lin Yu Wuhan Med. Col. (Wuhan)	Dr. Robert Miller NCI (Bethesda)	1 year	Methodology Training
Dr. Yi-wan Li Cancer Institute (Hospital) (Beijing)	Dr. William Blattner NCI (Bethesda)	3 months	HTLV Antibodies in the Peoples Republic of China

ANNUAL REPORT OF

OFFICE OF THE SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

A. Mission

This program unit comes under the Office of the Director, Division of Cancer Cause and Prevention (DCCP), National Cancer Institute, and serves as a focal point for development of program interests and activities and collaboration (intramural and extramural) in the area of environmental cancer. The cooperative projects and collaboration are with other Federal agencies, predominantly the Environmental Protection Agency (EPA) and the National Institute for Occupational Safety and and Health (NIOSH), on environmental/occupational cancer; state agencies; industrial and academic institutions; trade unions; consumer groups; and scientific and medical societies. It serves as the primary information resource for the Division and the Institute on the role of environmental pollution and industrial exposures in carcinogenesis. The coordination activities and efforts of this office fulfill one of the essential functions prescribed in the National Cancer Act of 1971 and as amended by the Biomedical Research and Training Amendments of 1978.

Since 1972 this unit has introduced and emphasized concepts of a holistic approach to the assessment of stress from environmental contaminants identified as carcinogens in environmental media. Thus, importance is placed beyond single exposures (as with traditional bioassays) on the actual multiple exposures. Here the evaluation is made of air and water pollutants, diet contaminants, cosmetics used, drugs prescribed, and occupational exposures received by man. To achieve these goals for appraisement of occurrence, frequency and concentration of such environmental carcinogens, close working arrangements are required and collaboration is continuing with EPA, NIOSH, the Food and Drug Administration (FDA), the Occupational Safety and Health Administration (OSHA), the U. S. Department of Agriculture (USDA), the Department of Defense (DOD), the U.S. Department of Interior (USDI) and many other agencies or subagencies. Data and information from these sources are transmitted to the Office of the Scientific Coordinator for Environmental Cancer (OSCEC) and these activities are extended through support of workshops, symposia, presentations at scientific meetings and issuance of reports and proceedings on environmental carcinogenesis. These activities are of mutual benefit in that NCI/DCCP exploits the use of these information resources at other agencies and institutions while filtering back data and information on identification and classification of environmental carcinogens.

The activities of the Interagency Collaborative Group on Environmental Carcinogenesis (ICGEC), sponsored by this office and now in its tenth year (70th meeting to be held in August 1983), really adds to the information exchange activity as well as fulfilling one of the missions encompassed within the earlier referenced legislation. The ICGEC grew from a constituency of ten Federal agencies to 28 agencies or subagencies with which NCI interacts. The mutual stimulation, dissemination, and exchange of information and data has advanced NCI competence and provided greater recognition for development of cause and prevention projects in

the area of environmental cancer. Prior to 1972, NCI lacked a strong initiative and mechanism for systematic acceleration of activities in this area of environmental cancer.

This unit also monitors and manages the NCI/EPA and NCI/NIOSH collaborative contractually supported programs on environmental and occupational carcinogenesis mandated by the Office of Management and Budget (OMB) and the U. S. Congress in 1976. This office also provides an interface with the National Toxicology Program (NTP) regarding chemical selection for carcinogenesis testing.

As a result of Congressional directives, a Task Force on Environmental Cancer and Heart and Lung Disease (EPA the lead agency) was established with NCI. This office continues to play an active role in the work requirements of some of the subcommittees and annual workshops.

B. Other Assigned Functions

This office continues to support the NCI Chemical Selection Working Group (CSWG) for NCI nominations to the NTP and the Chemical Evaluation Committee of the NTP; this support is provided by staff and by contractor (SRI International). This office also maintains a Chemical Selection Planning Group (CSPG) which works with a contractor to plan agendas and make prior decisions on chemicals to be submitted to the CSWG. The CSPG also serves to assist the project officer in providing, along with a Steering Committee, the necessary guidance for this significant information resource contract.

A resource contract with Technical Resources, Inc., Bethesda, Md., involves the preparation of the PHS-149 report (Survey of Compounds Which Have Been Tested for Carcinogenicity; 1974-75, 1976-77, 1979-80). NCI, through this office, maintains a cooperative agreement with Centers for Disease Control and collaborates with the Michigan State Department of Health on a follow-up of populations in Michigan exposed to polybrominated biphenyls (PBBs). A collaborative program on environmental carcinogenesis with the Environmental Protection Agency and an occupational carcinogenesis program with the National Institute for Occupational Safety and Health is conducted out of this office. Through a master agreement mechanism, information resources in the form of documentation on carcinogens in drugs and cosmetics are being developed. Plans are also underway for a resource document on foods.

C. Accomplishments

Interagency Collaboration

One of the most visible mechanisms for collaboration is the Interagency Collaborative Group on Environmental Carcinogenesis. This group consists of 28 agencies or subagencies and was set up by this office for NCI to fulfill one of the mandates of the National Cancer Act of 1971. The group meets every 6-8 weeks and by August 1983 there will have been 70 meetings. Topics of meetings held so far this year are as follows: 1) Health and Environmental Risk Analysis Program, DOE; 2) In Vitro Systems; 3) Chelation and Carcinogenesis; 4) Chemoprevention; 5) Mechanism of Benzene Toxicity; and 6) Diesel Fuel Emissions. Before the end of this reporting period, one or two more meetings will have been held. From time to time, proceedings are published from these meetings.

Previous reference was made to the Task Force on Environmental Cancer and Heart and Lung Disease and our participation in all Working Group Activities. A member of the Task Force Planning Group reviewed and planned the activities of Working Group meetings including preparation of the Sixth Annual Report to Congress. A member of our staff continues to serve as Chairperson and coordinator for the Project Group on Exposure and Metabolic Mechanisms.

The Subcommittee on Metabolic Mechanisms is preparing a report for the Task Force entitled "Strategies for Determining the Mechanisms of Toxicity." This report is structured in a manner which will allow various Federal agencies to follow a logical stepwise approach in order to develop their own effective and efficient protocols for toxicity studies.

The office is involved in the preparation of the DHHS report entitled: "Research Activities of Relevance to the Clean Air Act. Third Biennial Report to Congress; 1980-1981."

Assistance is rendered to the Department of Energy's Health and Environmental Effects Program.

A contract with the American Health Foundation entitled "The Genotoxic Potential of Medical Device Materials was jointly administered with FDA via an interagency agreement between FDA and NCI. This project was completed in December 1982 and was supported by pass-through funds provided to NCI by FDA.

The office continues, as a major activity, the coordination work and management of two collaborative programs, the NCI/EPA Program on Environmental Cancer and the NCI/NIOSH Program on Occupational Cancer.

Interfacing with Trade Associations, Industries and Trade Unions

The exchange of data and information with a wide spectrum of private sector and industrial identities--individual companies as well as trade associations--continues. The very large class study of dyes (for the purpose of making nominations to the NTP bioassay activity) being conducted with the collaboration of the relevant international trade association and the concerned trade union is proceeding well. One large component is near completion, and it is anticipated that the entire project will be completed in FY'84. These organizations have been involved in processes of chemical nomination and class studies in order to take full advantage of their expertise.

Our staff has provided the private sector with guidance in animal model selection, and protocol design, with access to Federally funded research so that their own toxicological efforts are maximized. Municipal and state governments, and other agencies, have requested and received a great deal of information from the various programs so that their legislative decisions reflect the most recent scientific information.

This office has cooperated with the Institute's epidemiologists and industrial organizations to effect collaborating projects. The study of formaldehyde workers is progressing very well and could be completed in FY'84. A feasibility study for another compound is almost completed and could well result in a request for permission to conduct a full blown study. Two other studies are still awaiting industrial initiative.

In one continuing contact, this office has provided to an international trade association access to U. S. agencies to facilitate their making scientific decisions that more readily comply with global regulatory compliance developments. A major effort has been the involvement of U. S. industry in the production of the IARC monographs so that the private sector's knowledge of processes, marketing trends, etc., can be incorporated into the most accurate documents possible.

The in vitro mutagenicity contracts have performed successfully during this time frame. Approximately 100 compounds have been tested and the results utilized by intramural scientists, the chemical selection process, other Federal agencies, and one international trade association.

Special Projects on Environmental Carcinogenic Contaminants: Air, Water, Food, Drugs and Cosmetics

BOA Report on Cosmetics

A report entitled: "The Potential Carcinogenicity of Cosmetic Ingredients" which contains a series of 64 IARC-style monographs was completed under contract in December, 1982. These monographs contain data on chemical/physical properties, production occurrence and use, pharmacokinetics as well as in vivo and in vitro carcinogenesis, general toxicology and human epidemiology.

Expert Panel Review of Monographs

Previously, a two-volume report on "Genotoxic Assessment of Selected Drugs and Medical Procedures" was also completed by a contractor and contained monographs on 109 drugs. Both reports (e.g. Cosmetics and Drugs) are now undergoing review by a panel of experts convened by FASEB.

Master Agreement: Natural Toxicants in Foods

A draft final report on the assessment of the carcinogenicity and mutagenicity of natural food toxicants and contaminants has been prepared for NCI by a contractor. This report summarizes the published literature for approximately 100 compounds in tables of in vitro and in vivo carcinogenesis results. Substances covered in this study include various chemical classes such as flavenoids, anthraquinones, hydrazines, allylic and propenylic arenes, metals, mycotoxins, polycyclic aromatic hydrocarbons, halogenated organics, nitrosamines and other unintentional contaminants. This report is currently undergoing review by both NCI and FDA staff.

Master Agreement: Monographs on Organic Air Pollutants

A series of air pollutants have been selected for preparation of individual or group IARC-style monographs depending upon the amount and quality of published data available. This project was a more detailed and expanded study than a previously published report entitled: "Organic Air Pollutants: Carcinogens, Mutagens and Promoters." Approximately 70 compounds have been selected for this report from an initial list of 770 on the basis of various prioritization criteria and will have more detailed discussions of environmental occurrence/fate data than normally appear in current IARC monographs. It should also be mentioned that compounds discussed at the February 1983 IARC Working Group Meeting were not chosen for the above study. This report will be completed in late September (1983).

Special Reports on Water Pollutants

A previous DHHS/PHS/NIH Report on Biorefractories in Water (Carcinogens, Mutagens and Promoters) concentrated on 767 organic contaminants in drinking water has now been expanded to include an identification and classification of organic contaminants (now 1565) in drinking water. This is an evaluative study and includes 52 carcinogens, 68 mutagens, and 23 tumor promoters. Similarly, a study was done on 15 major inorganic chemicals (13 metals and 2 non-metals or anions) in drinking water. Each of these inorganic chemicals was evaluated as to evidence for carcinogenicity based on evidence in animals, humans and from genotoxicity and in vitro tests.

Data Bases on Carcinogens, Mutagens, Cocarcinogens and Promoters and Bioassay Summary Reports on Chemicals Tested (NCI/NTP) for Carcinogenicity

Data bases developed by NCI/DCCP/OSCEC over the past few years in collaboration with a contractor (SRI) have now reached a point of maturity where they are very useful and significant. These data bases, built on surveys of water pollutants, air pollutants, cosmetics, dye class studies, other use and structural class studies, IARC monograph chemicals and NCI/NTP bioassay chemicals, have yielded a reference data base that NCI has made available to limited groups of people. A decision is pending on how to broaden distribution of this data base. Similarly, NCI/NTP Bioassay Summary Reports on approximately 224 entries were made available in hard copy as a ready reference source that will save many manhours of search since these technical report abstracts can be found according to CAS number, alphabetical listing, Technical Report Number, and other permutations. This compendium will be brought up to date as technical reports become available. Data on carcinogens, mutagens, promoters, cocarcinogens, carcinogen metabolites are now available in hard copy and are available for "online" search in PROPHET and CIS (Chemical Information System) which can be accessed by the scientific community-at-large.

Cooperative Project Between National Cancer Institute and National Toxicology Program (List of Chemicals for Testing and Research - Biomedical Research and Training Amendments of 1978)

To fulfill this Congressional requirement, within the National Cancer Institute, this office has been designated the task, each year, of compiling a list of chemicals for testing and research for carcinogenesis. Additionally, this compendium is to provide information on the extent to which Federal exposure standards on a substance decrease the risk to public health from exposure to that substance and how the Secretary or others responded to requests. Obviously, this effort requires considerable coordination, especially with NIOSH, on the latter information. These listings, comprising several hundred chemicals, are furnished to the National Toxicology Program for submission in an annual report to Congress.

Highlights

Work on the preparation of the PHS-149 report, "Survey of Compounds Which Have Been Tested for Carcinogenic Activity" was completed for the years 1976-77 in camera-ready copy for printing by GPO. Distribution will be made to over 750 scientists in the cancer research field in this country and abroad.

Last year reference was made to the development of data bases on carcinogens, mutagens, promoters and cocarcinogens. This compendium, in hard copy, was distributed to some NCI staff but steps have been taken to have this data base "on line" so that all agencies and interested scientists in this country can access it. Another important development was the preparation of the "Bioassay Summary Reports" which in abstract form, as a ready reference resource, provide information and data on approximately 224 entries for which NCI/NTP Technical Reports have been prepared and distributed. This data base is being incorporated into the data base referenced above so that "on line" access will be possible. Hard copies of this data base were distributed in limited numbers to NCI staff.

A new project was initiated with FASEB to evaluate the biological (in vitro, in vivo, epidemiological) data summarized in monographs on drugs and cosmetic ingredients prepared for this office by a contractor. The titles of these reports which have been completed during FY 1982 and 1983 are: "Genotoxic Assessment of Selected Drugs and Medical Procedures" and "Potential Carcinogenicity of Cosmetic Ingredients." These reports were prepared under a Basic Ordering Agreement, and FASEB will convene a panel of experts who will provide a summary evaluation of the data. In addition, FASEB will act as imprimatur for the quality and content of these monographs which will be published as a series of evaluated reports over the next two years. A New Master Agreement "Data Bank on Environmental Agents" was awarded in FY 1982 to three organizations: SRI, Int., TJI, Inc. and Syracuse Research Corporation.

Major contributions to the work of the Environmental Cancer and Heart and Lung Disease Task Force (lead agency EPA), mandated by the U.S. Congress, was made by representatives of this office. Specifically, this took the form of short chapters on metabolism, exposure and mechanisms of toxicology, all of which are part of the Annual Report to Congress.

An annual report was furnished to the National Toxicology Program by this office on chemicals specified for testing and research for submission to Congress in response to legislation enacted in 1978 under the Biomedical Research and Training Act (Maguire Amendment).

This office prepared a report on "Organic Air Pollutants, Carcinogens, Mutagens and Promoters" as part of a continuing series on the presence of environmental carcinogens in various environmental media. This effort resulted from collaboration with SRI International, supported under contract by this office.

An updated report was distributed on the organic contaminants in drinking water that were identified and classified as carcinogens, mutagens and promoters in drinking water from an evaluative study on 1565 contaminants. An additional report was made available on 15 inorganic chemicals in drinking water which were similarly evaluated for carcinogenicity.

Publications

Cameron, T. P., Lattuada, C. P., Kornreich, M. R. and Tarone, R. E.: Longevity and reproductive comparisons for male ACI and Sprague-Dawley rat aging colonies. Lab. Anim. Sci. 32: 495-499, 1982.

Helmes, C. T., Sigman, C. C., Kraybill, H. F. and Kelsey, M. I.: Evaluation and classification of the potential carcinogenicity of air pollutants. J. Environ. Sci. and Health A17(3): 321-389, 1982.

Kelsey, M. I.: In vitro effect of bile acids. In Autrup H. and Williams, G. M. (Eds.): Experimental Colon Carcinogenesis. CRC Press Inc., Boca Raton, FL, pp. 241-252, 1982.

Kraybill, H. F.: Multimedia exposure and concerns for a holistic approach toward assessment of risk for environmental carcinogens. J. Environ. Sci. and Health A17(4): 491-497, 1982.

Kraybill, H. F.: Assessment of human exposure and health risk to environmental contaminants in water with special reference to Cancer. In Clayson, D. B., Krewski, D. R. and Munro, I. E. (Eds.): Toxicological Risk Assessment. Boca Raton, FL, CRC Press, (In press).

Kraybill, H. F.: Assessment of human exposure and health risk to environmental contaminants in the atmosphere and water with special reference to cancer. J. Environ. Sci. and Health, (In press).

CONTRACT NARRATIVES

OFFICE OF THE SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

AMERICAN HEALTH FOUNDATION (NO1-CP-55705)

Title: Genotoxic Potential of Medical Service (Device) Material

Contractor's Project Director: Dr. Gary Williams

Project Officer (NCI): Dr. Morris I. Kelsey

Objectives: To provide specialized in vitro testing of monomeric and polymeric chemicals used in medical device materials including DNA repair, chromosomal damage and cytotoxicity in hepatic primary cultures (HPC).

Major Findings: Acrylonitrile was not genotoxic in primary hepatocytes (HPC) DNA Repair nor in Sister Chromatid Exchange (SEC) assay systems. A marginal effect of acrylonitrile was seen in SCE with Chinese Hamster Ovary (CHO) cells in culture. Both butadiene monoxide (BHO) and styrene gave positive responses in the HPC/DNA repair assay and BMO caused SCE with an epithelial rat liver cell line (ARL-18). All monomers appeared to cause cytotoxicity, whereas, polyacrylonitrile was negative in all assay systems. In general, however, these systems do not give reproducible results and may not be useful for routine testing at this time.

Significance to Biomedical Research and the Program of the Institute: Data on the genotoxicity of these compounds will constitute new important findings on the properties of such "leachable" compounds which are present in a wide variety of medical device materials. This information will expand the data base of our office on the occurrence of environmental carcinogens and will be important information to include in our other contract (NO1-CP-05633-01) concerning the carcinogenicity of drugs and medical procedures.

Proposed Course: This contract was completed during this reporting period.

Date Contract Initiated: September 29, 1981

Current Level of Funding: $99,037 (pass-through money from FDA)

ARTHUR D. LITTLE, INC. (NO1-CP-85677)

Title: Evaluation of the Transformation Assay Using C3H 10T-1/2 Cells for Use in Screening Chemicals for Carcinogenic Potential

Contractor's Project Director: Dr. Andrew Sivak

Project Officer (NCI): Dr. Thomas P. Cameron

Objectives: The objective of this project is to evaluate and determine the usefulness and reliability of this assay for inclusion in a battery of short-term assays.

Major Findings: It has been determined that intact rat hepatocytes will not function satisfactorily in this assay as a source of exogenous metabolic activation. In addition, it has been reaffirmed that cell synchronization is

an unnecessary and essentially unrewarding complication in this assay. Splitting the treated cultures, at confluence, a method developed by the parallel contractor, has also shown promise of transformation enhancement in this laboratory.

Significance to Biomedical Research and the Program of the Institute: This contract effort, checked by a parallel contract, will serve to sharply define the predictive value of this assay. In the event that this assay can be shown to reliably indicate the transformation potential of these coded compounds whose carcinogenic activity has been previously verified by long-term rodent bioassays, it will be a major step forward in substituting an inexpensive short-term test for the very expensive and time-consuming lifetime rodent studies heretofore the mainstay of our carcinogen detection efforts.

Proposed Course: This is the final year of the contract, and the remainder of the period will be devoted to testing coded samples, essentially in parallel with the other contractor, in order to confirm the most appropriate protocol. A final report will be rendered at the end of the contract and an effort made to integrate it with the report of the other contractor to produce a unified evaluation of the assay.

Date Contract Initiated: September 30, 1978

Current Annual Level: $233,023

CENTERS FOR DISEASE CONTROL (YO1-CP-60215)

Title: Human Health Consequences of Polybrominated Biphenyls (PBBs) Contamination of Farms in Michigan

Contractor's Project Director: Dr. Matthew Zack

Project Officer (NCI): Dr. Thomas P. Cameron

Objectives: As a consequence of the distribution of animal feeds contaminated with PBB, the inhabitants of at least 412 Michigan farms, as well as a wide surrounding belt of consumers, were accidentally exposed to this potentially threatening compound. This activity defines and recruits large cohorts of individuals (of several degrees of exposure) and will engage in a long-term follow-up primarily with regard to the incidence of cancer.

Major Findings: Maintenance of the cohort registry is now the primary function of the contract, with emphasis on following up critical events--illnesses, pregnancies, deaths. The comparative high exposure/low exposure clinical study of 390 subjects has been completed and the data are being analyzed. The two manuscripts submitted have been published. Five research areas (based on the exposed cohort) have been identified as suitable for grant support--one has been submitted and is in review at the present time, the other 4 are in various stages of preparation.

Significance to Biomedical Research and the Program of the Institute: This is a unique situation in which a large group of people had a known exposure to a potential carcinogen under sharply defined circumstances of geography and time.

Unfortunate subsequent rises in cancer incidence, if they occur, can thus be charted in this prospective study and correlated with modes and amount of exposure. Major scientific observations on this particular compound and structurally allied compounds can be developed.

Proposed Course: The contract will continue into its 7th year. Emphasis will be on cohort registry maintenance and the submission of complementary grant applications.

Date Contract Initiated: April 8, 1976

Current Annual Level: $165,000

ENVIRONMENTAL PROTECTION AGENCY (YO1-CP-80205) (Office of Toxic Substances and Office of Research and Development)

Title: Interagency Agreement for Performance of Collaborative Studies in Area of Environmental Cancer

Contractor's Project Director(s): Drs. William Farland (OTS) and Dr. Frode Ulvedal (ORD)

Project Officer (NCI): Dr. H. F. Kraybill

Objectives: Through a cooperative agreement, to conduct projects dealing with environmental cancer (experimental, epidemiological and information resources) which are of mutual interest to the National Cancer Institute and the Environmental Protection Agency. This program is responsive to requests from OMB and the U.S. Congress.

Major Findings: An example of the information resources aspect of this program is the annual publication of a comprehensive report on "Chemicals Found in Human Biological Media: A Data Base" which can be accessed by both Federal agencies and state health departments. Some elucidations on the role of environmental chemicals in cancer induction have resulted from our projects in the experimental and epidemiology areas. A project to study tumorigenesis in aquatic animals has shown that a small aquarium size fish (silversides) demonstrated a carcinogenic response (liver) to exposure to aflatoxin. In a series of epidemiological studies, it has been possible to identify certain chemical exposures with specific cancer sites and also by geographical regions not hitherto recognized as such.

Significance to Biomedical Research and the Program of the Institute: This collaborative program emphasizes and gives visibility to the importance of studying environmental chemicals in air, water, and diet, to determine their impact on human cancer. In addition to supporting the mission and program initiatives of the EPA in environmental health and regulation and control of environmental insults, the projects yield important data on environmental cancer which is critical to NCI's mission. This collaborative program includes studies on water carcinogens, UVB radiation, hexachlorobenzene, asbestos, and other atmospheric carcinogenic contaminants.

Proposed Course: Three-five years is the average duration for these projects. Suggestions for new areas are considered at the Workshops. While the EPA projects are slanted more to experimental studies, NCI projects are epidemiological,

experimental and information resources. To the extent possible, a balance in funding and initiation of projects between NCI and EPA is achieved.

Date Contract Initiated: June 22, 1978

Current Annual Level: $1,900,000 ($950,000 to NCI and $950,000 to EPA)

FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY (FASEB) (NO1-CP-31014-72)

Title: Expert Panel Review of Drugs and Cosmetics Monographs

Contractor's Project Officer: Dr. Kenneth Fisher

Project Officer (NCI): Dr. Morris I. Kelsey

Objectives: To provide an analysis of the in vitro and in vivo carcinogenicity data contained in 109 monographs on drugs and 64 cosmetic ingredients. Provide an evaluation and imprimatur which will be recognized by the national and international scientific communities.

Major Findings: This contract has just started and selection of the expert panel has been completed. No evaluations have been performed so far but plans for the administration of this project have been reviewed in depth with FASEB staff and the NCI project officer.

Significance to Biomedical Research and the Program of the Institute: The final report will be an authoritative review and evaluation of the biological data on drugs and cosmetic ingredients which will be published as a series of monographs on these compounds. These reports provide important resource information to this office as part of our mission in examining the occurrence and relevance of carcinogens in various environmental media including drugs and cosmetics. It is expected that these documents will be important resource information to scientists and administrators in the government and private sectors.

Proposed Course: Once the expert panel convenes they will review a total of 173 monographs over the next two years. However, evaluated monographs will be published as a series of volumes during this period of performance.

Date Contract Initiated: April 1, 1983

Current Level of Funding: $102,462

GULF COAST RESEARCH LABORATORY (NO1-CP-26008)

Title: Biochemical, Pharmacological, and Tumorigenic Studies on a Composite of Drinking Water Carcinogens and Mutagens Utilizing Aquatic Animals as a Bioassay Model

Contractor's Project Director: Dr. Robin Overstreet

Project Officer (NCI): Dr. H. F. Kraybill

Objectives: The project is directed to several goals: 1) To ascertain whether a spectrum of organic contaminants in drinking water (some of which have been characterized as carcinogens in reported rodent bioassays) will produce tumors in selected small fish species; 2) To establish whether the chemical(s) respond on a dose-dependent basis supported by biochemical/pharmacological data or evidence; and 3) To establish or reconfirm that aquatic animals are appropriate models for demonstration of tumor response similar to the rodent.

Major Findings: This project is about six months old and preliminary efforts have been focalized on setting up facilities, elimination of problems in adjustment of concentration of chemicals in flow through tanks, and modification of analytical and histological procedures.

Significance to Biomedical Research and the Program of the Institute: NCI has been associated with some epidemiological studies on the possible role of drinking water contaminants in cancer induction (bladder, etc.). However, some 25 studies conducted nationwide failed to demonstrate causality between such contaminants and cancer at various sites. To sharpen up this focus, one resorts to experimental studies to assess this environmental insult and the significance of water contaminants as a public health hazard. Regulatory agencies such as EPA are dependent on such experimental verification and revelations in ultimate support of standard setting on allowed levels of processed water contamination. Additionally, the work on fish will be useful to NCI in establishment of this model in experimental carcinogenesis research.

Proposed Course: This project, planned for four years, will endeavor to assess the carcinogenic response of 15 major contaminants in drinking water, both singly and in a mixture at doses that are multiples of the concentration in municipal drinking water. In the first phase an effort will be intensified on seven organic chemicals that are at the highest concentration. Biochemical and histological data will be obtained on control and test animals. Fish embryos and cultures will be resorted to in ancillary studies.

Date Contract Initiated: September 30, 1982

Current Annual Level: $137,000

MICROBIOLOGICAL ASSOCIATES (NO1-CP-85617)

Title: Evaluation of the Transformation Assay Using C3H 10T-1/2 Cells for Use in Screening Chemicals for Carcinogenic Potential

Contractor's Project Director: Dr. Leonard M. Schechtman

Project Officer (NCI): Dr. Thomas P. Cameron

Objectives: The objective of this project is to evaluate and determine the usefulness and reliability of this assay for inclusion in a battery of short-term assays. A secondary objective is the incorporation of an S-9 metabolic activation system as a routine protocol ingredient to detect transformation potential of chemicals, otherwise likely to be missed, which are not direct acting.

Major Findings: The testing of more coded compounds and the retesting of others has confirmed that the S-9 metabolic activation addition does add significantly to the sensitivity of this assay. Likewise, the splitting of the confluent exposed cultures (Phase II) does enhance the transformation to Type III foci and greatly increase the sensitivity of the compound--of 8 previously negative compounds, 5 were shown positive using this technique.

Significance to Biomedical Research and the Program of the Institute: This contract effort, checked by a parallel contract, will serve to sharply define the predictive value of this assay. In the event that this assay can be shown to reliably indicate the transformation potential of these coded compounds whose carcinogenic activity has been verified previously by long-term rodent bioassays, it will be a major step forward in substituting an inexpensive short-term test for the very expensive and time-consuming lifetime rodent studies heretofore the mainstay of our carcinogen detection efforts.

Proposed Course: This is the final year of the contract, and the remainder of the period will be devoted to testing coded samples, essentially in parallel with the other contractor, in order to confirm the most appropriate protocol. A final report will be rendered at the end of the contract and an effort made to integrate it with the report of the other contractor to produce a unified evaluation of the assay.

Date Contract Initiated: September 30, 1978

Current Annual Level: $241,134

MICROBIOLOGICAL ASSOCIATES (NO1-CP-15739)

Title: In Vitro Evaluation of Chemical Candidates for In Vivo Testing

Contractor's Project Director: Dr. Paul Kirby

Project Officer (NCI): Dr. Thomas P. Cameron

Objectives: The testing of chemical compounds in the mouse lymphoma in vitro assay provides valuable information that is utilized in the selection of candidates nominated for chronic in vivo testing by the National Toxicology Program. In addition, support is provided to intramural scientists who require mutagenicity information not otherwise available, as an adjunct to their own investigations.

Major Findings: As of the date of writing, 72 compounds have been reported upon and 35 compounds are in some stage of testing.

Significance to Biomedical Research and the Program of the Institute: This contract will serve to eliminate "gaps of knowledge" concerning the biological effects of specific chemicals. This will facilitate the accurate selection of candidates for bioassay and insure the optimal employment of that prolonged and extremely expensive test system. The same information supplied to intramural scientists will supplement their investigations in chemical carcinogenesis without diverting staff time and scarce laboratory space to setting up in vitro systems that would be used only intermittently.

Proposed Course: It is intended that this contract will be continued for its final year as candidate compounds are identified by a variety of mechanisms and organizations. The project officer has begun coordinating the writing of a series of publications comparing test results of this assay with those of a bacterial mutagenicity system.

Date Contract Initiated: March 20, 1981

Current Annual Level: $306,203

NATIONAL INSTITUTE FOR OCCUPATIONAL SAFETY AND HEALTH (Y01-CP-60505)

Title: Conduct of Research on Occupational Carcinogenesis

Contractor's Project Director: Dr. Kenneth Bridbord

Project Officer (NCI): Dr. Thomas P. Cameron

Objectives: This agreement was originated as a Congressionally mandated effort to integrate and thereby enhance the activities of both the National Cancer Institute and the National Institute for Occupational Safety and Health in investigating chemical-related occupational cancer.

Major Findings: To date 100 distinct projects have been initiated; 31 are still in progress--of which 14 are scheduled for completion in FY'83.

Significance to Biomedical Research and the Program of the Institute: The merging of the unique capabilities of each organization into a concerted investigation of a variety of industrial situations promises an acceleration of the response to the question of the quantification of occupationally caused cancer. In that quest, answers to the many questions concerning acute toxicity, safe ambient levels, etc., which are more germane to the NIOSH mission, will accrue. NIOSH contributes the capabilities of freely entering industrial operations, as well as considerable experience in surveying potentially dangerous situations therein.

Proposed Course: Ongoing projects were reported on and their continuation approved by an ad hoc review group of senior staff members of both Institutes. Two of the ongoing projects will be presented for approval to the September 1983 meeting of the Board of Scientific Counselors, DCCP, as will 10 new projects reviewed by the ad hoc review group. Funding for each Institute's initiatives is now at the $950,000 annual level.

Date Contract Initiated: September 23, 1976

Current Annual Level: $1,900,000

RESEARCH TRIANGLE INSTITUTE (N01-CP-15740)

Title: In Vitro Evaluation of Chemical Candidates for In Vivo Testing

Contractor's Project Director: Mr. Thomas Hughes

Project Officer (NCI): Dr. Thomas P. Cameron

Objectives: The testing of chemical compounds in the Salmonella/typhimurium in vitro assay provides valuable information which is utilized in the selection of candidates nominated for chronic in vivo testing by the National Toxicology Program. In addition, support is provided to intramural scientists who require mutagenicity information not otherwise available, as an adjunct to their own investigations.

Major Findings: As of the date of this writing, 100 compounds have been reported upon and 8 compounds are in some stage of testing.

Significance to Biomedical Research and the Program of the Institute: This contract will serve to eliminate "gaps of knowledge" concerning the biological effects of specific chemicals. This will facilitate the accurate selection of candidates for bioassay and insure the optimal employment of that prolonged and extremely expensive test system. The same information supplied to intramural scientists will supplement their investigations in chemical carcinogenesis without diverting staff time and scarce laboratory space to setting up in vitro systems that would be used only intermittently.

Proposed Course: It is intended that this contract will be continued for its next year as candidate compounds are identified by a variety of mechanisms and organizations. The project officer has begun coordinating the writing of a series of publications comparing test results of this assay with those of a mammalian cell mutagenicity system.

Date Contract Initiated: March 31, 1981

Current Annual Level: $103,999

SRI INTERNATIONAL (NO1-CP-95607)

Title: Resource to Support the Chemical, Economic and Biological Information Needs of NCI/DCCP and to Provide Chemical Process, Production and Economic Information to the International Agency for Research on Cancer (IARC).

Contractor's Project Director: Dr. C. Tucker Helmes

Project Officer (NCI) : Dr. H. F. Kraybill

Objectives: This project has four primary tasks. Task I is concerned with the acquisition of information and data to compose summary sheets and prepare class studies all requisite in NCI nomination through CSWG (Chemical Selection Working Group) of chemicals for testing in the NTP bioassay program. Task II is concerned with support of IARC through furnishing information and data essential in monograph preparation. Task III, or CCRIS (Chemical Carcinogenesis Research Information System) is the repository or data base developed to produce hard copies and "online" data relating to carcinogens, mutagens, cocarcinogens, promoters, metabolites, target sites, etc. for ready reference. Task IV refers to publication of class studies and special reports but primary task is research and development of special tasks or studies that will provide significant data on environmental carcinogens, mutagens, promoters, etc.

Major Findings: Through these four tasks elucidated above, the NCI/DCCP has developed several class studies for chemical selection; 15 or more summary sheets

on chemicals to be selected. Special reports have been developed on atmospheric carcinogens and water carcinogens; a listing of cocarcinogens and promoters in the environment. Class studies have been published on aryl sulfonamides, aromatic nitro compounds; metals and air pollutants. Under CCRIS (Chemical Carcinogenesis Research Information System) a resource has been developed on evaluative information and data on over 900 chemicals (3000 test results), on carcinogens, mutagens and cocarcinogens, or promoters. This compendium in hard copy is also "online" in retrievable systems such as PROPHET and CIS (Chemical Information System). In this data base is information on carcinogen metabolites, target sites for various chemicals and also bioassay summary reports for about 224 chemicals tested under NCI/NTP testing system. For the IARC, support information has been supplied on production, exposure, physical chemical properties on about 400 chemicals in four years. Under Task IV, about 5 special studies provide information on atmospheric and drinking water contaminants, carcinogen metabolites, metal containing drugs and carcinogenesis inhibitors (work is still continuing).

Proposed Course: The project is now in its fourth year and has one more year to go for the original five year commitment. We propose to recompete this contract for another 3 years after October 1984 to continue in the development of information and data relevant to the four tasks outlined above.

Significance to Biomedical Research and the Program of the Institute: While this information resource does not pretend to be an all inclusive resource, it is unique in that the information is not solely bibliographic but it is evaluative and hence is a valuable resource to scientists involved in carcinogenesis research. Major emphasis is placed on environmental chemicals which impact on man. These resources are ready references for NCI staff and since most of it is now online, especially in CIS, many Federal agencies, academia and industry can readily access this data.

Date Contract Initiated: September 30, 1979

Current Annual Level: $822,000

SRI, INTERNATIONAL (NO1-CP-26004-02)
(Part of Master Agreement NO1-CP-15811-72)

Title: Monographs on Organic Air Pollutants

Contractor's Project Director: Dr. T. Helmes

Project Officer (NCI): Dr. Morris I. Kelsey

Objectives: To prepare IARC-style monographs on selected organic air pollutants with emphasis on biological activity and environmental fate and occurrence of these compounds.

Major Findings: The contractor has selected approximately 70 compounds as subjects for individual or group monographs from an initial list of almost 800 compounds. These monographs contain IARC-style information with detailed discussions of environmental fate and occurrence.

Significance to Biomedical Research and the Program of the Institute: Results from this effort provide important detailed and new information on the role of air

pollutants as environmental carcinogens. This effort supplements the results summarized in tabular form in a previous report from this office entitled: "Organic Air Pollutants. Carcinogens, Mutagens and Promoters."

Proposed Course: The contractor has completed 14 monographs to date and the project is on schedule at this time. An outside review of the draft final report is planned in late summer, and the final report will be submitted in late September.

Date Contract Initiated: September 30, 1982

Current Level of Funding: $221,460

TECHNICAL RESOURCES, INC. (NO1-CP-15761)

Title: Survey of Compounds Which Have Been Tested for Carcinogenic Activity (PHS-149); Volumes for 1974-75, 1976-77, 1979-80

Contractor's Project Director: Mr. Anthony Lee

Project Officer (NCI): Dr. H. F. Kraybill

Objectives: This contract provides for the preparation of the PHS-149, "Survey of Compounds Which Have Been Tested for Carcinogenic Activity," for the years 1974-1977 and 1979-1980.

Major Findings: Publication of this survey was discontinued in 1973 and those missing years, except for 1978 which was accomplished in 1981, will be covered by this contract. A previous contract covered the literature for 1978. This survey of literature is done on a worldwide basis. At the time that the 1979-1980 Volume is published, a cumulative index will also be prepared and published, covering all entries in all previous volumes.

Significance to Biomedical Research and the Program of the Institute: This survey was first published in 1951 and has proved over the years to be an invaluable resource for scientists at NCI as well others involved in the field of carcinogenesis. The survey is a result of a search of approximately 450 journals, foreign and domestic, and the data on chemicals bioassayed are presented in a concise manner. Distrubtion is made from this office to approximately 750 scientists in this country and abroad.

Proposed Course: The second camera-ready PHS-149 report was made available in May 1983.

Date Contract Initiated: March 31, 1981

Current Annual Level: $268,220

TRACOR JITCO, INC. (NO1-CP-05633-02)

Title: Potential Carcinogenicity of Cosmetics Ingredients

Contractor's Project Director: Dr. Harold Seifried

Project Officer (NCI): Dr. Morris I. Kelsey

Objectives: This project provides resource information on the chemical and biological activity of cosmetic ingredients.

Major Findings: The contractor has prepared 52 IARC-style monographs on currently-used cosmetic ingredients that were approved by an outside steering committee and updated an additional 12 monographs prepared previously by SRI International (NO1-CP-05711).

Significance to Biomedical Research and the Program of the Institute: The bound report of monographs dealing with the potential carcinogenicity of cosmetic ingredients will be an important resource to the Office of Environmental Cancer in responding to inquiries from the Division, Institute, and other interested parties. Such information is crucial to the mission of our Office in gathering data on carcinogens present in various environmental media that result in multiple chemical insults to which humans are exposed.

Proposed course: This report along with the report on "Genotoxic Assessment of Drugs and Medical Procedures" (NO1-CP-05633-01) have been submitted to FASEB for review of the biological data by an expert panel. (see FASEB: Expert Panel Review of Monographs)

Date Contract Initiated: September 30, 1981

Current Level of Funding: $117,322

TRACOR JITCO, INC. (NO1-CP-26003-01)
(Part of Master Agreement NO1-CP-15811-72)

Title: Natural Toxicants in Foods

Contractor's Project Director: Dr. Harold Seifried

Project Officer (NCI): Dr. Morris I. Kelsey

Objectives: To provide a summary of in vitro and in vivo carcinogenicity on compounds occurring naturally in foods.

Major Findings: The contractor has summarized and evaluated the data on approximately 100 compounds which occur in foods naturally or enter the food chain inadvertently via contamination from other environmental media.

Significance to Biomedical Research and the Program of the Institute: Results from this project will provide important biological information relevant to the mission of our program which evaluates data on carcinogens present in various environmental media, including foods. This data may be used as the basis for more in-depth studies of quantitative levels of these agents consumed by humans.

Proposed Course: This contract was completed during this reporting period.

Date Contract Initiated: September 30, 1982

Current Level of Funding: $76,614

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP03509-20 OD

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Carcinogenesis, Chemotherapy and Biological Markers in Nonhuman Primates

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. M. Sieber Deputy Director, OD, DCCP, NCI

COOPERATING UNITS *(if any)*

Department of Pathology, Louisiana State University, New Orleans, LA; Hazleton Laboratories America, Inc., Vienna, VA

LAB/BRANCH

Division of Cancer Cause and Prevention

SECTION

Office of the Director

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Twenty-eight substances, including antitumor agents, contaminants of human food-stuffs, rodent carcinogens, pesticides, and artificial sweeteners have been or are being evaluated in four species of nonhuman primates for their potential carcinogenicity and other long-term toxic effects. Seventeen of these substances have not as yet demonstrated carcinogenic activity, although some have been on test for less than 4 years. Eight of the compounds are carcinogenic in nonhuman primates, producing tumors in 19-100% of the treated animals. 1-Methyl-1-nitrosourea induced squamous cell carcinomas of the oropharynx and esophagus, with the esophageal tumors possessing clinical and morphologic similarities to human esophageal carcinoma. Long-term treatment with procarbazine resulted in an increased incidence of malignancies, one-half of which were acute leukemia. The effects of 6 of the 8 compounds (DENA, DPNA, 1-nitrosopiperidine, aflatoxin B-1, MAM-acetate and urethane) were manifested primarily as hepatocarcinogenicity. Single cases of malignant tumors have been diagnosed in animals treated with adriamycin, butter yellow and sterigmatocystin.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

R. J. Parker	Visiting Associate	OD, DCCP, NCI
J. Whang-Peng	Head, Cyto. Oncology Section	MB, NCI

Objectives:

To obtain data on which to base a comparison of the response of nonhuman primates and rodents to materials known or suspected to be carcinogenic to man, and thereby to evaluate the relative merits of rodents and nonhuman primates in predicting carcinogenic risk for man.

To evaluate the carcinogenic potential and long-term adverse effects of clinically useful antineoplastic and immunosuppressive agents.

To develop model tumor systems in primates for evaluating the potential usefulness of new anticancer agents active against rodent tumors before these agents are administered to cancer patients.

To determine whether the nonhuman primate resembles the rodent in that it is more susceptible to chemical carcinogenesis as a neonate than as an adult.

To evaluate the possibility of preventing or reversing chemical carcinogenesis in nonhuman primates.

To use normal and tumor-bearing nonhuman primates for studying the pharmacological, toxicological, and chemotherapeutic properties of various anticancer, antiviral, and antimicrobial agents.

To carry out comparative biochemical and immunological studies using normal animals, animals with preneoplastic lesions, and animals with malignant neoplasms.

To develop methods for detecting preneoplastic changes and accomplishing the early diagnosis of tumors.

Methods Employed:

Compounds Under Investigation:

Twenty-seven substances are currently under investigation or have been investigated, including: antineoplastic and immunosuppressive agents (procarbazine, adriamycin, methylnitrosourea, melphalan, azathioprine and cyclophosphamide), food additives and environmental contaminants (aflatoxin B_1, methylazoxymethanol acetate; sterigmatocystin, cyclamate, saccharin, butter yellow, 3-methyl-DAB, DDT, arsenic and cigarette smoke condensate), "model" rodent carcinogens (urethane, 3-MC, 2-AAF, 2,7-AAF, copper chelate of N-OH-AAF, dibenzpyrene

and dibenzanthracene), and various nitroso- compounds (DMNA, DENA, DPNA, 1-nitrosopiperidine and MNNG).

The compounds are administered subcutaneously, intravenously, intraperitoneally, or orally. For oral administration to newborn monkeys, the compound is added to the Similac formula at the time of feeding; when the monkeys are six months old, carcinogens given orally are incorporated into a vitamin mixture which is given to monkeys as a vitamin sandwich on a half slice of bread. An alternate way of giving doses orally is to incorporate the compound into a prune or to intubate. The dose level chosen is dependent on the chemical under evaluation. Antineoplastic and immunosuppressive agents are administered at doses likely to be encountered in a clinical situation; other substances, such as environmental contaminants, are given at levels 10-40 fold higher than the estimated human exposure level. The remainder of the chemicals tested are administered at maximally tolerated doses which, on the basis of weight gain, blood chemistry and hematology findings, and clinical observations, appear to be devoid of acute toxicity.

Animals

The present colony, consisting of 525 animals, is comprised of four species: Macaca mulatta (rhesus), Macaca fasicularis (cynomolgus), Cercopithecus aethiops (African green) and Galago crassicaudatus (bushbabies). Seventy-nine of these monkeys are adult breeders which until recently supplied the newborns for experimental studies. The majority of the animals are housed in an isolated facility which contains only animals committed to this study, and with the exception of the breeding colony, most animals are housed in individual cages. The administration of test compounds was usually initiated within 24 hours of birth and is continued until a tumor is diagnosed or until a predetermined exposure period is completed. A minimum of 30 animals is usually allotted to each treatment group, since in a sample of this size it is possible to detect a tumor incidence of 10% within 95% confidence limits.

A variety of clinical, biochemical and hematological parameters are monitored weekly or monthly, not only to evaluate the general health status of each animal, but also for the early detection of tumors. Surgical procedures are performed under phenycyclidine hydrochloride, Ketamine or sodium pentobarbital anesthesia. All animals which die or are sacrificed are carefully necropsied and the tissues subjected to histopathologic examination.

Major Findings:

Carcinogenic Potential of Antineoplastic and Immunosuppressive Agents

Procarbazine

There is evidence that Hodgkin's disease patients receiving treatment with the MOPP regimen, one component of which is procarbazine, are at increased risk of developing AML. Although a causal relationship between cyotoxic drug therapy and the appearance of AML in these patients has not yet been established, there is ample evidence that procarbazine is a potent carcinogen in mice and rats. The carcinogenic potential of procarbazine in 3 species of nonhuman

primates has been under study for 17 years. A total of 48 monkeys have survived 6 months or longer after the first dose of drug. Fifteen of the 41 monkeys (31.2%) necropsied thus far have had malignant neoplasms, 8 of which were acute leukemia. The leukemias, all non-lymphocytic, arose in monkeys after latent periods ranging between 16 and 166 months (average 86.5 months). They developed in monkeys that had ingested an average cumulative procarbazine dose of 61.2 gm (range 2.7 to 170.4). Solid tumors were diagnosed in 7 monkeys on the probarbazine study. Three monkeys developed osteogenic sarcomas, 2 monkeys developed hemangiosarcomas, and single cases of lymphocytic lymphoma and astrocytoma were found. These tumors arose after latent periods of 68-192 months (average 111 months), and after an average cumulative procarbazine dose of 64.6 gm (range 23.8 to 154.4 gm). The 7 surviving monkeys in this study may represent a population at high risk for developing acute non-lymphocytic leukemia. For this reason, they are being utilized in hematologic and cytogenetic studies in order to determine whether a "pre-leukemic" phase is detectable prior to the development of frank drug-induced leukemia. For this purpose, sequential bone marrow samples from all of the monkeys receiving procarbazine are being examined for alterations in cellular morphology and for chromosomal aberrations. Since initiation of these studies, no specific chromosomal aberrations have been detected in bone marrow cells from the monkeys and no additional cases of acute leukemia have developed. However, a number of the toxic effects of procarbazine seen clinically are also noted in the monkeys, including vomiting and myelosuppression. Its most striking toxic effect, however, is on the reproductive system of the males. The majority of the adult males necropsied to date have had testicular atrophy with complete aplasia of the germinal epithelium.

Adriamycin

The potential carcinogenicity of adriamycin was also evaluated. A group of 10 monkeys received an iv dose of drug (12 mg/m^2) once each month for 23-27 months. At the end of the dosing period it was intended to hold the animals under observation for the remainder of their lives. However, approximately 2 months after the last dose of adriamycin 8 of 10 animals developed congestive heart failure. Histopathologic examination of cardiac muscle taken at necropsy revealed lesions characteristic of adriamycin-induced cardiomyopathy in humans, and in some cases these findings were confirmed by electron microscopy. In man, a cumulative adriamycin dose of 550 mg/m^2 has been associated with cardiac toxicity; nonhuman primates appear to be more sensitive to adriamycin-induced cardiomyopathy, as the monkeys in the present study had received an average cumulative dose of 310 mg/m^2 (range 276-336 mg/m^2). One of the 10 monkeys developed acute myeloblastic leukemia after receiving 324 mg/m^2 of adriamycin divided into 27 monthly doses. The 10th monkey in this series is alive and without evidence of illness. It received 25 injections of adriamycin totaling 300 mg/m^2, and received the last dose of adriamycin 62 months ago. This study is being repeated, using 2 groups of 10 monkeys each; the monkeys are receiving monthly iv injections of adriamycin at 2.4 and 4.8 mg/m^2; dosing will be terminated when a cumulative dose of 240 mg/m^2 is attained. Thus far, monkeys receiving adriamycin at 2.4 and 4.8 mg/m^2 have been given cumulative drug doses of 85.2 mg/m^2 and 115.2 mg/m^2, respectively. None of the monkeys have as yet developed signs of congestive heart failure or other indications of ill health.

N-methylnitrosourea (MNU)

This compound administered by iv injection has been reported by clinicians in the Soviet Union to be effective in Hodgkin's disease and undifferentiated carcinoma of the lung, and the nitrosoureas BCNU, CCNU and methyl-CCNU have been used in this country to treat a variety of human tumors. The latter 3 agents have been implicated in the development of second tumors in 2 patients receiving treatment for CNS neoplasms. Both patients were diagnosed with acute non-lymphocytic leukemia after receiving oral doses of nitrosoureas totalling approximately 1420 and 2700 mg/m^2, respectively. We have evaluated the carinogenic potenital of MNU in three species of nonhuman primates. A total of 44 monkeys have received oral doses of MNU for periods up to 195 months. Twelve of the 21 monkeys (57%) necropsied thus far have had squamous cell carcinoma (SCA) of the mouth, pharynx and/or esophagus; upper digestive tract lesions such as atrophy or dyskeratosis of the esophageal mucosa and esophagitis have been a consistent finding among the 21 monkeys necropsied to date. The tumors developed in monkeys ingesting cumulative MNU doses averaging 130 gm (range 53.2-246.4 gm) over an average of 111 months (range 57-168 months). A minimum of approximately 50 gm of MNU appears necessary for tumor induction by the oral route. Many parallels were noted between the esophageal SCA observed in the present series of monkeys and human esophageal carcinoma, including the clinical manifestations of the tumor, its complications, its radiographic appearance and its morphology. The MNU-induced lesions of the oropharynx and esophagus of primates may therefore be a valuable model for the study of human esophageal carcinoma.

Melphalan

There is some evidence that patients receiving long-term treatment with melphalan for multiple myeloma or ovarian cancer may also be at increased risk of developing AML. Although a causal relationship between melphalan treatment and the development of AML in these patients has not yet been established, the carcinogenic activity of this agent has been demonstrated in mice and rats. The carcinogenic potential of melphalan in nonhuman primates is therefore under study. Twenty monkeys are currently being treated by the oral route with melphalan (0.1 mg/kg) daily, 5 days a week. The average cumulative dose administered to these animals is 0.78 gm and the dosing interval averages 86 months. Women receiving prophylactic melphalan therapy for ovarian carcinoma would receive in the prescribed 18-month dosing period a total melphalan dose of 660 mg/m^2, a dose lower than that already ingested by these monkeys. None of the monkeys on this study have died and all appear to be healthy.

Azathioprine

Kidney transplant recipients and other patients under chronic immunosuppressive therapy with azathioprine appear to be at risk of developing malignancies, primarily lymphomas. Whether this increased risk is related to a direct oncogenic effect of azathioprine or is secondary to a prolonged immunosuppressed state is at present unclear. The carcinogenic potential of azathioprine is being evaluated in two groups of monkeys receiving the drug daily, 5 days every week at doses of 2 and 5 mg/kg, respectively. The 2 mg/kg group is

comprised of 14 animals that have thus far received an average cumulative azathioprine dose of 10.4 gm over the course of approximately 62 months. Fourteen animals are receiving azathioprine at 5 mg/kg, and have ingested an average cumulative dose of 15.0 gm over an average dosing interval of 41 months. None of the monkeys on this study have developed a malignancy and all appear to be healthy.

Cyclophosphamide

Cyclophosphamide is widely used as a single agent and in combination regimens for treating human cancer; it is also being used with increasing frequency for treating various non-malignant disorders such as rheumatoid arthritis. The association of transitional cell carcinomas of the urinary bladder and acute myelogenous leukemia with prolonged cyclophosphamide treatment of both malignant and non-malignant disease represents the basis for a recently initiated study on the carcinogenic potential of cyclophosphamide in nonhuman primates. Cyclophosphamide is being administered to a group of 20 monkeys orally, 5 days every week, beginning at 6-7 months of age. The initial dose (3 mg/kg) is increased to 6 mg/kg after 6 months. This study has been underway for an average of only 24 months; during this period an average cumulative cyclophosphamide dose of 6.78 gm has been administered. Three of the monkeys died during an outbreak of measles in the colony two years ago, but no evidence of malignancy was found upon histopathologic examination of their tissue.

Carcinogenic Potential of Food Additives and Environmental Contaminants

Aflatoxin B_1 (AFB_1)

AFB_1, a product of a mold (Aspergillus flavus) known to contaminate some human foodstuffs, is carcinogenic in a variety of experimental animals. The carcinogenicity of AFB_1 has been under evaluation in nonhuman primates for the past 16 years. A total of 39 Old World monkeys, chiefly rhesus and cynomolgus, has received AFB_1 by ip (0.125-0.25 mg/kg) and/or oral (0.1-0.8 mg/kg) routes for 6 months or longer, and 2 are currently alive. Twenty-one of the 37 monkeys necropsied to date developed a total of 28 malignant neoplasms, yielding an overall tumor incidence of 56.1%. Five of the 21 tumor-bearing monkeys developed hemangioendothelial sarcomas of the liver, 6 developed bile duct or gallbladder adenocarcinomas, and 2 cases of hepatocellular carcinoma were diagnosed. Two monkeys developed osteosarcomas and 6 were found at necropsy to have multiple primary tumors. All of the latter animals possessed adenocarcinoma of the pancreas and/or gallbladder or bile ducts as well as urinary bladder carcinoma, fibrosarcoma and osteosarcoma. The tumors diagnosed in the 21 monkeys developed after latent periods ranging from 49-186 months, and after cumulative AFB_1 doses of 99-1472 mg. Eleven of the 16 (69%) necropsied monkeys without tumor showed histologic evidence of liver damage, including toxic hepatitis, cirrhosis and hyperplastic liver nodules. Our results indicate that AFB_1 is a potent hepatotoxin and carcinogen in nonhuman primates and further support the hypothesis that humans exposed to this substance may be at risk of developing liver cancer.

Cycads (Cycad Meal, Cycasin and MAM-Acetate)

Cycasin, the active principle in the cycad nut, induces liver and kidney tumors in rats and may be a human carcinogen as well. The carcinogenic potential of cycasin and its aglycone, MAM-acetate, is under investigation in nonhuman primates. Old World monkeys (rhesus, cynomolgus, and African greens) received cycasin and/or MAM-acetate by po or ip routes for periods up to 12 years. Seventeen monkeys survived > 6 mo after initiation of treatment with cycasin (50-75 mg/kg) or MAM-acetate (1.5-3.0 mg/kg) given po daily 5 days/week, and 12 of the animals have been necropsied. Histopathologic examination of tissue from one of these monkeys revealed hepatocellular carcinoma. A second monkey was noted to have multiple tumors, including hepatocellular carcinoma, intrahepatic bile duct adenocarcinoma, renal carcinoma and adenomas, and adenomatous polyps of the colon. An adenocarcinoma of the pancreas was diagnosed in a third monkey. Although liver tumors were not observed in the other monkeys, all but one had hepatic lesions such as toxic hepatitis and cirrhosis. A group of 10 monkeys received MAM-acetate by weekly ip injections (3-10 mg/kg). Six of these animals developed tumors after receiving an average of 6.14 gm (range 3.58-9.66 gm) of MAM-acetate for an average of 75 mo (range 50-89 mo). Four of the monkeys developed hepatocellular carcinomas and 2 had multiple primary tumors including hepatocellular carcinomas, renal carcinomas, squamous cell carcinomas of the esophagus and adenocarcinomas of the small intestine. Our results show that MAM-acetate is a carcinogen in monkeys and add to the evidence that cycasin and its aglycone may be carcinogenic in man.

Sterigmatocystin

Sterigmatocystin has been under test for approximately 7 years. It is being administered po, 1 day/week at 1 mg/kg (15 monkeys) and 2 mg/kg (15 monkeys). Thus far, only one monkey in the 2-mg/kg group has been necropsied, and histopathologic examination of tissue from this animal revealed no evidence of tumor development, although severe toxic hepatitis with hyperplastic nodules was noted. One animal in the 1.0 mg/kg group has been necropsied, and histopathologic examination of its tissue revealed primary hepatocellular carcinoma. This animal had received a total of 1.13 gm of sterigmatocystin during the course of 74 months. Laparoscopic examinations of the livers of the remaining 28 monkeys are in progress.

Butter Yellow

A total of 7 animals survived 6 months or longer after receiving the initial oral dose of butter yellow, and all animals have been sacrificed at this time. One case with a highly invasive liposarcoma was found among the treated animals. Whether this tumor arose as a consequence of treatment with butter yellow or is a spontaneous tumor developing in an aged (20 year) animal is uncertain.

Cyclamate

Cyclamate has been under test for the past 12 1/2 years. Two groups of monkeys have received this compound orally, 5 days every week, at 100 and 500 mg/kg, respectively. The 100 mg/kg dose in monkeys corresponds, on an equivalent surface area basis, to a daily intake of 2.3 gm/day/70 kg man, and is equivalent

to drinking about 6 diet drinks per day. The 500 mg/kg dose in monkeys corresponds, on an equivalent surface area basis, to a daily intake of 11.6 gm/day/70 kg man, and is equivalent to drinking about 30 diet drinks per day. Two of 12 monkeys at the low dose, and 2 of 11 monkeys at the high dose have been necropsied, but no evidence of a malignant neoplasm was found.

Saccharin

Two groups of 10 monkeys each have been receiving oral doses of saccharin (25 mg/kg), 5 days every week. This dose corresponds, on an equivalent surface area basis, to a daily intake of 5 cans of diet soda by a 70 kg man. One group of monkeys has been receiving saccharin for an average of 138 months (range 137-140 months), and the second group of 10 monkeys began saccharin treatment approximately 5 years ago. Since the inception of the study, none of the monkeys have died, and there is no evidence of toxicity in any of the treated animals.

DDT

Similarly, long-term administration of DDT has not resulted in the development of tumors in our nonhuman primates. A total of 25 animals has received DDT by the oral route (20 mg/kg) daily, 5 days every week in a study that has been underway for the past 152 months. Administration of DDT is discontinued after a dosing interval of 130 months is completed. Although 7 of the monkeys have died thus far, none were found to have developed tumor. The apparent cause of death in 5 of these animals was DDT-induced CNS toxicity, as they experienced severe tremors and convulsions immediately prior to death. The 18 surviving monkeys appear to be in good health.

Arsenic

The carcinogenic potential of arsenic has been under evaluation for approximately eight years. A total of 20 monkeys has received sodium arsenate po (0.1 mg/kg) 5 days/week, and three monkeys in the group have died. The cause of death in the monkeys was unrelated to arsenic treatment, and the surviving monkeys are well and without signs of toxicity.

Cigarette Tobacco Smoke Condensate

Ten monkeys have received lung implants containing tobacco smoke condensate in a beeswax matrix; all are well and without evidence of toxicity approximately ten years after implantation of the material.

Carcinogenic Potential of "Model" Rodent Carcinogens

With the exception of urethane, none of the compounds in this category have demonstrated carcinogenic activity in nonhuman primates.

Urethane

Rhesus and cynomolgus monkeys received urethane (250 mg/kg) orally, 5 days every week beginning within one month of birth. They received continuous

urethane treatment for 5 years, during which time some monkeys also received 3-10 weekly courses of total body irradiation at 50 rads per course. Urethane administration was discontinued 13-16 years ago and since that time all animals have been held under close observation for development of tumor or other adverse effects of treatment. Thirty monkeys survived 6 months or longer after the first dose of urethane, and 23 of these animals have been necropsied. A total of 8 malignant tumors were found in 5 (16.7%) of the 30 treated monkeys; in comparison, 7 of a total of 219 (3.2%) control monkeys have developed tumors during this period. One or more primary liver tumors (3 cases of hemangiosarcomas, 1 case of adenocarcinoma of intrahepatic bile ducts, 1 case of hepatocellular carcinoma) were present in 4 monkeys; one of the monkeys with a liver hemangiosarcoma was also found to have an ependymoblastoma, and the fifth monkey developed a pulmonary adenocarcinoma. The animals with tumor had received an average cumulative urethane dose of 260 gm (range 230-339 gm); the latent period for tumor induction averaged 171 months (range 142-229 months). Two of the 5 monkeys developing tumors had received 9 and 10 courses of WBI, respectively. These results indicate that urethane, with or without WBI, is carcinogenic in monkeys; however, the latent period for tumor induction (> 14 years) is long, requiring approximately 50% of the usual lifespan of Old World monkeys in captivity.

Carcinogenic Potential of Nitroso- Compounds

Diethylnitrosamine (DENA)

DENA is highly predictable as a carcinogen in Old World monkeys, inducing hepatocellular carcinomas when given either orally or by ip injection. In one study, we are using DENA as a model hepatocarcinogen in Old World monkeys to examine the relationship between chronic (milligrams per kilogram) dose, cumulative dose, and latent period for tumor induction. To this end, groups of monkeys are being given bimonthly ip injections of DENA at doses of 0.1, 1, 5, 10, 20, and 40 mg/kg and are observed for the appearance of tumor. In the five groups of monkeys in which tumors have developed, we have found that the latent period increases as the milligram-per-kilogram dose decreases. Thus, the latent period at the 40 mg/kg dose averages 17 months, for the 20 mg/kg dose it is 26 months and at 10 mg/kg it is 38 months. A semilog plot of these three mg/kg doses of DENA against the latent periods for tumor induction yields a straight line which intercepts the y-axis at approximately 88 months. This point on the ordinate corresponds to a DENA dose of 0.1 mg/kg. Thus animals in the 0.1 mg/kg group should develop tumors after a latent period of 88 months if the relationship between the milligram-per-kilogram dose and latent period is strictly linear; however, the animals at this dose have only been on study approximately 25 months. Extrapolation from the curve to the ordinate for the 1 mg/kg group yields a latent period of 60 months, although 90% of the animals in this group remain tumor-free after an average of 111 months of observation. The tumors developing in the 9 animals receiving the 5 mg/kg dose required a latent period averaging 70 months, a figure which shows a marked deviation from the value (42 months) expected if the relationship between dose and latent period were linear. Our results thus far indicate that this relationship departs from linearity at lower chronic doses of DENA.

Although the dose rate has a significant effect on the length of the latent period, it does not appear to be of importance in determining the minimum cumulative DENA dose required for tumor induction. The averages for this figure range between 1.7 gm (40 mg/kg) and 2.3 gm (5 mg/kg), with cumulative DENA doses as low as 0.39 gm inducing tumors in individual animals. DENA is also carcinogenic in the more primative primate Galago crassicaudatus. All 10 treated animals have developed tumors after bimonthly ip injections of DENA at doses of 10-30 mg/kg. In contrast to the DENA-induced primary hepatocellular carcinomas in Old World monkeys, all 10 of the bushbabies developed mucoepidermoid carcinomas of the nasal cavity. In 2 of these 10 animals, carcinoma of the liver was also present, and in both cases metastases to the lungs or to intestinal lymph nodes was noted. The average total dose of DENA given the bushbabies was 0.747 gm, and ranged from 0.295-1.485 gm. It is considerably lower than that required to induce tumors in Old World monkeys and reflects the lower body weight of the bushbabies. The average latent period for tumor induction in this species (23 months) is comparable to that in Old World monkeys at the 20 mg/kg dose. No obvious reason exists for the marked difference in the site of DENA-induced tumors noted between Old World monkeys and the bushbabies. It may be related to differences in the metabolism or distribution of DENA, and this possibility will be investigated.

Dipropylnitrosamine (DPNA)

DPNA induced primary hepatocellular carcinomas in all 6 rhesus and cynomolgus monkeys given bimonthly ip doses of 40 mg/kg. The average total dose of DPNA was 7.0 gm; the average latent period for tumor development was 28.5 months.

1-Nitrosopiperidine (PIP)

This compound is also a hepatocarcinogen in macaques. Hepatic cell carcinomas developed in 11 of 12 monkeys receiving this compound by the oral route and in 5 out of 10 monkeys treated by the ip route. The average cumulative dose necessary for tumor induction by PIP given orally (1742.5 gm) was higher than for oral DENA (18.0-55.1 gm); similarly, the average cumulative dose of PIP given by the ip route (39.4 gm) exceeded that required for tumor induction by ip DENA (1.7 gm) or ip DPNA (7.0 gm).

N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG)

MNNG is being administered by the oral route (1.0 mg/kg, daily 5 days every week). A group of 21 monkeys has received this compound for periods of up to 8 years; thus far, 2 animals have died of causes unrelated to MNNG toxicity. The remaining 19 animals appear to be in good health and without signs of toxicity. However 3 additional monkeys were given MNNG as a colon implant; 2 of these monkeys have been necropsied and one monkey was diagnosed with a well-differentiated adenocarcinoma at the rectosigmoid junction. The latter monkey had received a total MNNG dose of 8.65 gm; it was administered in gelatin cubes containing 5.3-42.7 mg MNNG which were inserted into the colon twice every week.

Significance to Biomedical Research and the Program of the Institute:

The present colony has been in continued existence for 21 years; it supports the largest study of chemical carcinogenesis in nonhuman primates undertaken in this country, and as such it represents a national resource. In addition to providing data on the carcinogenicity of a variety of chemicals, including antitumor and immunosuppressive agents in clinical use, it has also made it possible for us to acquire information in other important areas of primatology. Such information includes the spontaneous tumor incidence in various species of nonhuman primates, their lifespan in captivity, their reproductive characteristics, the organization and management required to hand-rear 40-50 neonates per year in a nursery, and parameters of growth and development in simian primates. Normal animals of all ages as well as tumor-bearing animals are used in a variety of pharmacologic studies. The availability of monkeys receiving chronic treatment with chemicals has made it possible to identify some consequences of long term chemical exposures (other than tumor development) not previously recognized. Tumor-bearing monkeys have been employed in attempts to develop biological markers, such as α-fetoprotein, and other diagnostic tests for detecting premalignant lesions and early tumors. More recently, tumor-bearing monkeys have been used to develop new contrast media for computerized tomography of liver and spleen, and to compare the distribution of free and liposome-entrapped ara-C in tumor tissue and in adjacent normal tissue. Our accumulated experience with specific carcinogens has also enabled us to initiate studies on the prevention or reversal of chemically induced tumors. This project is of great significance to biomedical research in general and continues to serve well the objectives of the Program.

Proposed Course:

The studies described will be continued. Several studies on tumor promoters will be initiated, the first of which will involve the use of DENA as an initiator and DDT as a promoter.

Publications

Adamson, R. H. and Sieber, S. M.: Chemical carcinogenesis studies in nonhuman primates. In Langenbach, R., Nesnow, S. and Rice, J. M. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Publishing Corp., 1983, pp. 129-156.

Adamson, R. H. and Sieber, S. M.: Studies on the oncogenicity of procarbazine and other compounds in nonhuman primates. In Advances in Malignant Lymphomas: Etiology, Immunology, Pathology and Treatment. New York, Academic Press, 1982, Chapter 15, pp. 239-257.

CONTRACT IN SUPPORT OF THIS PROJECT

HAZLETON LABORATORIES AMERICA, INC. (NO1-CP-25601)

Title: Induction, Biological Markers and Therapy of Tumors in Primates

Current Annual Level: $561,288

Man Years: 9.2

Objectives: To maintain a breeding colony of various species of primates so that offspring are readily available for use in experimental studies. To make available normal and tumor-bearing animals for pharmacologic, toxicologic, biochemical and immunologic studies. To provide the facilities for maintaining and treating a large colony of nonhuman primates for studies of chemical carcinogenesis.

Major Contributions: This contract continues to satisfy the objectives of the project. Animals are bred, housed and dosed with test chemicals by the Contractor according to protocols specified by the Project Officer.

Proposed Course: Ongoing studies on chemical carcinogenesis in nonhuman primates will be continued and several new studies will be initiated during the coming year.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04493-05 OD

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Modifications of the Bioenergetic Pathways in Transformed Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

A. E. Kaplan, Research Chemist, OD, DCCP, NCI

COOPERATING UNITS *(if any)*

H. Amos, Harvard Medical School, Boston; D. Garland, NHLBI; R. Brown and L. Hoyer, Litton Bionetics, Inc.; Research Support Program, Program Resources, Inc.

LAB/BRANCH

Division of Cancer Cause and Prevention

SECTION

Office of the Director

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We seek to identify enzyme modifications which lead to the increased synthesis of lactic acid in neoplastic cells. Using a control and a transformed epithelial cell line, we identified the following modifications with neoplastic transformation: There are marked alterations in the kinetic properties of lactate dehydrogenase (LDH) which can account for the ten-fold increase in lactic acid produced in the transformed cells. This is accompanied by molecular modifications in the subtypes of LDH in the transformed cell. We are purifying LDH in order to determine the structural differences between subtypes from the two cell lines. Following exposure to alkaline phosphatase, molecular subtypes of LDH in the control cell are altered to give the appearance of the transformed type. Further investigation of this is under way with (serine-) protein kinase. The membrane transport properties of the transformed cell appear to be unaltered with respect to lactate, suggesting that the increased output of the acid in the transformed cell is due to the new kinetic properties of LDH. Since cells from the two lines have the same appearance by light microscopy, even though one produces carcinomas in vivo, we are examining the cytoskeletal proteins. Results show a normal alignment of tubulin in the transformed cell. The two cell lines do differ in ultrastructure, the transformed cell being very deficient in mitochondria. This may also serve to enhance the output of lactic acid through the incomplete oxidation of substrates. Thus both the glycolytic and mitochondrial system show modifications which can serve as points of attack in neoplastic cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

None

Objectives:

The altered bioenergetic metabolism observed in neoplastic cells of both clinical and experimental origin leads to an increased rate of formation of lactic acid. This represents an incomplete utilization of chemical energy from nutrients. Although the loss of available energy leads to cachexia and death in cancer patients, the underlying causes of this remain unclear even after almost sixty years of investigation.

The objective of this laboratory is to apply current biochemical and biophysical methods to develop an understanding of the molecular modifications in the transformed cell which result in the drain of chemical energy from the host organism to the tumor. We believe that a more precise understanding of the altered bioenergetic pathway in tumor cells will permit the development of new methods of preventing or suppressing the growth of tumor cells.

With this objective in mind, we developed three related areas of research to study bioenergetic modifications in neoplastic cells leading to elevated lactic acid production: (1) kinetic and molecular studies of the enzyme, lactate dehydrogenase (LDH); (2) synthesis and transport of lactic acid; and (3) morphological changes in cells. These studies are carried out with hepatocytes established from ten day old rat liver and a cell line transformed from them by exposure to nitrosomethylurea in vitro.

Methods Employed:

Physico-chemical methods employed in this study include stopped-flow kinetic measurements of LDH with parametric analyses of the data, electrophoretic separations of LDH subtypes, chromatography of LDH, including HPLC, a new method for direct measurements of intracellular pH in anchorage-dependent cells, fluorescence microscopy, scanning and transmission electron microscopy.

Major Findings:

Implementation of this project was delayed for several months due to the move from the NIH campus to the Frederick Cancer Research Facility. Some aspects of the work are still not in full operation.

We identified kinetic and molecular differences in LDH from control and transformed hepatocytes as well as NIL and NIL-PY fibroblasts. The latter cell was transformed with polyoma virus. Kinetic modifications in LDH support the 10-fold increase in lactic acid synthesis observed in the epithelial cells. In both transformed cells basic isoelectric forms of LDH increase. Treatment of LDH with alkaline phosphatase leads to a conversion of the control type of epithelial enzyme to the transformed type. The latter was unaffected by alkaline phosphatase. An additional control study was carried out with alkaline phosphatase and LDH-1 purified from rabbit red cells. LDH-1 was unaffected by alkaline phosphatase.

Preparatory to purifying LDH subtypes from control and transformed epithelial cells by HPLC, we found the protein content of the enzyme extracts extremely low relative to the enzyme activity. By column chromatography, 75% of the extraneous protein was eliminated from the enzyme preparation while retaining the stability of LDH. Preliminary studies for HPLC indicate that we will not be able to use reverse phase columns for further purification of the enzyme. While reverse phase columns are proving useful with a number of proteins, LDH appears to be too ionic to use this method.

The transport properties of the plasma membrane of the transformed cell appear unaltered with respect to the control cell. Thus the increased output of lactic acid appears to arise from the kinetic modification in LDH and not from membrane modifications.

Morphological differences between the two epithelial cell lines are only observed by electron microscopy because they look the same by light microscopy. In the transformed cells, a striking difference is seen in the diminished number of mitochondria as well as their fragmentary structure. This suggests that both the altered kinetics of LDH and the weakened function of oxidative phosphorylation foster imbalanced bioenergetic metabolism in transformed cells. Throughout however, the cell size and shape appear constant. For this reason, we have begun to study cytoskeletal structures with fluorescent antibodies. The first experiments indicate that the NMU-3 transformant has a normal alignment of tubulin.

Significance to Biomedical Research and the Program of the Institute:

The results suggest two possible sources of the increased synthesis and export of lactic acid in transformed cells. The first arises from the altered kinetic properties of LDH and related findings with respect to molecular modifications. These result in a 10-fold increase in lactic acid with transformation. The second is related to the observation of defective mitochondria in transformed cells which suggests that the normal pathway of oxidative phosphorylation is defective. No alterations in membrane physiology are apparent. The findings thus far suggest that the neoplastic cell may have several sites which are sensitive to inhibition with respect to its bionergetic system.

Proposed Course:

Purify LDH to separate subtypes and determine the amino acid sequence to see if the modifications in this enzyme are due to changes in the proportion of types synthesized or to the synthesis of new molecular types.

Continue studies to clarify the presence or absence of phosphate groups in LDH.

Extend cytoskeletal studies to see if all structures remain normal in this transformed cell line as compared to the altered properties of LDH in this cell.

New studies are planned to identify enzyme defects in the mitochondria of the transformed cell.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04548-11 OD

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Registry of Experimental Cancers/WHO Collab. Ctr. for Tumours of Lab Animals

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Harold L. Stewart Scientist Emeritus, OD, DCCP

COOPERATING UNITS *(if any)*

LAB/BRANCH

Office of the Director

SECTION

INSTITUTE AND LOCATION

NIH, NCI Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	2.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objectives of the Registry of Experimental Cancers are the storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents) and the use of such information for research and educational purposes. The Registry has acquired a total of 2,219 (90 since the 1982 report) single or group accessions from investigators outside the NCI. Approximately 56,285 records have been prepared for coding and coded. Thirty-nine investigators have come to the Registry for study and consultation on single or multiple visits. One foreign investigator was a guest at the Registry for one week.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Bernard Sass	Veterinary Medical Officer	DCCP	NCI
Margaret K. Deringer	Guest Researcher	DCCP	NCI
Cornelia Hoch-Ligeti	Guest Researcher	DCCP	NCI
Carel F. Hollander	Guest Researcher	DCCP	NCI
Annabel G. Liebelt (Beginning April 12, 1982)	Biologist-IPA Fellow	DCCP	NCI

Objectives:

1) The storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents), 2) the use of such pathologic material and data for research and educational purposes.

Methods Employed:

The methods employed in the work of the Registry involve the selection of protocols, pathologic material, including histologic slides, paraffin blocks, and illustrations in the form of lantern slides, gross photographs, and photomicrographs in black and white and in color. The work of the Registry also includes the collection of records of experiments, reprints and references on this material. The Registry of Experimental Cancers possesses a large collection of spontaneous and induced cancers and other lesions. The pertinent information on the collection is indexed. Many of the data have been prepared for and entered into the computer. The Registry accesses material from investigators at NCI, other institutes of NIH, other governmental agencies, industrial laboratories, and universities here and abroad. A total of 2,219 single or group accessions from investigators outside of NIH have been processed since 1971. The Registry has prepared and is preparing Study Sets of slides, with explanatory notes, relating to particular cancers of rodents.

The Registry has Study Sets of slides on "Comparative Pathology of Hematopoietic and Lymphoreticular Neoplasms," "Induced and Spontaneous Tumors of Small Intestine and Peritoneum in Mice," "Induced Tumors of the Liver in Rats," "Tumors and Nonneoplastic Lesions of the Lungs of Mice," "Mammary Tumors in Mice," "Pulmonary Metastases in Mice," "Neoplastic and Nonneoplastic Lesions of the Lymphoreticular Tissue in Mice," "Neoplasms and Other Lesions of Praomys (Mastomys) Natalensis," "Malignant Schwannomas of Rats," "Harderian Gland Tumors of Mice," and "Renal Tumors of Rats." These Study Sets, with descriptive material, are loaned to investigators who request them. Fifty-seven loans have been made this year.

Investigators come to the Registry for study and consultation. There have been single or multiple consultations with 39 individuals since the 1982 report. One foreign investigator has been a guest at the Registry for one week.

Major Findings:

The functions (outlined in objectives) of the Registry in the field of cancer research are such that there will be no major findings to report.

Significance to Biomedical Research and the Program of the Institute:

The availability of the wealth of material possessed by the Registry advances the knowledge of spontaneous and induced disease processes in animals. It is a national and international resource.

The existence of the Registry will contribute to the standarization of nomenclature of cancers and other lesions in laboratory rodents. Slides and protocols from the Registry are used to illustrate and describe lesions discussed at weekly slide conferences.

The members of the Registry serve as consultants in the monitoring of pathology from laboratories of NCI, of other institutes of NIH and elsewhere.

The Subcommittee on Rat Liver Tumors, appointed by the Institute of Laboratory Animal Resources, NRC, NAS, carried out its work at the Registry of Experimental Cancers where it met one or two days each month over a period of 18 months. The members of the Subcommittee studied the pathologic material in the Registry's Study Set on Rat Liver Tumors and other pathologic material accessed at the Registry. The publication of the report entitled "Histologic Typing of Liver Tumors of the Rat" appeared in January of 1980 (J. Natl. Cancer Inst. 64: 177-206, 1980). During the period from January, 1980 until April 30, 1983, the Registry has received 8,560 requests for reprints. This histologic classification and typing of rat liver tumors is calculated to promote uniformity of diagnoses from one laboratory to another in this country.

The Director General of the World Health Organization designated the Registry of Experimental Cancers as the WHO Collaborating Centre for Reference on Tumours of Laboratory Animals on 26 October 1976. This is the only such registry in the world to be so designated by the WHO. This will expand communications between scientists of this country and those of other countries, now numbering 153, which are members of WHO.

Proposed Course of the Project:

The Registry will continue and expand all of its activities (already set forth in this report).

Publications

Hoch-Ligeti, C., Congdon, C.C., Deringer, M.K., Strandberg, J.D., Sass, B., and Stewart, H. L.: Primary tumors and adenomatosis of the lung in untreated and irradiated guinea pigs. Toxicol. Pathol. 10: 1-11, 1982.

Sass, B., Vlahakis, G. and Heston, W.E.: Precursor lesions and pathogenesis of spontaneous mammary tumors in mice. Toxicol. Pathol. 10: 12-21, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP06134-08 OD

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Role of Lymphatic System in the Absorption and Distribution of Antitumor Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

S. M. Sieber — Deputy Director, OD, DCCP, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH: Division of Cancer Cause and Prevention

SECTION: Office of the Director

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of the lymphatic system in the absorption and distribution of free or liposome-entrapped antitumor agents, and monoclonal antibodies administered by iv, ip or sc routes in mice, rats and tumor-bearing guinea pigs and nonhuman primates is under investigation. Nonhuman primates bearing DENA-induced hepatocellular carcinomas have been used to compare the plasma clearance, tissue distribution (including uptake by tumor tissue), metabolism and excretion of free and liposome-entrapped cytosine arabinoside (ara-C). In mice, subcutaneous injection of radiolabeled monoclonal antibodies against normal cell types resulted in accumulation of radioactivity in regional lymph nodes to levels 600-fold higher than could be achieved by intravenous administration of the same antibody. Variables such as antibody dose, injection volume, injection site and concentration of carrier protein were studied in order to optimize lymph node uptake of antibody. Early lymph node metastases in guinea pigs of a dermal hepatocarcinoma that could not be imaged scintigraphically with a radiolabeled tumor-specific antibody injected intravenously were, however, clearly demonstrated after interstital injection of the same antibody. The lymphatic absorption and distribution of radionuclides, toxins or chemotherapeutic agents attached to antibodies and adminstered by iv and sc routes is also under investigation. The therapeutic effects of antibody attached to I-131 are being evaluated in hepatocarcinoma-bearing guinea pigs. Conjugation of antibodies to liposomes increased their lymph node uptake by 3-fold in mice given the liposome-antibody conjugates by sc injection.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

R. J. Parker	Visiting Associate	OD, NCI
J. N. Weinstein	Senior Investigator	LMB, NCI

Objectives:

The objective of this project is to obtain information on the role of the lymphatic system in the absorption and distribution of clinically useful anti-tumor agents and other materials such as monoclonal antibodies following their administration by subcutaneous or intravenous routes. The development of methods for producing a preferential and selective uptake of therapeutic or diagnostic agents by lymphatic vessels and lymph nodes is a major goal in this work. Efforts are currently focused on evaluating the use of monoclonal antibodies and/or liposomal encapsulation of drugs as a means of attaining this goal. The studies are designed to determine the relative rate and extent to which free and liposome-entrapped antitumor agents as well as specific and non-specific monoclonal antibodies are removed from the interstitial space by lymphatic versus blood routes.

Major Findings:

Nonhuman primates bearing DENA-induced hepatocellulaar carcinomas have been used to compare the plasma clearance, tissue distribution (including uptake by tumor tissue), metabolism and excretion of free and liposome-entrapped ara-C. Animals were dosed iv with free [^{3}H]Ara-C or [^{3}H]Ara-C entrapped in liposomes composed of phosphatidyl choline and labeled with [^{14}]cholesteryl oleate. At 2 hr after dosing, animals were sacrificed and various tissues removed and counted for radioactivity. In addition, plasma and urine were analyzed for unchanged Ara-C and its metabolite Ara-U by HPLC. Clearance of Ara-C equivalents from the plasma was found to be slower for liposome-entrapped Ara-C than for the free drug. The rate of metabolic breakdown of Ara-C, as indicated by the ratio of Ara-C to Ara-U in plasma, was found to be slower in the monkeys receiving the liposome-entrapped drug than in animals given free Ara-C. Urinary excretion of Ara-C and its metabolite was significantly reduced in animals receiving liposome-entrapped Ara-C (27%) as compared to monkeys given the free drug (40%). Liposome-entrapment also markedly altered the tissue distribution of Ara-C. Tissue concentrations of Ara-C equivalents were higher by 50- and 80-fold respectively in the spleen and liver of animals receiving the liposome-entrapped drug than in corresponding tissues of monkeys given free Ara-C. Tumor concentrations of Ara-C equivalents were significantly higher by 2-fold in animals treated with the liposome-entrapped drug as compared to monkeys receiving the free drug. This finding indicates that a modest increase in the concentration of an antitumor agent in liver tumors can be obtained by entrapping the agent in liposomes. In animals treated with free [^{3}H]Ara-C, tritium levels in liver tumors and surrounding normal liver tissues were essentially the same. In contrast, in monkeys given liposome entrapped Ara-C, levels of both [^{3}H] and [^{14}C] activity in tumor tissue were 5-fold lower than in surrounding normal

tissue. The possibility of exploiting the differential uptake of liposomes between tumor and surrounding normal tissue to detect tumors of the liver and spleen using CT scanning in conjunction with liposome-entrapped radioopaque material is being investigated.

Monoclonal antibodies against normal cell types were studied initially in order to describe the pharmacokinetic parameters which govern their lymphatic absorption, accumulation in lymph nodes and tissue distribution. A monoclonal antibody specific for the mouse histocompatibility antigen H-2K^{k} injected sc in H-2K^{k} positive (k^{k}-pos) mice was found to accumulate in regional lymph nodes to concentrations 50-fold higher than in K^{k} negative (K^{k}-neg) mice and 600-fold higher than when given by intravenous injection to either K^{k}-pos or K^{k}-neg mice. Similar results were obtained with a monoclonal antibody specific for the mouse histocompatibility antigen H-2D$^{b/d}$. In K^{k}-pos mice, regional lymph nodes draining the injection site could be visualized by gamma camera imaging as early as 30 min after sc injection of the antibody and remained visible up to 24 hr after dosing. However, in K^{k}-neg mice given the antibody sc and in both K^{k}-pos and K^{k}-neg mice receiving the antibody iv, regional lymph nodes were not visualized at any time up to 72 hr after dosing.

Various parameters likely to affect lymphatic uptake of antibodies given sc were studied including dose of antibody, site of injection, concentration of carrier protein, injection volume and the in vitro binding characteristics of the antibody in order to optimize lymph node accumulation of antibody. The dose of antibody injected and its in vitro binding characteristics were found to be the most important determinants of its uptake into lymph nodes. Uptake of anti-K^{k} by regional lymph nodes in K^{k}-pos mice increased linearly as the dose of antibody was increased from 0.1 to 0.3 µg. In contrast, the concentration of antibody in most other tissues did not increase over this dose range. While higher antibody doses of 0.5 to 3.0 µg gave no further increase in lymph node uptake of antibody, the concentration of antibody in blood, liver and several other tissues rose rapidly over this higher dose range. Measurement of in vitro binding characteristics showed that both the association and dissociation rate constants for anti-D$^{b/d}$ were faster than for anti-K^{k}. It was predicted from this finding that accumulation of anti-D$^{b/d}$ in lymph nodes would be less than for anti-K^{k}. This prediction was bourne out when anti-D$^{b/d}$ gave only a 4-fold advantage in nodal uptake for the D$^{b/d}$-pos strain over the D$^{b/d}$-neg as compared with a 50-fold advantage found in the corresponding study with anti-K^{k}. Similar studies are currently underway with monoclonal antibodies against antigens on lymphocytes (Lyt 2.2) and T-cells (Thy 1.2) since they may provide better models for antibody targeting to lymph nodes than do antibodies against histocompatibility antigens. Methods have been developed to distinguish intact radiolabeled IgG from free radioiodine in lymph nodes of animals receiving radiolabeled antibodies. SDS/PAGE and HPLC analysis of node digests indicate that most (70 - 90%) of the radioactivity in nodes is present as intact radiolabeled IgG.

In addition to antibodies against normal cell types, we studied the lymphatic delivery following sc injection of an radiolabeled antibody (D3) to lymph node metastases of a hepatocarcinoma implanted in guinea pigs. Gamma camera imaging, organ dissection and autoradiography combined to demonstrate highly specific antibody localization in early lymph node metastases. Co-injection of D3 with a non-specific antibody (MOPC-21) sc showed a ratio of specific to

non-specific antibody uptake of 100:1 in lymph node metastases. Detection of early lymph node metastases in guinea pigs injected sc with D3 by gamma camera imaging was possible at a time when tumor occupied only 3 - 6 % of the total weight of the node as determined by morphometry. Further improvement in imaging resolution of early lymph node metastases using radiolabeled D3 are currently being investigated by giving a second injection of a radiolabeled antibody to D3. Therapy of guinea pig lymph node metastases in animals with surgically excised primary tumors is under investigation using D3 labeled with I-131 and other radionuclides.

Studies aimed at increasing lymph node uptake of liposome entrapped antitumor agents by conjugation of various antibodies with the liposome membrane are now in progress. Initial experiments with liposomes conjugated to anti-K^K showed a 3-fold increase in lymph node uptake when compared with liposomes not conjugated to antibody in K^K-pos mice injected sc.

Significance to Biomedical Research and the Program of the Institute:

Monoclonal antibody conjugation and liposomal encapsulation are potentially useful delivery systems for therapeutic and diagnostic agents; this project represents an attempt to improve the selectivity of therapeutic agents by targeting them, through monoclonal antibodies or liposomal encapsulation, to specific sites. Tumor metastasis is a major clinical problem in treating cancer and presents a diagnostic challenge as well. Lymphatic channels are frequently the route by which tumors metastasize, with micrometastases lodging in regional and even distant lymph nodes. It would therefore be of potential therapeutic and diagnostic advantage in early metastases to selectively concentrate agents in lymph channels and lymph nodes. The results of our studies suggest that targeting with monoclonal antibodies or liposomal encapsulation are feasible methods for directing antitumor agents to these sites.

Proposed Course:

To continue to pursue the goals listed under "Objectives" above. Particular emphasis will be given to optimize the uptake and accumulation of antibodies in tumor cells metastatic to regional lymph nodes after interstitial injection of the antibodies. In addition, attempts will be made to further characterize the kinetics and distribution of monoclonal antibodies in normal and tumor-bearing animals.

Publications:

Flessner, M. F., Parker, R. J. and Sieber, S. M.: Peritoneal lymphatic uptake of fibrinogen and erythrocytes in the rat. Am. J. Physiol. 244: H89-H96, 1983.

Khato, J., Chirigos, M. and Sieber, S. M.: Antimetastatic effects of maleic anhydride-divinyl ether in rats with mammary adenocarcinoma. J. Immunopharmacol. (In Press) 1983.

Khato, J., del Campo, A. A. and Sieber, S. M.: Carrier activity of sonicated small liposomes containing melphalan to regional lymph nodes of rats. Pharmacology 26: 230-240, 1983.

Litterst, C. L., Sieber, S. M., Copley, M. and Parker, R. J.: Toxicity of free and liposome-encapsulated adriamycin following large volume, short term intraperitoneal exposure in the rat. Toxicol. Appl. Pharmacol. 64: 517-528, 1982.

Parker, R. J., Adamson, R. H., Douros, J. D. and Sieber, S. M.: Comparative pharmacologic studies of actinomycin D (NSC #3053) and pip-1β actinomycin (NSC #107660). Cancer Treat. Rep. (In Press) 1983.

Parker, R. J., Priester, E. A. and Sieber, S. M.: Effect of route of administration and liposome entrapment on the metabolism and disposition of adriamycin in the rat. Drug Metab. Dispos. 10: 499-504, 1982.

Weinstein, J. N., Parker, R. J., Keenan, A. M., Dower, S. K., Morse, H. C. and Sieber, S. M.: Monoclonal antibodies in the lymphatics: toward the diagnosis and therapy of tumor metastases. Science 218: 1334-1337, 1982.

CARCINOGENESIS INTRAMURAL PROGRAM

ANNUAL REPORT OF

THE LABORATORY OF BIOLOGY

NATIONAL CANCER INSTITUTE

OCTOBER 1, 1982 through SEPTEMBER 30, 1983

The goal of the Laboratory of Biology is to elucidate cellular alterations occurring during carcinogenesis in order to identify the series of steps that lead to malignancy. The primary objective is to determine the crucial molecular and physiological changes that occur in cells, which have been treated with chemical or physical agents, as they transform from the normal to the neoplastic state. Coordinated biochemical and biological studies are used (1) to characterize cellular alterations associated with carcinogenesis, (2) to evaluate relationships between DNA metabolism and carcinogenesis, (3) to determine effects of physiological host factors on carcinogenesis, and (4) to develop new in vitro cellular transformation systems which are pertinent for the study of the molecular mechanisms of carcinogenesis.

The research program investigates the causes of transformation and identifies and characterizes both exogenous and endogenous factors involved in the enhancement and inhibition of carcinogenesis. The Laboratory uses biological preparations, cells from animals and humans, and a variety of intact mammals. In the Laboratory's coordinated program, the Somatic Cell Genetics Section emphasizes heritable changes and the Tumor Biology Section is concerned with the physiological and immunological interactions during carcinogenesis. The primary emphasis is the study of target cells in vitro to understand the molecular changes (genetics and epigenetics) occurring during carcinogenesis.

The process of carcinogenesis is being studied in in vitro systems to ascertain how cells respond to the various stimuli that are associated with the process of transformation. As a result of carcinogen treatment, human cells can acquire a number of attributes that classically have been considered properties of experimentally transformed cells. For example, colony growth in agar after carcinogen treatment can occur in a dose-dependent manner. Nevertheless, it must be appreciated that carcinogen treatment of normal human cells in vitro does not ordinarily result in the full expression of malignancy. For example, although the transformed cells have an extended life span, they usually do not have the indefinite life span characteristic of rodent cell transformation. The inability to obtain full expression of malignancy even though human cells are capable of metabolizing a chemical carcinogen in a fashion identical to that of rodent cells, raises serious questions concerning the differences responsible for the difficulties in transforming human cells relative to animal cells in vitro. Cultured human fibroblasts differ from the commonly used animal cells in that spontaneous transformation does not occur; after a fixed number of cell doublings, the cells exhibit senescence, and cell division ceases. The transformed human cells also differ karyologically from experimentally transformed cells such as hamster cells. Whereas the chromosome constitution of cells of both species is relatively stable, hamster cells after transformation are frequently associated with a variety of chromosome changes. The experimentally transformed human

cells, however, differ in that the chromosome rearrangements, even when sophisticated G band techniques are used exhibit only minor chromosome rearrangements, in contrast to the major rearrangements in spontaneous human sarcomas which have been characterized by this laboratory. Although no specific chromosome rearrangement was associated with the surgical human sarcomas examined, they all exhibited a variety of new chromosomes. The occurrence of new chromosomes is consistent with the long-term course associated with the development of human cancers and suggest that the regulation of suppressors by human cells may differ from that of the few animal species that have been found to be readily transformable. As a consequence, a considered effort is being made to force the evolution of genomic changes with the hope that it will be possible to channel the gene alterations in the direction leading to the permanent production of gene products essential to tumor progression.

Other studies of this laboratory have provided conclusive evidence concerning the importance of DNA metabolism to the process of carcinogenesis of both human and animal cells. A comparison of the effects of different methylating agents indicates that the transformation frequency in HEC correlates with the level of O^6- and not N^7-methylguanine. HEC have a limited capacity to remove the former. This is consistent with the hypothesis that O^6-guanine is the critical site for in vivo carcinogenesis resulting from methylating carcinogens. The effects of MNNG and MMS at concentrations that induce equivalent numbers of O^6-methylguanine per genome on the size of both parental and nascent daughter DNA as well as the rate of DNA replication were studied to determine how this lesion might affect DNA metabolism and thus contribute to the initiation of carcinogenesis. Because no change occurred in the size of either parental or daughter DNA due to O^6-methylguanine and because the inhibition rate of DNA replication did not correlate with O^6-methylguanine levels, the initiation of carcinogenesis by O^6-methylguanine is probably not mediated directly by effects of the rate of DNA replication but is more likely due to the miscoding properties of this lesion.

From these studies it is evident that understanding the nature of carcinogen-induced changes in DNA sequences is critical to understanding the molecular basis of the carcinogenesis process. Recent progress in recombinant DNA research has demonstrated that the DNA sequences from human tumor cells responsible for the transformed phenotype can be isolated. This approach, however, has not elucidated the mechanism(s) responsible for the formation of these sequences. The ability to isolate and characterize transformed human, guinea pig, and hamster cells obtained as a result of chemical or physical carcinogen insult represents an ideal opportunity for determining how the sequences responsible for the origin of cancer develop during carcinogenesis.

The Laboratory of Biology resources are unique because its repository contains preserved cells that have been characterized for tumor producing ability as well as other neoplastic characteristics. Thus far, a number of transformed cell lines have been isolated from foci derived from 3T3 cells transfected with DNA from hamster and guinea pig tumorigenic cell lines. When the 3T3 DNA obtained after transfection with hamster DNA was probed with known retroviral oncogene sequences an additional sequence homologous to the Kirsten ras gene was found. Subsequently, this DNA was used to create a library to isolate the specific DNA fragment homologus to the Kirsten ras. This purified DNA sequence will be utilized to further analyze its role as a transforming sequence in an original carcinogen transformed line. The guinea pig model presents unequivocal distinct

preneoplastic stages of carcinogenesis that can be identified and isolated. Definitively transformed stages that produce tumors contain DNA sequences capable of transforming 3T3 cells. On the other hand, an anchorage independent colony that is negative in all tumorigenicity assays is also negative as a donor in 3T3 transfection assays. Studies are underway with guinea pig cell strains that exhibit only some preneoplastic stages; with such a sequential study it will be possible to determine at what stage oncogenic DNA sequences are formed.

Because of the concern associated with the transformation of NIH 3T3 cells by transfection with DNA from various sources, the use of human cells for DNA transfection experiments and characterization of DNA sequences responsible for maintenance of the neoplastic stage has been investigated. Successful transfection experiments have been performed using non-malignant, diploid fibroblasts derived from a skin biopsy of a patient with Bloom's syndrome. DNA has been extracted from 3T3 cells that contain a single copy of the Harvey murine sarcoma virus (HA-MuSV) genome and that do not produce virus. As a result of transfection, a number of transformed foci have been found that exhibit characteristics of malignant cells. When the DNA extracted from one of these was digested with Bam H1 and analyzed by the Southern blot protocol, evidence for DNA sequences from the Ha-MuSV as well as a sequence typical of the parental human DNA was obtained. Thus, the transformed phenotype occurred after incorporation of a transfected Ha-MuSV sequence had occurred. These experiments demonstrate that transformation by transfection with DNA from tumor cells can occur in non-malignant tumor cells. These cells can be used to study the role of DNA transforming sequences in human malignancy.

The susceptibility of normal hamster cells to transformation by environmental agents has made it possible to determine the carcinogens' action and the cells response. For example, a number of inorganic metals have been shown to induce transformation of HEC and to enhance the transformation associated with Simian adenovirus-7. The results with arsenic, for example, represent the first reproducible experimental data that supports the epidemiologic conclusions concerning arsenic. The data indicate that the co-carcinogenic effect of inorganic metals and of virus is caused by the metals rendering the cells more susceptible to viral transformation by presumably increasing the amount of viral genome incorporation into cellular DNA. The increased integration of virus as a result of pretreatment by chemical is responsible for the higher transformation efficiency. Biochemical as well as cytologic detection of virus-specific DNA indicates that in the final analysis, the transformed cells have the same amount of viral DNA, on the average of one genome per cell, independent of whether they were transformed formed by combination of chemical and virus or chemical alone. Thus, it is concluded cluded that the chemical acts upon the individual cells to make them more sensitive to viral transformation. A series of related chloroalkenes was tested for transforming ability of HEC because the parental compounds are carcinogenic in animals and because vinyl chloride caused malignant transformation only after exposure of the pregnant hamsters to the compound but not by direct treatment of the cells. All six epoxides tested were active with the potency of the various compounds being influenced by the difference in stability of the compounds. The data suggest that these compounds are potential carcinogens and that if the epoxides are metabolic intermediates of the parental chloropropanes, the epoxides are probably proximate carcinogens. Bisulfite was studied because it is a nonmutagenic agent which induces transformation. This compound does not induce any excision, post-replication repair responses, sister chromatid exchanges (SCE) or chromosome

aberrations. The only immediate effect of bisulfite is to induce a dose-dependent decrease in the rate of DNA replication per cell. The transformed lines that have been derived as a result of bisulfite treatment have been examined using high resolution banding of prometaphase and prophase chromosomes. With this sophisticated approach, small interstitial deletions of chromosomes one and six have been detected. These permanent alterations are considered to be the result of secondary steps in the neoplastic development.

Lastly, a series of nitrated polycyclic aromatic hydrocarbons were examined because the nitrated derivitives are potent bacterial mutagens and many of the parental compounds such as fluoranthene and pyrene are non-carcinogens. This class of polycyclic hydrocarbons is of increasing interest because a number of them are found in cigarette smoke, diesel exhaust, photocopier fluids, etc. The transforming potential of the nitro derivatives varied from compound to compound. None was as active as benzo(a)pyrene; however, 1,8-dinitropyrene, the most active compound tested, was one order of magnitude less effective than benzo(a)pyrene. Because of the dose-dependent transformation frequencies and the ubiquitous distribution of these nitrated compounds, these materials probably have deleterious effects on human health. In general, these compounds induced frame shift mutations and are active in reductase-deficient bacteria. Because the nitrated compounds are able to stimulate DNA repair synthesis in cultured human cells, there is an implication of a possibility of a reductase activity occurring in mammalian cells to account for their transforming ability.

Carcinogen-DNA interactions may also result in damage visible on metaphase chromosomes as structural aberrations or SCE. These two forms of DNA damage are considered independent cellular responses and their significance to the neoplastic transformation process may differ. The results of a split dose protocol indicate that SCE induction is dependent of the type and persistence of DNA lesion. The SCE frequency may be either increased or decreased depending upon the growth conditions and/or type of carcinogenic insult suggesting that a relationship exists between SCE and unrepaired DNA damage which in turn may influence the induction of transformation. For example, a split dose of N-acetoxyfluorenylacetamide given within 12 hours caused a significantly higher transformation frequency than that caused by one treatment at twice the concentration. Ultraviolet and N-methyl-N'-nitro-N-nitrosoguanidine caused significantly lower transformation frequencies as split doses than did a single treatment of either carcinogen. X-irradiation which is a very poor carcinogen in vitro does not significantly increase the SCE frequency when given either as a split dose of radiation or as a single radiation dose. The X-irradiation SCE results paralleled the transformation results; only rare transformations were noted in either instance. To further determine the persistence of SCE, cells were exposed to a carcinogen followed by 5-bromodeoxyuridine (BrdUrd) at different intervals and after two rounds of replication the SCE's were determined on metaphase chromosomes. The carcinogen-induced DNA damage leading to sister chromatid exchanges can persist or be partially or completely removed. The induction and persistence of SCE after exposure to a variety of chemicals and ultraviolet irradiation did not correlate with transformation induction. For example, methyl methanesulfonate induced lesions leading to SCE were removed more rapidly than any other carcinogen tested. It was also the least effective transforming agent. On the other hand, compounds in which the lesions were partially or completely removed as reflected by the SCE gave approximately the same frequency of transformation. The studies mentioned have led to a study responsible for SCE induction. The

diversity of compounds that induced SCE has made it difficult to determine the underlying mechanisms for their induction. A partial explanation may involve protein synthesis because amino acid deficient media results in an increase in SCE; but because cycloheximide or chloranphenicol do not influence the SCE frequency, protein synthesis alone cannot be responsible for the amino acid deprivation effect. The increase in SCE caused by amino acid deprivation can be suppressed by deoxycytidine. Furthermore amino acid deprivation did not induce SCE in a mutant 3T6 mouse cell line deficient in deoxycytidine deaminase showing the importance of deoxycytidine metabolism in SCE formation.

Alteration of HEC surface by lymphotoxin causes a persistent resistance to neoplastic transformation induced by carcinogens. Effectiveness of lymphotoxin prior to carcinogen treatment is not due to inhibition of HEC growth because there is no evidence of cytostasis of HEC with the lymphotoxin concentrations used. Lymphotoxin can be anticarcinogenic if given before or after carcinogenic insult or after TPA addition. The degree of sensitivity of different steps in carcinogenesis as the cells underwent the physiological changes associated with transformation was examined with six hour lymphotoxin treatments. Lymphotoxin treatment before irradiation and TPA caused a transient cellular change. When the cells were initiated within two days after lymphotoxin exposure, the induction of promoted transformation was inhibited. Results were similar with nonpromoted transformation. Lymphotoxin became a more effective anticarcinogen as the interval between the lymphotoxin pulse and carcinogen insult or TPA addition was reduced. When added during the last six hours of the experiment, lymphotoxin was equally inhibitory whether or not TPA was present. These results indicate that lymphotoxin induces an anticarcinogenic physiological state that is short lived or transient and that the temporal relationship between lymphotoxin and carcinogen exposure is important for preventing initiated or promoted transformation by lymphotoxin.

A major observation involving the anticancer activities of hamster and human lymphotoxin is that each consists of two distinct activities. These two proteins are separable by isoelectric focusing; the more acidic one contains nearly half the anticarcinogeneic as well as other anticancer activities. These activities include the cytostatic as well as the activity that sensitizes target cells to increased destruction by naturally immune lymphocytes. The other protein, in addition to anticarcinogenic, tumor cell cytostatic, and natural killer cell sensitizing activities, also has cytolytic activity which is generally measured by lysis of L929 cells. Whether the lytic activity is the same as that also seen against transformed cells in the same species has not been established. Lymphotoxin cytolytic activity is more susceptible to degradation by proteases including trypsin, chymotrypsin and pronase and to neuraminidase treatment which cleaves sialic acid off of the oligosaccharide side chains than are the other anticancer activities. Furthermore, the ratio of cytolytic activity to the other forms of anticancer activity secreted by lymphotoxin preparations is not constant providing further evidence that the cytolytic activity of the second class of lymphotoxin may represent another form of this immunological hormone. Additional evidence for this concept is the recent finding that if the lymphotoxin is prepared in the presence of an oligosaccharide inhibitor, the antibiotic tunicamycin, only the cytolytic activity is altered in its electrical charge. These observations are particularly important because they emphasize the need to select the appropriate assay or combination of assays when studying the individual lymphotoxin components for their various anticancer activities.

Two observations concerning the specificity of interaction of lymphotoxin with the cell surface are that insulin susceptible rat mammary carcinoma cells become resistant to the growth inhibitory activity of lymphotoxin and that lymphotoxin reverses the results of interferon treatment of target cells which ordinarily decreases their sensitivity to naturally immune lymphocyte destruction. It will be important to establish whether the insulin and interferon effects are surface related and operative when considering anticarcinogenic lymphotoxin activity because insulin and interferon may normally be present.

In another area, the fluorescence activated cell sorter was used to identify the normal lymphocyte subpopulations capable of producing lymphotoxin and responding with enhanced cytolytic activity to target cells previously treated with lymphotoxin. Fluorescein labeled monoclonal antibodies were used to identify the leu-7 positive subpopulation of natural killer cells that produces lymphotoxin and that can also respond with enhanced cytolytic activity after lymphotoxin treatment of the target cells. Therefore, the cell sorter can be used to isolate functionally active effector populations and to study changes occurring in the target cells. The data also provide further evidence that a lymphotoxin-like soluble mediator is secreted by natural killer cells and is involved in the destruction of the target cells. With available monoclonal antibodies it will be possible to identify the lymphocyte subsets that produce lymphotoxin and to determine which lymphotoxins are secreted by different lymphocyte populations. Identification of antigenic and structure membrane changes will permit sorting of cells for biochemical analysis of the lymphotoxin induced changes. The latter will permit correlation of biochemical properties of sorted cells with their susceptibility to carcinogenesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04629-18 LB

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents.

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Joseph A. DiPaolo Chief, LB, NCI

COOPERATING UNITS *(if any)*

NONE

LAB/BRANCH

Laboratory of Biology

SECTION

Somatic Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	2.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanism of carcinogenesis is studied with in vitro models to ascertain how mammalian cells respond to the various stimuli that are responsible for induction of transformation or that modify the frequency of transformation. The approach is to understand the nature of interrelationships between DNA metabolism, chromosome structure, biological reagents, and carcinogenesis. Human cells transformed by aflatoxin, UV, and by combination of X-ray and UV have been compared to non-treated cells and to human sarcoma specimens. The growth rate of all these cells was essentially the same; however, the chromosomal constitution varied from normal to minimal deviations to extensive chromosomal alteration for normal, experimentally transformed, and spontaneous sarcomas, respectively. Diploid fibroblasts from a patient with Bloom's syndrome were transformed by transfection with DNA from cells having single copy of the Harvey murine sarcoma virus (Ha-MuSV) per genome. Malignant characteristics of the cells and the identification by restriction enzyme analysis of the Ha-MuSV transforming sequence were evidence of the transformed phenotype. A comparison of effects of different methylating agents in hamster cells (HEC) indicates correlation of the transformation frequency with the level of O6- but not N7-methylguanine, determined by high pressure liquid chromatography. Sister chromatid exchange (SCE) frequency varies with growth conditions and/or class of carcinogen, indicating a relationship between SCE, unrepaired DNA damage, and transformation. The mechanism for SCE's is unkown, but protein synthesis appears critical and the level of spontaneous SCE is sensitive to changes in nucleotide pools. Lymphotoxin, an immunologic hormone, inhibits HEC transformations when added prior to or after carcinogen insult. The shorter the interval between lymphotoxin and carcinogen treatments, the greater the inhibition. Identification of DNA transforming sequences in guinea pig transformed cells is related to tumorigenicity. DNA transforming sequences have been identified in transformed HEC cell lines. Restriction enzyme analysis indicates several different sequences. One sequence, homologous to Kirsten ras, is being isolated.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

J. Doniger	Senior Staff Fellow	LB NCI
C.H. Evans	Chief, Tumor Biology Section	LB NCI
N.C. Popescu	Senior Staff Fellow	LB NCI

Objectives:

The overall approach to problems in carcinogenesis is to investigate factors and mechanisms responsible for the modulation of neoplastic transformation of human and other cells, vital aspects in the etiology and prevention of cancer. Although cell biology was emphasized in the past, biochemical and molecular biological approaches are increasingly important in the elucidation of the objectives of the Somatic Cell Genetics Section. The specific objectives are (1) to define the role of chemical, physical, and biological agents pertinent to the process of carcinogenesis; (2) to characterize the cellular and chromosomal alterations associated with carcinogenesis; (3) to evaluate the relationships between DNA repair and metabolism and carcinogenesis; and (4) to probe the somatic mutation aspects of experimental carcinogenesis.

Methods Employed:

All procedures performed are with the view of quantitating the progression of the transformation phenomenon. Such an approach is required to determine whether or not the observed transformation is due to the direct or indirect effect of the carcinogen and in order to study the early events associated with in vitro transformation. Cultures are made with freshly isolated cells from animals and humans and human cell strains obtained from patients with metabolic disorders that as controls have many of the attributes of "normal" cells, and from cell lines which are known to exhibit some of the properties associated with nontransformed cells. Cells derived from whole embryos or specific organs are grown in complete medium in the presence or absence of irradiated cells (feeder cells) and exposed to carcinogen transplacentally or prior to or subsequent to seeding the cells in plastic dishes. The transformation frequency takes into consideration the observed rate of transformation on a per cell basis or on the number of colonies obtained.

Procedures utilized for detection of acute carcinogen-induced damage include sister chromatid exchange (SCE), chromosome aberrations and micronuclei identification. Structural chromosome alterations are determined by high resolution analysis of G band of prophase or metaphase chromosomes in conjunction with procedures for localization of constitutive heterochromatin (C band) and nucleolar activity (Ag-AsNOR). Autoradiography and liquid scintillation procedures are used for DNA, RNA and protein metabolism.

DNA repair is measured by sucrose sedimentation, equilibrium density, and high-pressure liquid chromatography analysis of DNA extracted from carcinogen treated cells. DNA replication is assessed by thymidine incorporation, DNA equilibrium

density analysis, cytofluorographic analysis of cell cycle, cell autoradiography, and DNA fiber autoradiography. Isolation of DNA sequences responsible for neoplastic transformation is accomplished by DNA transfection, agarose gel electrophoresis, Southern blotting, DNA restriction analysis, and gene cloning.

Major Findings:

During the past year emphasis has continued to focus on the problems associated with transforming human cells. At the same time, studies have progressed utilizing Syrian hamster embryo cells (HEC) because they are readily transformed in an inductive manner by a variety of carcinogens. Furthermore, in addition to being used for determining the carcinogenicity of a variety of agents, hamster cells are useful for studying factors which are responsible for either potentiating or inhibiting the carcinogenesis process. Unfortunately, attempts to utilize the information learned with the various experimental systems, including hamster to human cells, have either been unsuccessful or have given semi-quantitative results, or a transitory type of transformation. As a consequence, a dilemma has developed in which the number of known potential human carcinogens that should be tested increases while utilization of human diploid cells to dissect the process leading to cancer remains underdeveloped. In the final analysis, the difficulty in transforming human cells, as compared to the difficulty in transforming animal cells, results from three primary differences: inexperience in culturing human material, a control mechanism that is responsible for the relatively stable human phenotype, and the heterogeneity of humans. The utilization of a completely defined medium compared to a serum supplemented medium did not result in extension of the lifespan of human cells nor in an increase in the transformation frequency. In terms of cell cycle, it appears that the best medium is Eagle's minimal essential medium supplemented by fetal bovine serum, pyruvate, serine, and aspartic acid. With this medium it was possible to obtain a doubling time of approximately 24 hours with fibroblasts derived from human foreskin. The use of a selective metabolic block to inhibit DNA synthesis has resulted in a higher efficiency of anchorage independent growth, accomplished by treating the cells with either aflatoxin B_1 or ultraviolet irradiation (UV) and immediately suspending them in agarose containing medium. After approximately four weeks, the tranformed colonies can be isolated from the agarose, grown in mass culture, and the neoplastic potential demonstrated by injecting them into the brain of nu/nu nude mice. A comparative cytogenetic analysis of cells from surgical human sarcoma specimens and of in vitro chemically transformed fibroblast cells was done to assess the significance of aneuploidy of experimentally induced transformation and spontaneous human neoplasia. The specimens were dissociated and single cells suspended in agarose containing medium. Colonies were isolated and propagated in a manner identical to that of the carcinogen treated cultures. The human sarcomas, compared to carcinogen-induced cell transformation, were characterized by a higher degree of chromosomal heterogeneity in terms of both numerical and structural alterations. The sarcomas had 2-3 distinct populations with near diploid, triploid or tetraploid number and contained complex rearrangements involving chromosomes 1, 2, 3, 9, 13, and 14. In addition, preferential nondisjunctions affected several chromosome pairs: 7, 17, 19, and 22. The in vitro transformed human cells had relatively few chromosome alterations. In fact, some transformations induced by carcinogens such as with ethyl methanesulfonate or propane sultone did not have any chromosome variations. Therefore, the

transformation of normal human cells by chemical or physical carcinogens can occur in the absence of visible chromosome alterations, or with only minor structural changes. It is postulated that secondary complex rearrangements that subsequently are found in cancer are necessary for the maintenance and the progression of neoplasia. Eventually, transformed cells, even though they produce tumors in nu/nu mice, usually develop characteristics associated with senescence; in order to further characterize the cells, additional populations must be taken from liquid nitrogen storage. Because pretreatment with X-irradiation or an alkylating agent resulted in enhancement of transformation associated with a potent carcinogen in the HEC system, X-irradiation has been used in combination with ultraviolet irradiation. These human cell transformations are being characterized and studied to determine whether they have characteristics of cancer not exhibited as a result of transformation by a single agent. Concurrent cells from cancer-prone humans with rare genetic diseases that have an increased sensitivity to one type or another of carcinogen have been studied. For example, fibroblast cells from neurofibroma patients are being subjected to X-irradiation, and cells derived from Fanconi's anemia are being treated with benzene. Evidence indicates that the evolution of a human tumor cell population occurs as a result of step wise genetic alteration. Various possibilities exist for rearranging the genome, all of which may alter gene dose. The human tumor cell, however, is characterized by a very stable phenotype; it does not exhibit the aneuploidy associated with most rodent lines as they undergo extensive subculturing. Consequently, the evolutionary process leading to highly malignant human cells appears to be slower and may be responsible for the long latent periods of growth associated with many human tumors. Therefore, attempts have been made to force the evolution of tumor cells by inducing chromosome changes. The results obtained with cytochalasin D, colcemid, or cell fusion have not shown an increase of chromosome numbers such as is associated with spontaneous human sarcomas.

HEC are particularly appropriate for the study of the transformation phenomenon. The induction of the carcinogen-induced morphological transformation is observed in a quantitative 7-day colony assay. In vitro morphological transformation occurs in a dose-dependent manner and is characterized by random criss-crossing and piling up of cells; it correlates with tumorigenicity because individually transformed cell colonies can be isolated, cell lines developed, and the formation of tumors demonstrated after the injection of the transformed cells into either Syrian hamsters or athymic nude mice. HEC can also be used to investigate stages of carcinogenesis, initiation and promotion, that were first demonstrated on the skin of mice.

Because of special studies involving carcinogen-cell interactions, the induction of transformation in HEC by nitrated polycyclic aromatic hydrocarbons, (PAH) chloroalkenes, inorganic metals, and bisulfite was examined. Five nitrated PAH synthesized from benzo(a)pryene (BP) fluoranthene, pyrene, and chrysene induced dose-dependent transformation of HEC. BP, a known carcinogen, induced transformation while the other parental compounds, which are non-carcinogens, were not effective. The transforming potential of the nitroderivatives varied from compound to compound; on a molar basis, 1,8-dinitropyrene was the most effective followed in order by 3-nitrofluoranthene, 1-nitropyrene, 6-nitrochrysene, and 6-nitrobenzo(a)pyrene (6NBP). 6NBP has mutagenic activity comparable to that of BP in the Ames test. In the present hamster assay system, however,

both compounds induced a dose-dependent transformation frequency and neither required external exogenous metabolic activation. In contrast to bacterial mutation studies, BP was one order of magnitude more active than 6NBP in inducing transformation. Because of their ubiquitous distribution and their ability to induce morphological transformation in mammalian cells, nitrated PAH must be considered as potential carcinogens. The activity of nitro PAH derivatives implies serious deleterious effects on human health; however, the carcinogenicity of these compounds remains to be established.

Malignant transformation of HEC by vinyl chloride occurs only after pregnant hamsters are exposed to the compound at fairly high concentrations of 20% (vol/vol) in air and not by direct treatment of the cells. The results indicate that vinyl chloride gives rise to metabolites responsible for mediating the various deleterious effects. The highly reactive chloroethylene oxide is considered the most plausible of these metabolites and is probably responsible for the carcinogenic action of vinyl chloride. The transforming ability was determined for six epoxides of structurally related chloroalkenes, cis-1-chloropropene oxide (c-CPO), trans-1-chloropropene oxide (t-CPO), cis-1,3-dichloropropene oxide, trans-1,3-dichloropropene oxide, trichloroethylene oxide (TCEO), and tetrachloroethylene oxide (PCEO). All six epoxides induced morphologic transformation of Syrian hamster embryo cells and caused cell lethality as reflected in the reduced cloning efficiency; in all instances, transformation was observed with less than 25% toxicity. The potency of the various epoxides was influenced by the difference in stability of the compounds. The results with c-CPO, t-CPO, TCEO, and PCEO were consistent with a linear dose response. The transformation results reflect the carcinogenicity of the parental chloroalkenes tested thus far. Furthermore, if the epoxides are metabolic intermediates of the chloropropene parental compounds, the epoxides are probably proximate carcinogens and provide further confidence concerning the explanation for the parental chloroalkenes.

The interaction of carcinogens with DNA appears to be a requirement for the induction of cancer by carcinogens that are also considered mutagens. Bisulfite, a chemical that at neutral pH does not induce mutations at two loci in Chinese hamster V-79 cells, induces transformation of HEC. Although bisulfite affects DNA metabolism, there was no evidence of bisulfite-induced DNA damage. Bisulfite induced no excision repair replication, caused no DNA strand breaks detectable in alkaline sucrose gradients, had no effect on the size distribution of DNA nascent daughter strands, and did not affect excision or post-replication repair of UV induced damage. However, bisulfite did induce a dose-dependent decrease in the rate of DNA replication per cell, apparently due to a reduction in the number of functioning replicons. The data indicate that bisulfite causes no detectable DNA damage. Permanent transformed lines have evolved as a result of bisulfite treatment of HEC. These transformed lines have been further characterized in terms of chromosome changes.

Evidence is accumulating that G bands of metaphase chromosomes can be further resolved and consequently subdivided into additional bands. The available methods for obtaining elongated chromosomes have utilized peripheral lymphocytes. The high resolution banding of prometaphase and prophase chromosomes has resulted in the resolution of an increased number of bands and sub-bands and permitted the conclusion that many acute nonlymphocytic leukemias are abnormal. The

fibroblast cultures have some inherent difficulties because of considerably lower mitotic rate which limits the number of dividing cells available for study, and the lack of natural synchrony prevents accumulation of large numbers of cells in the desired stage of the cell cycle. A procedure developed in our laboratory, utilizing a combination of G_1 cell arrest with arginine and glutamine deficient medium followed by excess thymidine, was effective in blocking the cells for release in early metaphase or prophase. With this procedure it was possible to obtain high quality chromosome preparations of various human, rat, or Syrian hamster cells transformed in vitro by chemical or physical carcinogens. For example, several cell lines with characteristics of neoplastic cell transformation were isolated from single colonies developed after HEC treatment with sodium bisulfite. Conventional G band analysis of metaphase chromosomes showed a unique deletion of the chromosome 10. G band of prophase chromosomes, however, revealed two additional abnormalities involving small interstitial deletions in the long arm of chromosomes 1 and 6. These permanent chromosome alterations associated with sodium bisulfite carcinogenesis are of a special significance because this compound failed to induce sister chromatid exchanges or chromosome aberrations after acute exposure. Thus, the aneuploidy which is observed need not be the result of a direct interaction between bisulfite and target cell genetic material but probably represents a secondary step in the neoplastic development.

In view of the various hypotheses concerning viruses, chemicals, physical agents, and cancer, and due to the inherent difficulties in studies using the intact animal, it is advantageous to study the various interactions between viruses and other potential carcinogens in a controlled in vitro system. Studies with a series of organic carcinogens and a Simian adenovirus, SA7, have shown that treatment of HEC with chemicals before, or shortly after, virus inoculation causes an increase in the viral transformation frequency. The enhanced viral transformation frequency results from an increase in both the absolute numbers of foci and a relative increase based upon survival data. The accumulated data suggest that carcinogen treatment renders the cells more susceptible to viral transformation presumably by increasing the quantity of viral genome incorporation into cellular DNA. In these studies of 56 metal salts, arsenic, cadmium, chromium, mercury, manganese, and molybdenum were considered positive. Except for nickel and beryllium, correlation was obtained between breakage of cellular DNA and enhancement of SA7 transformation. Both the positives and negatives in the enhancement assay are in excellent agreement with results of in vitro infidelilty of DNA synthesis assays. Carcinogenic chemicals increase not only the frequency of adenovirus transformation, but also the absolute number of SA7 foci per dish. This increase nullifies the supposition that the chemicals act by selecting cells sensitive to viral transformation; in addition, the viral transformation is increased in the absence of cell killing. Data strongly suggest that the chemical acts upon the individual cells, rendering them more sensitive to viral transformation.

Increased transformation frequencies occurring after 12-O-tetradecanoylphorbol 13-acetate (TPA) promotion of X-irradiation or UV of diploid HEC can be inhibited at different steps in the carcinogenesis process by either phytohemagglutinin (PHA), a naturally occurring mitogen, or lymphotoxin (LT), an immunologic

hormone. Although both compounds interact with the cell surface through glycoproteins, it is hypothesized that the inhibition of transformation occurs as a result of different mechanisms. HEC can also be used to investigate stages of carcinogenesis initiation and promotion, that were first demonstrated on the skin of mice. One day after being seeded for colony formation, HEC were X-irradiated (300 R). Two days later, the tumor promoter, TPA, was added. The transformation frequency increased from 0.1% of the surviving colonies to 7% in a linear fashion as the TPA concentration was increased from 0 to 160 nM. Above this concentration of TPA, the transformation frequency remained at 7%. The increase in transformation frequency was not accompanied by any change in the relative cloning efficiency after X-ray. Phorbol-12,13-dihexanoate, another in vivo tumor promoter, also increased the X-ray initiated transformation. No increase in transformation was observed with 3 different phorbol ester derivatives that have no tumor promoting activity in vivo. Furthermore, when HEC were treated with 80 nM TPA 2 days after X-irradiation, there was a linear increase in the transformation frequency as the X-ray dose increased from 0 to 350 R. These results indicate that in vitro promotion by phorbol esters is quantitative and mimics in vivo promotion. Sensitivity of Syrian hamster cells to the anticarcinogenic action of hamster LT depends on the stage of transformation, either initiation or promotion. LT was produced by mitogen stimulation of hamster leukocytes and purified by 10,000 MW exclusion ultrafiltration and pH 4.5-5.5 isoelectric focusing. LT irreversibly inhibits chemical or irradiation induced in vitro morphologic transformation of HEC when added at any point subsequent to carcinogen treatment. Galactose, a specific LT inhibitor, prevented the anticarcinogenic activity of LT, confirming that LT rather than another lymphokine was responsible for inhibiting transformation. Twice as much LT is required to obtain a 50% reduction in TPA promoted transformation than in nonpromoted transformation, suggesting a difference in sensitivity to LT. In another study of stages of promoted transformation, a 48 hour LT treatment inhibited transformation when added either immediately before or after X-irradiation, or during TPA exposure. The degree of sensitivity of different steps in carcinogenesis as the cells undergo the physiological changes associated with transformation was examined more precisely with 6 hour LT treatments. If added 72 hours before radiation, LT is ineffective, but is effective at 48 hours prior to irradiation. Cells became more sensitive to LT as the interval between the pulse and carcinogen insult or TPA addition was reduced. When added during the last 6 hours of the experiment, LT was equally inhibitory whether or not TPA was present. LT pretreatment before irradiation and TPA caused a persistent but nonpermanent effect unless fixed by the carcinogen treatment. Results were similar with nonpromoted transformation. These results indicate that LT induces an anticarcinogenic physiological state in noncarcinogen treated cells that is short lived or transient; the temporal relationship between LT and carcinogen exposure is critical for preventing transformation. Whereas LT, an immunologic hormone, has a persistent anticarcinogenic effect, independent of whether added prior to or after TPA, PHA is inhibitory only if added during TPA treatment. Moreover, PHA in conjunction with LT causes additional inhibition of TPA promoted transformation. Thus, PHA and LT affect the biological activity of TPA by diverse mechanisms; LT alters the physiological state of the cell, causing a change in the cellular response to TPA; PHA may either affect the binding of TPA to a critical cellular receptor for promotion or a later step in promotion.

The effect of repeated exposure to carcinogens would be expected to differ from that of a single exposure. Cells undergoing or completing DNA repair may respond differently to further carcinogen insult. With the quantitative HEC system, the induction of morphological transformation by split doses of physical and chemical carcinogen was compared to that produced by a single exposure. Four chemical carcinogens, N-acetoxyfluorenylacetamide (AcAAF), N-methyl-N'-nitro-N-nitroso-guanidine (MNNG), methylmethanesulfonate (MMS), and Mitomycin C (MMC), as well as UV light induced a dose-dependent increase in transformation frequency in HEC. X-irradiation was the least effective in inducing transformation and its effect was dose independent. The transformation frequencies induced by split doses of AcAAF (0.25 μg/ml + 0.25 μg/ml medium) were significantly higher than those caused by a single (0.5 μg/ml) treatment. Increases of 13%, 92%, and 40% greater than that obtained with one treatment were observed with split doses separated by 4, 7 and 12 hours, respectively. No enhancement was observed when 24 hours separated the split doses. With the exception of UV light at the 2 hour interval, the transformation caused by split doses of UV and MNNG was significantly lower than that induced by a single treatment. Split doses of UV (15 + 15 ergs/mm^2) separated by 2 hours induced a slight increase in transformation compared to a single treatment with 30 ergs/mm^2. However, within 4 hours a reduction in the transformation frequency occurred as a function of time. At 24 hours for example, split doses of UV induced 48% fewer transformations than a single exposure. Split doses of MNNG (0.1 + 0.1 μg/ml) separated by only 2 hours reduced the transformation frequency by 32% compared to a single treatment compared to one exposure of 0.2 μg MNNG/ml. A further gradual decline occurred at 4, 7, 12 and 24 hours. In fact, when the split doses of MNNG were separated by 24 hours, the transformation frequency was less than that induced by a single treatment with 0.1 μg MNNG/ml medium. Split doses of MMS (20 μg/ml + 20 μg/ml) separated by 2, 4, 7 or 12 hours induced transformation frequencies similar to that induced by a single exposure of 40 μg/ml. Split doses of MMS treatments separated by 24 hours, however, induced a lower transformation frequency compared to single treatment. Consistent with previous results X-irradiation was a poor carcinogen and induced only rare transformations. No dose response in transformation frequency was observed after 100 or 200 R of X-irradiation. Split doses of X-irradiation (100R + 100R) did not alter the results. These results demonstrate that, dependent upon the carcinogen and the time interval between treatment, split doses may have an additive, enhancing, or reducing effect on the transformation frequency. In addition the induction of SCE by split doses of carcinogen parallels the transformation response.

The persistence of DNA lesions accompanying the formation of SCE's and its relationship to the induction of transformation in HEC was examined. The available evidence indicates that SCE induction is a sensitive indicator of DNA damage and its repair. Previously the persistance of SCE's was determined on tetraploid or diploid metaphases from cells which have divided up to three generations in the presence of bromodeoxyuridine (BrdUrd). Our protocol consisted of sequential SCE analysis after carcinogen exposure. Exponentially growing HEC cultures were exposed to BrdUrd 1, 24 or 48 hours after carcinogen treatment for two rounds of replication required for visualization of SCE's during metaphase. The induction and persistence of SCE after exposure to MMC, AcAAF, MNNG, MMS, and UV were correlated with the induction of transformation. The SCE values showed that for a period of 72 hours after carcinogen insult, during which time the cells undergo

at least 6 replicative cycles, the carcinogen-induced DNA damage leading to SCE can persist or be partially or completely removed. MMC (3ng/ml) is the only carcinogen which caused long lasting SCE's; the SCE level (40 SCE per cell) remained elevated throughout the 72 hour interval examined. However, this sustained increase in SCE level after MMC was not observed if the cells were pulsed for 1 hour with MMC. Lesions caused by AcAAF (0.5µg/ml) were only partially removed as reflected in the decrease in the number of SCE from 39.1 to 28.4 and 20.9 after 1, 24, and 48 hours, respectively, after AcAAF. At the last interval approximately twice the number of SCE's compared to the control value (10 ± 2) still persisted. The lesions caused by the other carcinogens, MNNG (0.2 µg/ml), MMS (40 µg/ml), and UV (30 ergs), were completely removed within 72 hours as reflected in the SCE values which approached control level. MMS and UV induced lesions, however, were more rapidly removed than those caused by MNNG as indicated by the SCE values obtained when BrdUrd was added 24 hours after the carcinogen. Comparison of SCE values to the transformation frequencies determined using the quantitative HEC colony model did not show a correlation between the persistance of SCE and the induction of transformation for the majority of the carcinogens. The lesions caused by AcAAF, for example, were only partially removed, whereas those induced by MNNG or UV were completely removed as indicated by SCE assay. MNNG and UV produced 2.76 and 1.58% transformation frequencies, respectively, and AcAAF resulted in a lower transformation frequency, 1.1%. MMS induced lesions leading to SCE's were completely removed more rapidly than those induced by the other carcinogens (i.e within 24 hours). MMS was the least effective transforming agent inducing a transformation frequency of 0.93%. For this carcinogen, the efficient removal of lesions causing SCE was associated with a low transforming potential.

The variety of repair processes known to remove DNA damage caused by diverse classes of carcinogens has made it difficult to elaborate the mechanism(s) responsible for SCE induction. Because SCE's are induced by a variety of chemical and physical carcinogens, understanding the mechanism(s) of SCE formation may facilitate the elucidation of the role of DNA lesion(s) to the induction of neoplasia. There is a possibility that either direct or indirect influences on replication fork progression may be responsible for SCE's. In this study some factors that influence the formation of SCE in the absence of a DNA damaging agent were identified. A 48-72 hour incubation in arginine-glutamine deficient medium of either normal or malignant cells from various species, including human, resulted in an increase of 8-10 SCE's per cell over control values. Similar results were also obtained with cultured HEC using isoleucine deficient medium, density inhibited growth cultures, or cultures inhibited by serum depletion. It is possible that suboptimal levels of arginine and glutamine, or isoleucine may suppress the synthesis of histone as well as nonhistone proteins and in turn be responsible for an increase in SCE. Although specific inhibitors of protein synthesis such as cycloheximide or choramphenicol would be expected to induce SCE, neither increased the SCE frequency in HEC. Thus, protein synthesis alone cannot be responsible for the amino acid deprivation effect. The role of deoxycytidine metabolism was studied because SCE's induced by increasing BrdUrd concentrations were suppressed by excess deoxycytidine. The increase in SCE's caused by amino acid deprivation was also suppressed by deoxycytidine. Further evidence for the role of deoxycytidine metabolism in SCE formation was obtained with the 3T6 mouse cell line and a mutant 3T6 subclone with less than 1% deoxycytidine deaminase

activity. Both normal and mutant 3T6 cells had increasing SCE frequencies with increasing BrdUrd concentrations but different responses in the presence of arginine-glutamine deficient medium; after BrdUrd, no increase in SCE frequency occurred in the deoxycytidineless 3T6 mutant cells.

Although DNA repair and metabolism are considered relevant to carcinogenesis, the underlying processes are still obscure. Slow removal of O^6-methylguanine has been the basis for the hypothesis that this lesion is critical for carcinogenesis induced by methylating agents. For example, a positive correlation exists between the persistence of O^6-methylation in various tissues of the rat and susceptibility of these tissues to tumor induction by N-methylnitrosourea or dimethylnitrosamine, the brain being the most susceptible organ followed by the kidney and then the liver. The Syrian hamster embryo system allows for a quantitative comparison of the induction and repair of methylated DNA lesions with the induction of transformation and lethality by a variety of methylating carcinogens. We determined that MNNG, methyl nitrosourea or MMS concentrations that induce equivalent transformation frequencies in HEC also induced similar levels of O^6-methylguanine but not of N^7-methylguanine. Therefore, it is highly probable that O^6-methylguanine is the lesion responsible for the initiation of carcinogenesis induced by methylating agents. To determine how these lesions might effect DNA metabolism and thus contribute to the initiation process of carcinogenesis, the effects of MNNG and MMS at concentrations that induce equivalent levels of O^6-methylguanine on the size of both parental and nascent daughter DNA were examined by alkaline sucrose sedimentation analysis. The number of single strand breaks in parental DNA is greater in cells treated with MMS than in those treated with MNNG. Because the O^6-methylguanine level is the same with the conditions used for the two chemicals but the level of N^7-methylguanine is 30-fold high after MMS treatment, we concluded that the N^7-methylguanine lesion is responsible for the breakdown of parental DNA. We calculate, however, that there are approximately 250 N^7-methylguanine lesions for each single strand break in parental DNA. These breaks probably represent incomplete repair of these lesions at the time of sampling. The N^7-methylguanine lesions do not appear to have any biological consequences in terms of carcinogenesis because transformation is induced in MNNG-treated cells without the appearance of these breaks. At a concentration of MNNG that induces approximately one O^6-methylguanine per four replicons and one N^7-methylguanine per third of a replicon, there is no effect on size distribution of nascent daughter DNA. Therefore, this level of lesions has no effect on either replicon initiation or chain elongation. The rate of DNA replication, however, is 20% lower than that of untreated cells. Therefore, a group of replicons exists that do not function. When four times as many lesions are present, a slight effect on chain elongation occurs as well as a further reduction in the rate of DNA synthesis. Because both the number of lesions remaining as well as the DNA replication rate are lower 24 hrs after MNNG treatment, it is unlikely that the inhibition of the rate of DNA synthesis is directly related to the number of lesions in DNA. The rate of DNA synthesis is much lower in cells treated with MMS having the same level of O^6-methylguanine as those treated with MNNG. This further reduction in rate could be due to N^7-methylguanine because there are approximately 100 per replicon. However, 24 hours after carcinogen treatment, the rate of DNA synthesis recovers to about 50% of untreated controls while there are still at least 25 N^7-methylguanines remaining per replicon. Therefore, it is also possible that the reduction in the rate of DNA synthesis

is due to methylation of RNA and/or protein in the cell as opposed to direct inhibition due to the presence of DNA lesions in the DNA. These results indicate that the initiation of carcinogenesis by O^6-methylguanine is probably not mediated directly by effects on the rate of DNA replication but is more likely due to the miscoding properties of this lesion.

An understanding of the nature of the DNA sequence of the daughter strand DNA replicated from carcinogen damaged parental DNA is critical in the study of carcinogenesis. Therefore, DNA sequences putatively responsible for induction and maintenance of the transformed state are being isolated and characterized. DNA isolated from six different Syrian hamster embryo tumorigenic cell lines, five originally treated with 3-methylcholanthrene and one with BP, contain DNA sequences that can transform 3T3 cells by DNA transfection. Five of these DNA's have been analyzed for sensitivity to inactivation of their transforming sequences by a battery of restriction endonucleases. Since DNA from different tumorigenic cell lines appear to be inactivated by a different set of restriction enzymes, the DNA transforming sequences in these different cell lines are different.

Transformed cell lines have been isolated from foci derived from 3T3 cells transfected with DNA from the hamster tumorigenic cell lines. The DNA from these foci have been analyzed for the presence of hamster "middle repeat" DNA sequences using Southern blot analysis. Unfortunately, because of some homology between the "middle repeat" sequences from hamster DNA and those from mouse DNA, the presence of these "middle repeat" hamster sequences in the DNA of the transformed foci cannot be confirmed. Therefore, another technique was used to examine transformed foci for the presence of DNA transforming sequences; the DNA was probed with known retroviral oncogene sequences. One transformed focus, isolated from 3T3 cells transfected with DNA from a BP transformed hamster cell line and cut by restriction endonuclease Eco R1, was found to contain an additional sequence homologous to the Kirsten ras gene. This sequence resides in a DNA fragment that is approximately 3.5 Kb long when the DNA from the transformed focus is cut with Eco R1. Subsequently, 3-4 Kb DNA sequences resulting from Eco R1 restriction of DNA from this transformed focus have been isolated. They are being used to create a library to isolate the specific DNA fragment homologus to Kirsten ras. This purified DNA sequence will then be used to further analyze its role as a transforming sequence in the original BP transformed line.

We have been examining a variety of human cell strains to determine their suitability as recipients in DNA transfection experiments for isolation and characterization of DNA sequences responsible for maintenance of the neoplastic state. Transformed foci have been isolated from cells transfected with DNA extracted from a Harvey murine sarcoma virus (Ha-MuSV) transformed NIH 3T3 cell line (Ha-8). These cells contain a single copy of the Ha-MuSV genome and do not produce virus. The recipient cells (GM3498B) are non-malignant, diploid fibroblasts derived from a skin biopsy of a patient with Bloom's syndrome. These cells have a limited life span in vitro and a high level of spontaneous SCE indicative of this syndrome. No focus was observed in calcium phosphate treated control cultures. Four weeks after transfection with Ha-8 DNA, foci were observed. Of 12 foci isolated and subcultured, 10 failed to grow beyond 10 population doublings: two (GM3498B-HV1 and GM3498B-HV2) have an extended life span, form colonies in 0.3% agarose, and produce neurologic disorders and death 6-8 weeks after intracranial

injection into nude mice (parental control cells have no effect on nude mice), characteristic of neoplastic transformation. They also have the high level of spontaneous SCE's characteristic of the parental cells. The DNA extracted from one of these cell lines (GM3498B-HV2) was digested with Bam HI and analyzed by the Southern blot protocol with nick translated mouse v-bas sequence as a probe. V-bas specifically hybridizes to the Harvey ras sequence of Ha-MuSV. DNA from GM3498B-HV2 had two DNA sequences (6.4 and 5.6 Kb) homologous to v-bas in addition to the 6.6 Kb sequence observed in the parental GM3498B DNA. DNA from both GM3498B-HV1 and GM3498B-HV2 transformed foci as well as Ha-8 contain a 4.5 Kb fragment after digestion with Sst 1 that is homologous to v-bas as well as p-14 (a fragment of the Ha-MuSV genome containing the LTR but not Harvey ras). This demonstrates that the transformed phenotype was the result of incorporation of the Ha-MuSV sequence into the human recipient cell DNA. These experiments demonstrate that transformation of NIH 3T3 cells by transfection with DNA from human tumor cells is accomplished in a manner analogous to transfection of nonmalignant human cells by Ha-MuSV DNA.

Cells at distinct preneoplastic stages of carcinogenesis can be identified and isolated in an in vitro guinea pig transformation system. These cells have been utilized for studying various aspects of transformation leading to malignancy and are considered particularly appropriate material for analysis by DNA transfection. In collaboration with Dr. Mariano Barbicid, Z01CP04948-08-LCMB, we have found that isolated tumorigenic clones derived from carcinogen treated guinea pig cells contain DNA sequences capable of transforming 3T3 cells. In addition, an anchorage independent colony isolated from agar but negative in all tumorigenicity assays is also negative as a donor in 3T3 transfection assays. To examine further the relationship between tumorigenicity and oncogenic DNA sequences, other similar clones capable of growing in agar but not of producing tumors are now being investigated to determine whether they are negative in the 3T3 assay. DNA isolated from a tumorigenic cloned cell line, as well as cell strains of its preneoplastic stages, are also under investigation for their ability to transform 3T3 cells to determine at which stage oncogenic DNA sequences are formed. Finally, attempts will be made to isolate the transforming DNA sequences from the various tumorigenic guinea pig cell lines to understand their role during carcinogenesis.

Significance to Biomedical Research and the Program of the Institute:

The prevention of cancer in humans depends to a large extent on understanding the process that is responsible for the development of transformation and on removing potentially harmful environmental agents. The determination of factors responsible for transformation serves as the basis for the study of molecular mechanism(s) involved in transformation. In this way, it will be possible to intervene with or prevent the development of cancer. The target cell approach with human and animal cells makes possible the study of biochemical events responsible for blocking, reversing, or eliminating transformed cells. In the process, biologically valid techniques for identifying potential carcinogens relevant to humans are also being developed.

Proposed Course:

The goal of this project is to establish conditions and methods for in vitro quantitative study of transformation by chemical and physical carcinogens to determine the underlying biochemical and molecular processes responsible for the somatic changes resulting in malignancy. The project will continue to define the conditions necessary for the quantitative transformation of mammalian cells with specific emphasis on human cells. Because the mechanism(s) involved in transformation are probably independent of cell type, various sources of fibroblasts will be used including those obtainable from patients with genetic disorders as well as those derived from fresh normal foreskin. After determining the conditions necessary for increasing the susceptibility of primary human fibroblasts, epithelial cells, particularly those derived from breast will be used because of the possibility of relating markers associated with differentiation to carcinogenesis. Because of the progressive (steps/stages) nature of cancer, preneoplastic cell populations will be identified and isolated by a fluorescence activated cell sorter. This will permit the characterization of cell surface changes, chromosome alterations, and DNA changes. Human cells will be utilized to isolate and characterize DNA sequences responsible for carcinogenesis induced by chemical or physical agents.

Publications:

Casto, B. C. and DiPaolo, J. A.: Enhancement of viral-induced neoplastic transformation by inorganic metal salts. In Stich, H. F. (Ed.): Carcinogens and Mutagens in the Environment, Naturally Occurring Compounds. Cleveland, CRC Press Inc., Vol. 2. (In Press).

DiPaolo, J. A.: Evaluation of carcinogenic potential of chemicals: Short term tests of induction of neoplasia. J. Environ. Pathol. Toxicol. (In Press).

DiPaolo, J. A.: Factors that modify in vitro transformation. In Mirand, E. A., and Mihich, E. (Eds.): 13th International Cancer Congress, Alan R. Liss, Inc., New York. (In Press).

DiPaolo, J. A.: Modulation of induced quantitative mammalian cell transformation. In Kolber, A. R., Wong, T. K., Grant, L. D., DeWoskin, R. S., and Hughes, T. J. (Eds.): In Vitro Toxicity Testing: Current and Future Possibilities, (Part A). New York, Plenum Press Corp., 1983, pp. 317-335.

DiPaolo, J. A.: Relative difficulties in transforming human and animal cells in vitro. JNCI 70: 3-8, 1983.

DiPaolo, J. A., DeMarinis, A. J., Chow, F. L., Garner, R. C., Martin, C. N. and Doniger, J.: Nitration of carcinogenic and non-carcinogenic polycyclic aromatic hydrocarbons results in products able to induce transformation of Syrian hamster cells. Carcinogenesis 4: 357-359, 1983.

DiPaolo, J. A., DeMarinis, A. J. and Doniger, J.: Asbestos and benzo(a)pyrene synergism in the transformation of Syrian hamster embryo cells. Pharmacology. (In Press).

DiPaolo, J. A. and Doniger, J.: Neoplastic transformation of Syrian hamster cells by putative epoxide metabolites of commercially utilized chloroalkenes. JNCI 69: 531-534, 1982.

DiPaolo, J. A., Evans, C. H., DeMarinis, A. J. and Doniger, J.: Phytohemagglutinin inhibits phorbol diester promotion of UV irradiation initiated transformation in Syrian hamster embryo cells. Int. J. Cancer 30: 781-786, 1982.

DiPaolo, J. A., Evans, C. H. and Milo, G. E.: The susceptibility of normal hamster and human cells to transformation by environmental agents. In Davis, W. (Ed.): Host Factors in Human Carcinogenesis, IARC, Lyon, Scientific Publications, 1982, pp. 561-569.

DiPaolo, J. A., Popescu, N. C., Casto, B. C. and Miyagi, M.: Cytologic detection of virus specific DNA in situ hybridization in Syrian hamster embryo cells transformed by viral or combined chemical and viral treatment. Cancer Genet. Cytogenet. 7: 197-207, 1982.

Doniger, J., O'Neill, R., Noguchi, P. and DiPaolo, J. A.: Initiation of carcinogenesis dependence upon MNNG induced release of the G_1 block of density inhibited Syrian hamster cells. Teratogen. Carcinogen. Mutagen. J. 3: 123-132, 1983.

Evans, C. H., DiPaolo, J. A., Heinbaugh, J. A. and DeMarinis, A. J.: Immunomodulation of the lymphoresponsive phases of carcinogenesis by mechanisms of natural immunity. JNCI 69: 737-741, 1982.

Evans, C. H., Heinbaugh, J. A. and DiPaolo, J. A.: Comparative effectiveness of lymphotoxin anticarcinogenic and tumor cell growth inhibitory activities. Cell. Immunol. (In Press).

Greiner, J. W., Evans, C. H. and DiPaolo, J. A.: Carcinogen-induced phenotypic alterations in mammary epithelial cells accompanying the development of neoplastic transformation. Cancer Res. 43: 273-278, 1983.

McCabe, R. P., Evans, C. H. and DiPaolo, J. A.: Relationship of neoantigens induced by 3-methylcholanthrene treatment of Syrian hamster embryo cells to antigens expressed on fetal and 3-methylcholanthrene transformed neoplastic cells. Oncodevelop. Biol. (In Press).

Popescu, N. C., Amsbaugh, S. C. and DiPaolo, J. A.: Human and rodent transformed cells are more sensitive to in vitro induction of SCE by N-methyl-N'nitro-N-nitrosoguanidine (MNNG) than normal cells. Hum. Genet. 63: 53-57, 1983.

Ransom, J. H., Evans, C. H. and DiPaolo, J. A.: Lymphotoxin prevention of diethylnitrosamine carcinogenesis in vivo. JNCI 69: 741-744, 1982.

Ransom, J. H., Evans, C. H., Jones, A. E., Zoon, R. A. and DiPaolo, J. A.: Control of the carcinogenic potential of ^{99m}technetium by the immunologic hormone lymphotoxin. Cancer Immunol. Immunother. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04673-12 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Immunobiology of Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

C. H. Evans Chief, Tumor Biology Section, LB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biology

SECTION

Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Whether the normal immune system can prevent or control carcinogenesis, i.e., the development of cancer, is central to mechanisms of homeostasis, surveillance, prevention, and interventive management of neoplastic, mutated, infected, or otherwise altered cells. Naturally occurring lymphocytes, macrophages, and other leukocytes and their secretory products, e.g., lymphokines, interleukins, and other immunologic hormones, are being studied to define their effective anticarcinogenic and tumor cell growth inhibitory activities. Lymphotoxin, one of the few lymphokines with cytotoxic activity can prevent carcinogenesis and inhibit tumor cell growth. Anticarcinogenic action is direct and irreversible and occurs without cytotoxicity. Inhibition of tumor cell growth is primarily reversible but can become irreversible due to increased susceptibility of preneoplastic and neoplastic cells to cytolytic destruction by natural killer cells resulting from lymphotoxin target cell interaction. Lymphotoxin at very high concentrations is also directly cytolytic for tumor cells. The direct acting anticarcinogenic activity of lymphotoxin is more potent than the tumor cell inhibitory activity but by also being able to increase target cell sensitivity to the cytoreductive action of naturally cytotoxic lymphocytes the lymphokine may be an effective homeostatic mechanism for control of carcinogenesis at its later stages of development. Lymphotoxin anticarcinogenic, tumor cell growth inhibitory, and cytoreductive sensitizing activity co-purify into two glycoprotein classes, lymphotoxin I and lymphotoxin II with differing electrical charges. Lymphotoxin I has an isoelectric pH of 4.6 and 5.0 and lymphotoxin II has isoelectric pH's of 5.0 and 7.1 for hamster and human lymphotoxin, respectively. The anticancer actions of lymphotoxin are distinct from other lymphokines including interleukin 1, interleukin 2, macrophage migration inhibitory factor, and interferon. Lymphotoxin alters cell surface conformation, membrane fluidity, and large molecular weight membrane glycoprotein synthesis with changes in the latter correlating directly in time and quantity to lymphotoxin induced establishment of the anticarcinogenic state.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

R. P. McCabe	Senior Staff Fellow	LB, NCI
J. A. DiPaolo	Chief	LB, NCI
J. H. Ransom	Staff Fellow	LB, NCI
J. P. Fuhrer	Expert Scientist	LB, NCI

Objectives:

The primary objective of this project is to elucidate at the target cell and host levels the relationships between cell surface alterations accompanying the development of carcinogenesis and host mechanisms that prevent, otherwise inhibit or even enhance the development of cancer. Specific objectives include (1) identification of somatic cell alterations during carcinogenesis using in vitro model systems to allow study of membrane and other phenotypic changes at specific steps or stages in carcinogenesis and (2) investigation of host interactions with specific cell surface alterations during carcinogenesis in vitro and in vivo. Particular emphasis is placed on natural and induced cellular and humoral immunobiological interactions due to the frequent occurrence of neoantigens, reexpression of fetal antigens, and alterations in alloantigens on tumor cells.

Methods Employed:

Guinea pig, human, mouse, rat, and Syrian hamster cells are utilized in this study. The quantitative in vitro chemical carcinogenesis system developed within the Laboratory of Biology employing Syrian hamster embryo cells (Project Z01-CP-04629, Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents) forms the basic methodology for obtaining normal, preneoplastic and neoplastic cells for study. Freshly isolated cells are obtained from from embryos, fetuses or adult animals, exposed in utero or in vitro to chemical or physical carcinogens. The cells are cultured and studied for somatic cell changes such as altered morphology, morphological transformation, growth in agar, tumorigenicity and interaction with components of the immune system. Immunobiological techniques including direct and indirect immunofluorescence, complement fixation, colony inhibition, radionuclide uptake and release assays, delayed hypersensitivity skin reactions, and tumor transplantation rejection are employed in analyzing cell membrane changes and in assessing host interactions to the changes.

Major Findings:

Investigations during the past research project year were directed at defining the antineoplastic activity spectrum of the immunologic hormone, lymphotoxin. Previously we had demonstrated that this lymphokine possessed direct acting tumor cell growth inhibitory activity as well as being able to directly and irreversibly prevent radiation and chemical carcinogen-induced morphologic transformation, an early event in carcinogenesis. Specific studies were

aimed at examining the mechanisms for the anticarcinogenic and tumor cell growth inhibitory activities of lymphotoxin and establishing whether this naturally occurring immunomodulator can inhibit or control the development of cancer in vivo.

The anticarcinogenic and tumor growth inhibitory activities of lymphotoxin operate through diverse mechanisms. Radionuclide uptake and release measurements, cell enumeration, and colony formation assays demonstrate that lymphotoxin inhibition of tumor cell growth is predominantly cytostatic (reversible); cell proliferation ceases in the presence of lymphotoxin but resumes following removal of the hormone. Lymphotoxin, however, increases susceptibility of preneoplastic and neoplastic cells to cytolytic destruction by natural killer cells. Thus, lymphotoxin is also, through indirect mechanisms, a cytolytic lymphokine with the potential to irreversibly inhibit tumor cell growth as well as prevent carcinogenesis. At much higher concentrations lymphotoxin also directly causes lysis of preneoplastic and neoplastic cells.

The indirect tumor cell growth cytolytic activity of lymphotoxin may be an important part of the anticarcinogenic activity spectrum of this lymphokine as lymphotoxin's direct tumor cell growth inhibitory activity is often ten-fold or more less effective on a unit activity basis than is the anticarcinogenic activity of the hormone. For example, six units of lymphotoxin per ml culture medium prevents fifty percent of chemical carcinogen or ultraviolet or gamma radiation induced morphologic transformation in golden Syrian hamster cells. In comparison 130 or more units of lymphotoxin are required to inhibit the growth of morphologically transformed tumor producing cells. If, however, the tumor producing cells are treated with lymphotoxin followed by exposure to natural killer cells then there can be increased cytolytic destruction of the tumor cells compared to cytolysis resulting from natural killer cell attack alone. In this fashion lymphotoxin may act as an amplification mechanism for the control of cancer development and thus be potentially as effective at early as well as at later stages of carcinogenesis.

Lymphotoxin is produced when lymphocytes are stimulated by the presence of antigen or mitogen. Histoincompatible cells and tumor cells themselves can stimulate lymphotoxin secretion. Stimulation results, in all likelihood, due to interaction with antigens on the surface of the histoincompatible normal or tumor cells. The presence of altered or new cell surface antigens during carcinogenesis could, therefore, potentially serve to activate lymphocytes to produce lymphotoxin which in turn could control tumor cell development. Recent experiments have demonstrated that 18 hour 3-methylcholanthrene treatment of golden Syrian hamster cells is followed by appearance of neoantigens before tumor cell development and presumably early in the process of carcinogenesis. These neoantigens or other carcinogen-induced cell surface alterations would provide a stimulus for lymphotoxin production should sufficient lymphocytes be present in the area where the cells bearing neoantigens are undergoing transformation to the malignant state. The lymphotoxin then might prevent the further development of carcinogenesis or increase the susceptibility of the cells to natural killer cell, macrophage, or other natural immunobiological effector cytolytic destruction. The control of lymphotoxin synthesis or secretion is, however, complex and the lymphokine may not always be present in sufficient quantity to effect control of carcinogenesis due to possible negative feedback control of lymphotoxin secretion. Additional

observations indicate that the biphasic inhibition of carcinogenesis and tumor cell growth frequently observed in vivo and in vitro in the presence of increasing numbers of normal lymphocytes may reflect immunomodulation of lymphotoxin by the lymphocytes or other cells in the leukocyte populations. The biphasic inhibition of carcinogenesis is eliminated and inhibition becomes proportional to the leukocyte number if the lectin and lymphotoxin stimulator, phytohemagglutinin, or if lymphotoxin itself is added to the admixture of lymphocytes and cells undergoing carcinogenesis. Phytohemagglutinin or lymphotoxin also eliminates the biphasic inhibition of tumor cell growth when added to the admixture of lymphocytes and tumor cells. This type of modulation of natural lymphoid cell cytotoxicity may be one means by which the individual host or developing cancer cell may circumvent and escape any inherent homeostatic mechanisms for the prevention and control of cancer.

Lymphotoxin induces synthesis of high molecular weight (approximating 200,000 daltons) cell surface membrane glycoproteins in nontransformed cells and inhibits the synthesis of high molecular weight glycoproteins in the membranes of tumor cells. These are the first biochemical alterations to be identified resulting from lymphotoxin interaction with mammalian cells that correlate with the anticarcinogenic and tumor cell growth inhibitory activities of the hormone. The membrane changes, moreover, occur within hours following binding of lymphotoxin to the cell and develop coincident with establishment of the anticarcinogenic state by lymphotoxin. The possible involvement of cell surface glycoproteins in the prevention of carcinogenesis is, furthermore, intensely interesting because cell surface glycoproteins and/or glycolipids are intimately involved in natural killer cell cytolysis. For these reasons, it is very important for understanding the mechanisms of carcinogenesis as well as that of natural killer cell and other immunobiological cytotoxicity, to identify the specific glycoproteins induced and/or inhibited by lymphotoxin and the biochemical interrelationships of the glycoproteins to carcinogenesis and tumor cell growth.

The aforementioned anticarcinogenic and tumor cell growth inhibitory activities of lymphotoxin have been defined in in vitro model systems developed for the study of carcinogenesis. In vitro systems offer the ability to study interactions during carcinogenesis unencumbered by host homeostatic or other influences. Continued dissection of the individual components and mechanisms of lymphotoxin-mediated prevention and control of cancer, however, retains validity only if lymphotoxin is present and exerts antineoplastic activity in vivo. Both have been shown to be true. The injection of lymphotoxin in guinea pigs retards the growth of transplanted tumor producing morphologically transformed cells. Injection of lymphotoxin simultaneously with either diethylnitrosamine or with the gamma-emitting radionuclide, ^{99m}Technetium, in golden Syrian hamsters prevents morphologic transformation induced by the chemical carcinogen or by the gamma radiation in vivo. These results demonstrate that lymphotoxin exerts anticarcinogenic and tumor cell growth inhibitory activities in vivo. Furthermore, intraperitoneal administration of phytohemagglutinin or the antigens, keyhole limpet hemocyananin or sheep erythrocytes, is followed by the appearance of lymphotoxin in the peritoneal cavity of golden Syrian hamsters within forty-eight hours. High performance liquid chromatography analysis of the in vivo induced lymphotoxin demonstrates that it is of the same molecular class as the anticarcinogenic and tumor cell growth inhibitory lymphotoxin induced by the

same mitogenic and antigenic stimulation of peritoneal leukocytes in vitro. The ability of lymphotoxin to inhibit the growth of tumor cells in vivo, furthermore, is specifically mediated through the natural killer cell and not through macrophages. This also confirms in vitro findings that the enhancement of the susceptibility of preneoplastic and neoplastic cells to natural cytotoxicity is specific for the large granular lymphocyte or natural killer cell rather than the macrophage.

Fluorescence activated microflowcytometry cell sorting indicates that the lymphocyte subpopulation with enhanced cytotoxicity demonstrable after lymphotoxin treatment of preneoplastic or neoplastic human cells is Leu-7 antigen positive and thus is a member of the natural killer subset. Lymphocytes positive for the OKT11 antigen are also enhanceable but suppressor lymphocytes positive for the Leu-3a antigen are not cytotoxic. The Leu-7 positive lymphocytes have also been identified as a lymphotoxin producing population. Several molecular species of hamster and human lymphotoxin characterized by differing isoelectric points and anticancer activities have also been identified through the use of isoelectric focusing and high-pressure liquid chromatography. The anticarcinogenic, the tumor cell growth inhibitory, and the natural cytotoxicity enhancing activities co-purify during fractionation with these procedures. The activity, however, resulting in lysis of the alpha L929 murine tumor cells fails to co-purify with the other activities and appears to represent another lymphotoxin molecular species. The synthesis of cytolytic compared to the other anti-cancer activities of lymphotoxin is also not constant. The cytolytic activity, moreover, is also more sensitive to protease and to neuramindase degradation than are the other activities. The strongest evidence that the cytolytic activity is a lymphotoxin form distinct from those with the anticarcinogenic and tumor cell growth inhibitory actions is that inhibition of oligosaccharide synthesis by tunicamycin affects the isoelectric pH of the cytolytic but not the other anticancer actions of lymphotoxin. We now are in the process of establishing the biochemical and biological interrelationships among these physically distinct forms of lymphotoxin. In addition to separating and evaluating the activities of the various lymphotoxin components specificity studies in terms of interactions with other cell surface active agents, e.g. hormones, antibodies, and lectins, are in progress. For example, insulin can render lymphotoxin responsive cells resistant to the growth inhibitory action of lymphotoxin. In an opposite manner lymphotoxin can reverse the desensitizing action of interferon resulting in an increased susceptibility of the target cell to the cytoreductive action mediated by naturally immune lymphocytes. Investigations are in progress to determine the molecular character of these stimulatory and opposing actions. It may be through a balance between such stimulatory and antagonistic activities that lymphotoxin exerts its normal regulatory role. Lymphotoxin is, therefore, an immunologic hormone with direct and indirect anticarcinogenic and tumor cell growth inhibitory activity. The hormone binds to cell surface oligosaccharide receptors and its action may be mediated through alterations in cell surface glycoproteins that directly block the carcinogenesis process at its early stages or indirectly prevent cancer by increasing the susceptibility of preneoplastic and neoplastic cells to control or destruction by immunological or other homeostatic mechanisms.

Significance to Biochemical Research and the Program of the Institute:

This project provides a means to study and understand how the individual, through the mechanisms of natural immunity, intervenes to suppress, inhibit or even enhance the growth of an incipient tumor cell during carcinogenesis. Natural cytotoxicity of macrophages, lymphocytes and lymphokines alone or in combination can now be studied at various stages of carcinogenesis to provide new insights into the immunobiology of cancer. As the host mechanisms and the target cell structures with which the immune effectors interact are delineated, it will be possible to investigate how the natural and induced immunity of the individual host may be augmented to suppress and even prevent the final aspects of carcinogenesis--the transition from the preneoplastic to the neoplastic state.

Proposed Course:

Investigations will continue to define the mechanisms whereby neoplastically transformed rodent and human cells are preferentially susceptible to the cytotoxic activity of naturally immune host-derived cellular and humoral effectors. A major thrust will be a multidisciplinary investigative analysis of cell membranes relevant to differentiating tumor cells from normal cells. The approaches will include membrane structure changes and macromolecular differences in cellular behavior. This program will, in part, seek to explain how lymphotoxin preferentially binds to and inhibits tumor cells and why cell to cell contact is necessary in most natural cellular immunocytotoxicity. New technology in cell surface topography and cell separation using computerized cytometers and cell sorting cytofluorographs will enable resolution of the relationships between the cytostatic and cytocidal activities of macrophages, lymphocytes, and lymphokines to tumor cells. Definition of these relationships will clarify the species specificity of the effector mechanisms and our understanding of the role of *in vivo* natural immunity in the phenomenon of carcinogenesis.

Publications:

Basu, S., Basu, M., Higashi, H. and Evans, C. H.: Biosynthesis and characterization of globoside and Forrsman glycosphingolipids in guinea pig tumor cells. In Makita, A., and Hsanda, S. (Eds.): Glycolipids in Biology, New York, Plenum Press, pp. 131-137, 1982.

DiPaolo, J. A., Evans, C. H., DeMarinis, A. J. and Doniger, J.: Phytohemagglutinin inhibits phorbol diester promotion of UV-irradiation initiated transformation in Syrian hamster embryo cells. Int. J. Cancer 30: 781-786, 1982.

DiPaolo, J. A., Evans, C. H. and Milo, G. E.: The susceptibility of normal hamster and human cells to transformation by environmental agents. In Davis, W. (Ed.): Host Factors in Human Carcinogenesis, Lyon, IARC Scientific Publications, 1982, pp. 561-569.

Evans, C. H.: Lymphokines, homeostasis, and carcinogenesis. JNCI 70: 1983.

Evans, C. H. and DiPaolo, J. A.: Independent expression of chemical carcinogen-induced phenotypic properties frequently associated with the neoplastic state in a cultured guinea pig cell line. JNCI 69: 1175-1182, 1982.

Evans, C. H., DiPaolo, J. A., Heinbaugh, J. A. and DeMarinis, A. J.: Immunomodulation of the lymphoresponsive phases of carcinogenesis by natural immunity. JNCI 69: 737-741, 1982.

Evans, C. H., Heinbaugh, J. A. and DiPaolo, J. A.: Comparative effectiveness of lymphotoxin anticarcinogenic and tumor cell growth inhibitory activities. Cell Immunol. 7: 295-303, 1983.

Fuhrer, J. P. and Evans, C. H.: Rapid separation of biologically active Syrian hamster lymphotoxin in high yield by size exclusion high performance liquid chromatography. J. Chromatog. 248: 427-433, 1982.

Fuhrer, J. P. and Evans, C. H.: The anticarcinogenic and tumor growth inhibitory activities of lymphotoxin are associated with altered membrane glycoprotein synthesis. Cancer Lett. (In Press).

Fuhrer, J. P., Evans, C. H., Ransom, J. H. and Heinbaugh, J. A.: Identification of β-lymphotoxin as the predominant molecular class of in vitro and in vivo Syrian hamster lymphotoxin. Cell. Immunol. 75: 63-70, 1983.

Greiner, J. W. and Evans, C. H.: Temporal dynamics of cortisol and dexamethasone prevention of benzo(a)pyrene-induced morphological transformation of Syrian hamster cells. Cancer Res. 42: 4014-4017, 1982.

Greiner, J. W., Evans, C. H. and DiPaolo, J. A.: Carcinogen-induced phenotypic alterations in mammary epithelial cells accompanying the development of neoplastic transformation. Cancer Res. 43: 273-278, 1983.

McCabe, R. P. and Evans, C. H.: The regulatory role of extracellular proteases in tumor growth. Surv. Syn. Pathol. Res. (In Press).

McCabe, R. P., Evans, C. H. and DiPaolo, J. A.: Relationship of neoantigens induced by 3-methylcholanthrene treatment of Syrian hamster embryo cells to antigens expressed on fetal and 3-methylcholanthrene transformed neoplastic cells. Oncodevelop. Biol. Med. (In Press).

Pintus, C., Ransom, J. H. and Evans, C. H.: Endothelial cell growth supplement: A cell cloning factor that promotes the growth of monoclonal antibody producing hybridoma cells. J. Immunol. Meth. (In Press).

Ransom, J. H., Evans, C. H. and DiPaolo, J. A.: Lymphotoxin prevention of diethylnitrosamine carcinogenesis in vivo. JNCI 69: 741-744, 1982.

Ransom, J. H., Evans, C. H., Jones, A. E., Zoon, R. A. and DiPaolo, J. A.: Control of the carcinogenic potential of ^{99m}technetium by the immunologic hormone lymphotoxin. Cancer Immunol. Immunother. (In Press).

Ransom, J. H., Pintus, C. and Evans, C. H.: Lymphotoxin amplification of tumor growth inhibition is specific for natural killer cells not macrophages. Int. J. Cancer. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05145-04 LB

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Surface in Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Richard P. McCabe Senior Staff Fellow, LB, NCI

COOPERATING UNITS *(if any)*

NONE

LAB/BRANCH

Laboratory of Biology

SECTION

Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Expression of cell surface associated properties during chemical carcinogenesis is being investigated in animal and human in vitro model systems in order to identify cellular characteristics useful in isolating preneoplastic cell populations and for use in preventing neoplastic disease through interventive treatment early in carcinogenesis. Fibrinolytic activity, lymphotoxin sensitivity, induction of a sustained natural tuberculin-like skin response, and threshold tumorigenic inoculum in adult syngeneic animals have been found to be quantitatively related properties. A system of acid stable, autocrine-like protease inhibitory molecules functioning as regulators of local tissue protease activity has been described. New surface antigens associated with preneoplastic development expressed during carcinogenesis in the hamster are also found on a few neoplastic cell lines and on 10-day gestation hamster embryo cells. Monoclonal antibodies produced to 10-day embryo cells are being characterized with regard to the nature of the antigens recognized and their expression during carcinogenesis. Studies include protease stimulation of lymphotoxin production, oncofetal antigen expression during carcinogenesis and relationship of cell surface properties to the developmental state of the cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Charles H. Evans	Chief, Tumor Biology Section	LB NCI
Joseph A. DiPaolo	Chief	LB NCI

Objectives:

The progression toward neoplastic transformation following chemical carcinogen treatment often occurs with identifiable stages of preneoplastic development. During each stage alterations in the expression of many cell properties occur with the combined effect of conferring the capacity to form a progressively growing tumor. The evidence indicates that no single change by itself is sufficient for tumorigenicity, but rather the importance of each change lies in its relationship to the other altered properties expressed. In this study the molecular nature of such changes occurring at the cell surface or in the extracellular area is approached with regard to their expression during carcinogenesis, their relationship to coordinately expressed alterations, and their usefulness in investigations to arrest the course of preneoplastic development.

Specific objectives of this project are (1) to identify cell surface changes associated with neoplastic transformation which occur as cells progress through the stages of carcinogenesis; (2) to study the relationships among these changes for the purpose of identifying conditions of phenotypic expression necessary for tumor growth; (3) to determine which changes are most useful for selecting an enriched population of preneoplastic cells and which are appropriate targets for arresting preneoplastic development; and (4) to relate the significant phenotypic changes to specific alterations in cellular regulatory mechanisms associated with carcinogenesis. The research focuses on alterations in the carbohydrate, protein and lipid structure of the cell membrane and matrix, changes in susceptibility to natural host defenses, expression of novel immunogenic cell surface structures and their relationship to the differentiated state of resulting tumors, and disturbances in the expression of proteolytic and protease inhibitory activities which control the cascades of host responses decisively influencing the growth of nascent tumor cells.

Methods Employed:

In the guinea pig the stages of preneoplastic development are distinct and extended permitting the identification and isolation of cells at various points along the pathway to neoplastic transformation. These isolated cells are a unique resource for the investigation of the various changes occurring during carcinogenesis. Guinea pig cells have been assessed for tumorigenicity in syngeneic adult animals, the ability to elicit a sustained intradermal delayed type skin response in syngeneic nonimmune animals, sensitivity to homologous lymphotoxin in an inhibition of colony formation assay, expression of the Forssman antigen, other glycolipid antigens and common tumor-specific cell surface antigens by a protein A antibody radiobinding procedure, secretion of matrix components by immunofluorescence, and production of enzymes involved

take as long as a year or more. The Syrian hamster is, therefore, well suited for rapidly assessing the quantitative effects of various treatments on the course of neoplastic transformation.

Major Findings:

The relative ability of tumorigenic guinea pig cell lines to produce a progressively growing tumor in adult syngeneic animals is directly related to their fibrinolytic activity, their capacity to elicit a sustained tuberculin-like response in nonimmune animals and their sensitivity to guinea pig lymphotoxin. The close relationship of these properties to the tumorigenic capacity of the cells suggests that they may be among the important determinants of tumor growth. Other properties investigated, such as fibronectin production and cloning efficiency in soft agar, do not correlate with tumorigenicity. Nontumorigenic cells exhibit a markedly greater expression of Forssman cell surface glycolipid than do tumorigenic cells but this property appears unrelated to the tumorigenic ability of the cells. A cytokine that inhibits fibrinolysis is produced by guinea pig and hamster fibroblasts. The cytokine inhibits the proteinase activity of human urokinase, soluble TPA stimulated guinea pig plasminogen activator, and the cell associated plasminogen activator of tumorigenic guinea pig cells. Tumorigenic guinea pig and hamster fibroblasts as well as non-transformed guinea pig fibroblasts produce the inhibitory cytokine; the amount of inhibitor secreted is independent of the tumorigenic potential of the cells. Production of the inhibitor of normal cells may be related to contact inhibition of growth and the cytokine may contribute to the fine regulation of local proteolysis within tissues. Unique cell line specific tumor-associated neoantigens were not produced by all the tumorigenic cell lines confirming that this property is likewise unrelated to tumorigenic ability. In the hamster after 18 hour treatment of full term embryo cells with 3-methylcholanthrene, a portion of the treated cells expressed at least one cell surface neoantigen. The neoantigens(s) expressed were common to several neoplastic cell lines and to mid-gestation embryo cells. Expression of the neoantigen(s) preceded neoplastic transformation. These data indicate that neoantigen expression may be a property of preneoplastic cells; the antigens may provide a means of recognizing premalignant cell populations.

Significance to Biomedical Research and the Program of the Institute:

Many cell surface molecules or associated activities are altered on tumor cells. When these alterations occur as a consequence of chemical carcinogen treatment, they do not reflect the introduction of new genetic material but rather are due to disturbances in the regulation of normal gene products or are due to a direct action of the chemical agent on structural or regulatory genes. Altered expression of properties associated with the cell surface and involved in interactions with the cell's environment are being studied with immunochemical and physical biochemical techniques capable of identifying and isolating specific molecular components that can be functionally, as well as physicochemically, identified and their expression related to changes in specific regulatory components.

These studies will determine the importance of specific tumor cell surface and intracellular factors in carcinogenesis and in progressive in vivo growth of

tumor cells. Identification of these factors and the mechanisms(s) of their function and interrelations will provide insight into the necessary cellular changes which occur. Through this insight, points of intervention may be identified at which the carcinogenesis process may be halted or at which tumor growth may be reversed.

Proposed Course:

This is the final report for this project. It has been terminated due to the departure of the principal investigator.

Publications:

McCabe, R. P., and Evans, C. H.: The regulatory role of extracellular proteases in tumor growth. Surv. Syn. Pathol. Res. (In Press).

McCabe, R. P., Evans, C. H., and DiPaolo, J. A.: Relationship of neoantigens induced by 3-methylcholanthrene treatment of Syrian hamster embryo cells to antigens expressed on fetal and 3-methylcholanthrene-transformed neoplastic cells. Oncodevelop. Biol. and Med. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05206-03 LB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Characterization of Normal and Malignantly Transformed Cell Membranes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. P. Fuhrer Expert Scientist, LB, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biology

SECTION

Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cell surface glycoproteins and glycolipids are key recognition molecules involved in cell-cell interactions, differentiation and the cellular determination of tissue and species-specific phenotypic differences. Structural alterations in cell membrane glycoproteins and glycolipids are a direct consequence of the malignant transformations of a cell and are the likely cause of abnormal cell-cell, cell-substratum, or cell-ligand interactions characteristic of malignant cells which permit the establishment and metastatic spread of tumors. An understanding of the structures of normal membrane glycoproteins and glycolipids and of the comparable but structurally altered glycoproteins and glycolipids from chemically or virally transformed cells will identify the relevent biosynthetic lesions and guide investigations of the basic genetic alterations underlying the malignant process. Current research involves isolation of membrane glycoproteins from normal, preneoplastic, and neoplastic cell membranes by immunoprecipitation with monoclonal antibodies, characterization by computer facilitated analysis of two-dimensional IEF/SDS gel electrophoresis, and structural determination by high performance liquid chromatography (HPLC) and mass spectrometry. In the course of this work, monoclonal antibodies specific for human epithelial cells, for hamster fetal cells, and for chemically transformed hamster cell lines have been produced and a reproducible two-dimensional gel electrophoresis system for the analysis of glycoproteins has been developed. The anticarcinogenic lymphokine, lymphotoxin, has been shown to modulate glycoprotein biosynthesis in normal and transformed cells and to stimulate the synthesis in transformed cells of glycoproteins unique to normal cells. These studies have shown for the first time that transformed, tumorigenic cells synthesize normal and abnormal glycoproteins simultaneously. These results suggest that the transformation process has two different but significant effects on glycoprotein biosynthesis: one effect may cause molecular weight and negative charge, the other may cause control mechanisms to regulate inappropriate biosynthesis of structurally normal molecules.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

C. H. Evans Chief, Tumor Biology Section LB NCI

Objectives:

The primary objectives of this project are to identify changes in the molecular composition and structure of the membranes of normal and malignantly transformed cells that occur as a prerequisite to, or as a necessary accompaniment of, the transformed (neoplastic) state of a cell and to determine the molecular basis by which immunobiological modifiers participate in the control of the proliferation of neoplastic cells. Specific objectives include (1) quantitative and qualitative characterization of membrane proteins, glycoproteins, lipids, and glycolipids to differentiate normal, preneoplastic, morphologically transformed, and tumorigenic cells; (2) demonstration of quantitative and qualitative compositional and structural changes in membranes that are associated with the progress of cells toward the neoplastic state; (3) quantitative determination of compositional and/or structural changes in membranes that occur in response to the treatment of cells with chemical carcinogens, UV and X-irradiation, tumor promoters and other cocarcinogens, lymphokines and other immunological effectors; and (4) isolation, quantitation, and structural analysis of cell surface antigens and receptors from normal and transformed cells. Particular emphasis is placed on the specific nature of structural alterations that occur in the oligosaccharide portions of glycoproteins derived from cells during their progress toward neoplasia following the initiation of carcinogen-induced transformation.

Methods Employed:

This study utilizes Syrian hamster, guinea pig, and human normal, preneoplastic and neoplastic cells. Cells are metabolically labeled with radioactive protein and carbohydrate precursors or externally labeled with ^{125}I or NaB [$^{3}H_4$]. Membranes or membrane extracts are prepared from labeled cells and are analyzed for composition and structure by one and two dimensional IEF/SDS gel electrophoresis, HPLC, TLC, and other analytical and preparative biochemical and physicochemical techniques. Isolation of specific cell surface antigens for comparative quantitation or structural analysis is accomplished by indirect immunoprecipitation through the use of monoclonal antibodies. Monoclonal antibodies are produced with specificities for unique transformed cell surface antigens, for antigens present on both normal and transformed cells, for antigens expressed by cells in response to treatment with carcinogens, cocarcinogens, lymphokines, and other factors, and for antigens expressed on fetal cells that cross-react with transformed cells. One and two dimensional gels are quantitated and compared by computer-assisted data acquisition systems. Preparation of unique cell populations and studies of the distribution of cell surface antigens are accomplished by combinations of electrophoretic, centrifugal, chromatographic and cytofluorographic techniques through the use of monoclonal antibodies reactive with cell surface determinants.

Major Findings:

Major findings in this project are currently derived from two-dimensional electrophoresis studies of the expression of membrane glycoproteins of normal and transformed cells and of the effects of lymphotoxin on their biosynthesis. Lymphotoxin is a lymphokine which possesses the ability to inhibit carcinogenesis and modulate the growth of tumor cells.

Two-dimensional electrophoresis of membrane proteins and glycoproteins from a benzo(a)pyrene-induced tumorigenic hamster fibroblast cell line and non-tumorigenic hamster fetal cells demonstrated that significant differences occur in the number, molecular weight, charge, and heterogeneity of glycoproteins while non-glycosylated membrane proteins derived from the two cell types exhibit only quantitative differences. Tumorigenic cells synthesize a large number of "abnormal" glycoproteins which occur with increased negative charge and altered molecular weight but also synthesize "normal" glycoproteins which comigrate with non-tumorigenic glycoproteins on two-dimensional gels. These studies demonstrate that despite the synthesis of abnormal glycoproteins, tumorigenic cells retain the biosynthetic systems necessary to produce normal glycoproteins.

Previous studies in which normal and tumorigenic hamster cells were treated with the anticarcinogenic lymphokine, lymphotoxin, indicated that quantitative increases in high molecular weight glycoproteins occurred in normal cells while comparable glycoproteins in transformed cells decreased. Further examination of these changes by two-dimensional electrophoresis demonstrated that while high molecular weight glycoproteins increased quantitatively and other glycoproteins decreased or remained unchanged, lymphotoxin-induced changes in normal cells were only quantitative. In contrast, treatment of tumorigenic cells caused additions and deletions, as well as quantitative changes in glycoprotein expression. The most striking result of lymphotoxin treatment was the de novo synthesis in tumorigenic cell membranes of three glycoproteins previously unique only to non-tumorigenic cells and the increased synthesis of three additional glycoproteins shared by both cell types. In addition to the synthesis of these "normal" glycoproteins, lymphotoxin caused a decrease in the synthesis of a number of other glycoproteins unique to tumorigenic cells. As a result of lymphotoxin treatment, the transformed glycoprotein complement of the tumorigenic cell was reordered towards that of a normal, non-tumorigenic cell. This normalizing effect on the transformed cell glycoprotein phenotype exerted by lymphotoxin occurred at the same time as the prevention of carcinogenesis induced by lymphotoxin in normal carcinogen-treated cells and may be responsible for a reversal of carcinogen-related cell surface alterations that underly morphological transformation.

Significance to Biomedical Research and the Program of the Institute:

Detailed molecular investigations of the membranes of normal and transformed cells will elucidate the structures and mechanisms through which the immune and other physiological systems of the host interact with normal, preneoplastic, and neoplastic cells in vivo. Systematic characterization of compositional and structural changes in the cell surface and/or its components that occur as a result of treatment with chemical and physical carcinogens, modulators, effectors, etc., will provide insight into the mechanism(s) responsible for neoplastic

transformation and its control. Understanding the molecular mechanisms that underly neoplastic transformation and host control of cancer ultimately will provide the background for novel approaches to the detection, prevention and control of cancer.

Proposed Course:

The initial phase of this project encompasses qualitative and quantitative evaluations of the structural components of the membranes of normal cells which will serve as a background for characterizations of membrane changes that occur during the transition of cells from the growth-controlled to the neoplastic state. These studies are being conducted simultaneously with experiments designed to define and quantitate compositional and structural changes in membranes that occur in response to treatment of cells with carcinogens, cocarcinogens, and immunological effectors, specifically lymphokines.

Monoclonal antibodies to normal, fetal and transformed cell antigens will be used for the sorting of cell populations, analyses of antigen distribution and turnover, and for isolation of transformed cell surface antigens for structural comparison to cross-reacting normal and fetal antigens. This biochemical and immunochemical approach will permit intensive analyses of the mechanisms of carcinogenesis and of the responses of cells to host effectors.

Publications:

Fuhrer, J. P., and Evans, C. H.: The anticarcinogenic and tumor growth inhibitory activities of lymphotoxin are associated with altered membrane glycoprotein synthesis. Cancer Lett. (In press).

Fuhrer, J. P., and Evans, C. H.: Rapid separation of biologically active Syrian hamster lymphotoxin in high yield by size exclusion high-performance liquid chromatography. J. Chromotogr. 248: 427-433, 1982.

Fuhrer, J. P., Evans, C. H., Ransom, J. H., and Heinbaugh, J. A.: Identification of β-lymphotoxin as the predominant molecular class of in vitro and in vivo Syrian hamster lymphotoxin. Cell Immunol. 75: 63-70, 1983.

ANNUAL REPORT OF

THE LABORATORY OF CARCINOGEN METABOLISM

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

The major goals of the Laboratory of Carcinogen Metabolism (LCM) are to elucidate mechanism(s) of malignant transformation in human and animal cells by chemical carcinogens, to determine critical cellular and genetic factors involved in initiation, promotion and progression of these transformed cells, and to apply, whenever possible, the knowledge obtained from studying animal models towards effective prevention of cancer in man. In order to obtain these goals LCM plans, develops and conducts a research program that includes: (1) studies on chemistry, metabolism and genotoxicity of carcinogenic aromatic amines and amides as well as nitrosamines; (2) identification and characterization of exogenous and endogenous factors controlling initiation, promotion and progression in chemically induced murine hepatomas; (3) studies on regulation of gene expression and differentiation in both human and animal neoplasia, and (4) definition of the mechanism by which modifiers of cellular differentiation may inhibit and/or promote the neoplastic process.

Research in the Office of the Chief is primarily focused on studying the mechanism of chemical carcinogenesis. Several new research projects have been started which, in addition to classical biochemical methods, employ advanced computer and recombinant DNA technologies, as well as nuclear magnetic resonance measurements of changes in conformation of nucleic acids upon interaction with chemical carcinogens and mutagens. The technique of quantitative two-dimensional gel electrophoresis has been employed to study protein changes during both chemical carcinogenesis (i.e., hepatoma formation) and chemical differentiation of neoplastic cells (i.e., human leukemias and B-cell lymphomas). This type of electrophoresis allows the simultaneous separation of hundreds of polypeptides in a single polyacrylamide gel. We have acquired, and have significantly expanded, a computer-based system to automatically analyze autoradiograms of these gels. This system automatically finds and measures the amount of radioactivity in any polypeptide resolved by these electrophoretograms. The "automatch" program matches together the spot patterns found in different gels. Still other programs link together a series of gels which may constitute an experiment and allow the investigator to quantitatively follow the synthesis of any resolvable protein through that experiment, or a series of experiments. The investigator may specify various parameters and direct the computer to list those spots whose pattern of synthesis may lie within those parameters. Finally, several sophisticated computer-graphic programs allow the investigator to visually compare and follow various polypeptides which may be matched on a virtually unlimited number of electrophoretograms.

The studies on regulation of gene expression and differentiation in neoplasia employ both the techniques of molecular biology and quantitative measurement of total cellular proteins by two-dimensional gel electrophoresis. Our aim is to identify and characterize both cellular and genetic factors that are of critical importance in the oncogenic process. The experimental systems presently under study are: a variety of undifferentiated human B-cell lymphomas and human acute lymphocytic and myeloid leukemias; the human promyelocytic leukemia cell line

HL60; and chemically induced rat hepatoma cell lines and primary rat hepatocytes. The results obtained and the experiments in progress in the human lymphoma/leukemia system includes: (1) characterization of the transcriptional activity of c-mos and c-myc oncogenes in human B-cell lymphomas; (2) chemical transformation of lymphoblastoid cell lines; (3) further characterization of the role of EBV in human B-cell lymphomas; and (4) two-dimensional gel analysis of proteins associated with acute lymphocytic and myeloid leukemias using the NIH 3T3 transfection system. The results obtained and the experiments in progress in the human promyelocytic leukemia cell line HL60 includes: (1) delineation of the role of c-myc RNA expression respectively in growth and differentiation, (2) myristic acid regulation in the expression of differentiation specific proteins, and (3) two-dimensional gel analysis of HL60 whole cell proteins after chemically induced differentiation by 12-O-tetradecanoyl phorbol 13-acetate (macrophage) or by dimethylformamide (granulocyte). The results obtained and the experiments in progress in the rat hepatoma and primary hepatocytes includes: (1) partial construction of cDNA libraries using the expression vector system to trans-stilbene oxide induced rat liver for the cloning and subsequent characterization of epoxide hydratase, as well as to partially hepatectomized rat livers for the cloning and subsequent characterization of genes associated with cellular proliferation in the liver; (2) characterization of changes in transcriptional levels of genes coding for differentiation specific mRNA, specifically gamma-glutamyltranspeptidase as well as alpha-fetoprotein and albumin genes, after in vitro treatment of rat hepatoma cell lines with sodium butyrate and dexamethasone. Also the cloning of the gamma-glutamyltranspeptidase gene from rat hepatoma line 7777 is near completion.

The current research involving the identification and characterization of endogenous and exogenous factors that may control initiation, promotion and progression of chemically induced murine hepatomas includes both in vivo and in vitro studies. The in vivo studies have focused on isolation and characterization of preneoplastic cell populations. We have established a centrifugal elutriation method for isolation of different populations (based on size) of parenchymal liver cells from untreated and carcinogen treated rats. This elutriation separation has also been combined with a cell surface receptor based separation. Since an early event in the hepatocarcinogenesis is a highly significant reduction of the surface receptors for asialoglycoproteins, we have used asialofetuin (coated on tissue culture plates) as a ligand to selectively bind normal hepatocytes and thereby partially purify the preneoplastic cell population. Furthermore, we have developed an in vivo "bioassay" system for testing the growth potential, as well as the maligancy potential (invasiveness, metastasis, etc.) for the preneoplastic cells. This is accomplished by injecting the cells into the anterior chamber of the rat eye in order to observe both the growth and malignant characteristics of the different cell populations. The biochemical characterization of the preneoplastic hepatocytes includes computer assisted analysis of total cellular proteins measured by two-dimensional gel electrophoresis, and comparison to normal hepatocytes. To date, we have found relatively few (5-8) qualitative protein differences between the preneoplastic and normal hepatocytes, whereas 10-15% of the cellular proteins in the preneoplastic cells are undergoing at least a 50% quantitative change during the initiation stage when compared to normal hepatocytes.

Heme metabolism has been studied in the preneoplastic cells, and we have demonstrated in vitro that heme, in contrast to the situation in normal cells, does not cause a feedback inhibition of the delta-aminolevulenic acid synthetase activity in the transformed cells.

The in vitro studies on chemical hepatocarcinogenesis have focused on possible early events in this process. Cultured hepatocytes were treated with non-toxic concentrations of the hepatocarcinogen N-hydroxy-2-acetylaminofluorene (N-OH-AAF) in order to obtain only "initiated" cell populations. Using these cells we have begun studies concerning (1) early changes in gene expression as measured by quantitative two-dimensional gel electrophoresis of total cellular proteins; (2) the in vitro activities of sulfotransferase, deacetylase and acyltransferase, enzymes implicated in the in vivo activation of N-OH-AF to its ultimate carcinogenic form; and (3) identification and quantitation using P-32 postlabeling analysis of cellular carcinogen bound (fluorene) DNA adducts formed during initiation. Two-dimensional electrophoretic analysis of total cellular proteins from treated and untreated cells revealed few (6-8) qualitative protein changes between the groups. In contrast 10-15% of the 600-800 readily detectable proteins were undergoing at least a 50% quantitative change following treatment with N-OH-AAF. In order to examine the relationship between the covalent binding of carcinogens (aromatic amines) to cellular DNA with gene expression in early initiated cells, we have synthesized the major known or proposed deoxyguanosine and deoxyadenosine DNA adducts of N-OH-AAF. In addition, the respective 3-monophosphate and 3',5'-diphosphate adducts have also been prepared and we are currently developing a high pressure liquid chromatographic procedure for the separation and quantitation of the in vitro formed cellular DNA adducts.

We are currently attempting to construct a receptor mediated gene transfer system to utilize the highly efficient endocytosis process whereby asialoglycoproteins are taken up by normal rodent hepatocytes. This should enable us to examine the biological effects of introducing specific genes (i.e., oncogenes) into a normal highly differentiated cell. Technically, this is accomplished by covalently coupling the asialoglycoprotein to the DNA by using two reagents, N-acetyl-N'-(p-glyoxylbenzoyl)cystamine and 2-iminothiolane. The former reacts specifically with nonpaired guanine residues and upon reduction generates a free sulfhydryl group. The latter reacts with the protein to provide another sulfhydryl group which is subsquently conjugated to DNA by an intermolecular disulfide interchange reaction. The experimental model currently under study is the rat liver. The initial coupling has been done using two transformation specific viral DNAs, namely the bovine papillomavirus DNA and the cDNA clone of the Harvey RNA tumor virus. Both tumor viruses have been well characterized in terms of transforming ability. The bovine papillomavirus DNA was prepared from pBR322 recombinants and tailed with approximately 50 residues of dGTP using terminal deoxytransferase. G-tailed viral DNA was coupled to modified asialofetuin by incubation under appropriate conditions. Harvey cDNA is presently being prepared and modified in a similar manner. Uptake and expression of viral DNA coupled with asialofetuin into primary rat hepatocytes is currently being determined.

Studies on metabolism and mutagenicity of chemical carcinogens continue to be focused on the metabolic processing and the genotoxicity of selective chemical carcinogens in intact cells. Emphasis is placed on carcinogenic aromatic amines and amides and environmental contaminants such as nitrosamines. A sensitive in vitro test system, measuring bacterial mutation frequency and DNA damage in mammalian cells (Salmonella/hepatocyte system), is employed. The research is at present focused on the following areas: (1) the relative roles of metabolic activation and detoxification in determining both the mutagenic and carcinogenic potential of aromatic amines and amides; (2) the relationship between DNA damage, measured by alkaline elution technique in host cells, and the bacterial mutation frequency when known or suspected chemical carcinogens are tested in the

Salmonella/hepatocyte system; (3) the regulation of cytochrome P-450 dependent monooxygenase(s) induction during chemical hepatocarcinogenesis; and (4) the modification of both epoxide hydrolase and flavin-dependent monooxygenase activity during chemical hepatocarcinogenesis and the relationship of these enzyme activities with cell growth and differentiation. Results so far obtained include: (1) kinetic analysis of oxidative metabolism of 2-acetylaminofluorene (AAF) in liver microsomes from rat, rabbit and human subjects demonstrated that C-hydroxylation (detoxification) is catalyzed by several cytochrome P-450 isoenzymes, whereas N-hydroxylation (toxification) is catalyzed by a single cytochrome P-450 isoenzyme in all three species; (2) metabolic activation of N-acetylarylhydroxamic acids, such as N-hydroxy-2-acetylaminofluorene and N-hydroxy-phenacetin to genotoxic agents in isolated and intact liver cells from rats and mice, occurs via deacetylation; and (3) studies on epoxide hydrolase in both intact rat liver, isolated primary hepatocytes and rat hepatoma cells indicate that both microsomal and cytosolic epoxide hydrolases are under different regulatory control and that multiple forms of the microsomal enzyme exist.

The research activities in the Analytical Chemistry Section for the past year have focused on two main areas: 1. Chemistry of N-nitroso compounds. Specific emphasis includes: (a) development of new or improved synthetic methods useful in carcinogenic chemistry, and preparation of novel nitrosamines and their derivatives for chemical and biological studies; (b) the biological chemistry of nitrosamines, both in vivo and in vitro with special attention to the properties of suspected metabolic intermediates, as well as to the rate modifying effects of deuterium substitution; (c) mechanistic studies on N-nitrosation reactions of environmental interest, particularly those promoted by electrophilic species, including transition metal complexes; (d) chemical reactivity investigations, especially those aimed at developing decontamination and disposal methods useful for controlling the hazards associated with carcinogenesis research; (3) determination of the structures and stereodynamic properties of various nitrosamines, nitrosating agents, and nitrosatable precursors, using methods such as x-ray diffraction, circular dichroism, and nuclear magnetic resonance spectrometry. Possible implications of this work with respect to the overall goal of human cancer prevention are sought.

2. Chemical structure studies in carcinogenesis research. The research is focused in the following areas: (1) spectral characteristics and chemical reactivity pattern of carcinogenic aromatic amines, amides, and their N-hydroxylated and N-acetoxylated derivatives provide useful information, which may serve to explain their biological mode of action. The 2-aminofluorene derivatives are examined in detail. (2) Synthesis of stable isotope labeled open chain dialkylnitrosamines and subsequent mass spectral studies allowed a thorough rationalization of the gas phase cation radical behavior of this class of carcinogens. It was found that the significant hydroxyl radical lost in the mass spectra of nitrosamines does not involve hydrogens on carbon-1, -2 or from more distant sites but in all probability specifically from carbon-3. There is now good analogy to this finding in radical oxidation reactions in solution and in the microsomal metabolism of nitrosamines. (3) Chemical and mass spectrometric studies on various labeled analogs of several derivatives of diols, triols and tetrols of polycyclic aromatic hydrocarbons revealed selective chemical reactivities and diagnostic mass spectral patterns. (4) An active area of interest is the development of methods for derivatization and analysis of carcinogens and other bioactive materials. (a) Thus, fast atom bombardment

mass spectra on guanethidine N-oxide and electron impact spectra on its hexafluoroacetylacetone derivative provided full structural information for this enzyme substrate for flavin containing monooxygenase. (b) Improved ionization conditions were developed for analysis of nonvolatile biological materials with 4-cyanopyridinium hydrochloride. (c) Plans are initiated to adapt and improve the existing technology of sequencing polypeptides using fast atom bombardment ionization mass spectrometry and to apply it to structure studies of cell modulating factors.

LCM is involved to a minimal extent in the scientific direction of collaborative projects at present. Although the major emphasis of LCM is on intramural research, personnel of the LCM are involved as consultants or advisors on various interagency, national and international committees in the area of chemical carcinogenesis and environmental carcinogens. They furnish advice to other government agencies, to the industrial or academic world, and serve on various panels, boards or committees to deal with current problems relating to chemical carcinogens and laboratory safety. Staff members are also project monitors for various aspects of the program at FCRF.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04542-11 LCM

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemistry of N-Nitroso Compounds

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Larry K. Keefer Chief, Analytical Chemistry Section LCM, NCI

COOPERATING UNITS *(if any)*

P. Swann and R. Mace, Courtauld Inst.; M. Castegnaro, IARC; C. Day, Crystalytics; L. Jones, G. Lunn and E. B. Sansone, FCRF; A. Croisy, Institut Curie; T. Hansen, Drexel University; R. Loeppky, University of Missouri

LAB/BRANCH

Laboratory of Carcinogen Metabolism

SECTION

Analytical Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.3	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Data concerning the chemical and physical properties of carcinogenic N-nitroso compounds have been collected. Specific emphases have included: (a) development of new or improved synthetic methods useful in carcinogen chemistry, and preparation of novel nitrosamines and their derivatives for chemical and biological studies; (b) the biological chemistry of nitrosamines, both in vivo and in vitro, with special attention to the properties of suspected metabolic intermediates, as well as to the rate modifying effects of deuterium substitution; (c) mechanistic studies on N-nitrosation reactions of environmental interest, particularly those promoted by electrophilic species, including transition metal complexes; (d) chemical reactivity investigations, especially those aimed at developing decontamination and disposal methods useful for controlling the hazards associated with carcinogenesis research; (e) determination of the structures and stereodynamic properties of various nitrosamines, nitrosating agents, and nitrosatable precursors, using methods such as x-ray diffraction, circular dichroism, and nuclear magnetic resonance spectrometry. Possible implications of this work with respect to the overall goal of human cancer prevention are sought.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Shui-mei Wang	Visiting Fellow	LCM, NCI
Vanessa Vu	Staff Fellow	LCM, NCI

Objectives:

(1) To learn about mechanisms of nitrosamine formation so that strategies for preventing environmental contamination by these compounds can be developed. (2) To gather information on the chemistry of nitrosamine destruction so that procedures for intercepting them before human exposure can occur may be devised. (3) To study the interactions between N-nitroso compounds and organisms exposed to them, with the aim of inferring ways of protecting victims of unavoidable nitrosamine exposure from their carcinogenic effects. (4) To characterize the fundamental physical and chemical properties of the carcinogenic N-nitroso compounds as a means of contributing to the general fund of knowledge about such materials.

Methods Employed:

The standard methods of synthetic and mechanistic chemistry have been employed in these studies.

Major Findings:

1. We have continued to devote a strong effort to development of chemical degradation methods useful in the decontamination and disposal of carcinogenic laboratory waste. In collaboration with E. B. Sansone and G. Lunn of the Frederick Cancer Research Facility's Environmental Control and Research Program, a reductive procedure previously found to leave carcinogenic nitrosamines quantitatively to presumable innocuous products such as amines and ammonia has now been applied to other carcinogen types. It has proven equally useful for degrading hydrazines and their residues. It has also induced complete destruction of nitrosoureas, nitrosourethanes, and other nitrosamides. However, although the amount of diazoalkane produced from the nitrosamide was substantially smaller in the presence of the aluminum-nickel powder reductant than in aqueous alkali alone (currently the most commonly used reagent for laboratory destruction of nitrosamides), the existence of this toxic, explosive, and carcinogenic compound type among the degradation products was a potentially serious disadvantage of both base-induced decomposition methods.

Two alternative procedures which did not suffer this limitation were developed for the nitrosamides. Both involve dissolution of the carcinogen in strong acid, a treatment which by itself does not lead to complete disassociation of the nitroso group under all conditions. When either iron filings or sulfamic acid was added to react irreversibly with the intermediate nitrosating agent, degradation of the nitrosamide to noncarcinogenic products was quantitative in every case studied.

2. In collaboration with P. F. Swann and R. Mace of the Courtauld Institute of Biochemistry, new information concerning the metabolism of dimethylnitrosamine (DMN) has been found after administering this carcinogen and its fully deuterated analog (DMN-d_6) to rats by intraperitoneal injection of toxic doses (40 mg/kg). Complete deuteration reduced only slightly the maximal rate of metabolism when the two substrates were administered separately (V^H/V^D = 1.2). However, much larger (approximately 4-fold) deuterium isotope effects were observed when mixtures of DMN and DMN-d_6 were injected. These results have been interpreted as evidence that C-H bond cleavage is not rate-limiting in overall metabolism, but that the complex between DMN and the enzyme(s) metabolizing it in vivo freely equilibrates with unbound substrate.

Small oral doses similar to those leading to liver tumorigenesis on prolonged administration were also studied. Oral administration of 54 µg/kg of DMN-d_6 produced 1/3 less alkylation of liver DNA than did an equimolar dose of DMN. However, alkylation of DNA in the kidney was 3 times as great from DMN-d_6 as it was from DMN in these experiments. The reduction in alkylation of liver DNA correlates well with, and possibly explains, the decrease in ability of DMN-d_6 to induce liver tumors in rats. The associated increase in the alkylation of kidney DNA is consistent with a decrease in the amount of nitrosamine removed from the portal blood on the first pass through the liver.

Publications

Castegnaro, M., Eisenbrand, G., Ellen, G., Keefer, L., Klein, D., Sansone, E. B., Spincer, D., Telling, G. and Webb, K.: Laboratory decontamination and destruction of carcinogens in laboratory wastes. Some N-nitrosamines. IARC Scientific Publications No. 43. Lyon, International Agency for Research on Cancer, 1982, 72 pp.

Day, C. S., Hansen, T. J. and Keefer, L. K.: Stereochemistry of thialdine. J. Heterocyclic Chem. 19: 1301-1304, 1982.

Hansen, T. J., Croisy, A. F. and Keefer, L. K.: N-Nitrosation of secondary amines by nitric oxide via the 'Drago complex.' In Bartsch, H., O'Neill, I. D., Castegnaro, M. and Okada, M. (Eds.): N-Nitroso Compounds: Occurrence and Biological Effects. IARC Scientific Publications No. 41. Lyon, International Agency for Research on Cancer, 1982, pp. 21-29.

Keefer, L. K. and Hansen, T. J.: Primary amine use and other strategies for preventing human exposure to N-nitroso compounds: Application to cutting fluids. In Bartsch, H., O'Neill, I. K., Castegnaro, M. and Okada, M. (Eds.): N-Nitroso Compounds: Occurrence and Biological Effects. IARC Scientific Publications No. 41. Lyon, International Agency for Research on Cancer, 1982, 245-256.

Loeppky, R. N., Hansen, T. J. and Keefer, L. K.: Reducing nitrosamine contamination in cutting fluids. Food Chem. Toxicol. (In Press)

Lunn, G., Sansone, E. B. and Keefer, L. K.: Reductive destruction of hydrazins as an approach to hazard control. Environ. Sci. Tech. 17: 240-243, 1983.

Lunn, G., Sansone, E. B. and Keefer, L. K.: Safe disposal of carcinogenic nitrosamines. Carcinogenesis 4: 315-319, 1983.

Sansone, E. B., Lunn, G., Jonas, L. A. and Keefer, L. K.: Approaches to hazard control in the carcinogenesis laboratory: N-Nitroso compounds. In Bartsch, H., O'Neill, I. K., Castegnaro, M. and Okada, M. (Eds.): N-Nitroso Compounds: Occurrence and Biological Effects. IARC Scientific Publications No. 41. Lyon, International Agency for Research on Cancer, 1982, pp. 137-149.

Swann, P. F., Mace, R., Angeles, R. M. and Keefer, L. K.: Deuterium isotope effect on metabolism of N-nitrosodimethylamine in vivo in rat. Carcinogenesis (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05259-02 LCM

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemical Structure Studies in Carcinogenesis Research

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Peter P. Roller Chemist LCM, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Carcinogen Metabolism

SECTION

Analytical Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	1.9	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project involves both independent and collaborative work in chemical carcinogenesis where mass spectrometry, nuclear magnetic resonance and other chemical spectral methods can be applied to determine the structure and to confirm the identity of organic molecules. The research is focused in the following areas: (1) Spectral characteristics and chemical reactivity pattern of carcinogenic aromatic amines, amides and their N-hydroxylated and N-acetoxylated derivatives provide useful information, which may serve to explain their biological mode of action. The 2-aminofluorene derivatives are examined in detail. (2) Synthesis of stable isotope labeled open chain dialkylnitrosamines and subsequent mass spectral studies allowed a thorough rationalization of the gas phase cation radical behavior of this class of carcinogens. It was found that the significant hydroxyl radical lost in the mass spectra of nitrosamines does not involve hydrogens on carbon-1, -2 or from more distant sites but in all probability originates from carbon-3. There is now good analogy to this finding in radical oxidation reactions in solution and in the microsomal metabolism of nitrosamines. (3) Chemical and mass spectrometric studies on various labeled analogs of several derivatives of diols, triols and tetrols of polycyclic aromatic hydrocarbons revealed selective chemical reactivities and diagnostic mass spectral patterns. (4) An active area of interest is the development of methods for derivatization and analysis of carcinogens and other bioactive materials. (a) Thus, fast atom bombardment mass spectra on guanethidine N-oxide and electron impact spectra on its hexafluoroacetylacetone derivative provided full structural information for this enzyme substrate for flavin containing monooxygenase. (b) Improved ionization conditions were developed for analysis of nonvolatile biological materials with 4-cyanopyridinium hydrochloride. (c) Plans are initiated to adapt and improve the existing technology of sequencing polypeptides using fast atom bombardment ionization mass spectrometry and to apply it to structure studies of cell modulating factors.

PROJECT DESCRIPTION

Name, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Snorri S. Thorgeirsson	Chief	LCM, NCI
Narayanswami Sundaram	Visiting Fellow	LCM, NCI

Objectives:

(1) To study, in some detail, the mass spectral behavior of carcinogens such as aromatic amines, N-nitroso compounds and aromatic hydrocarbons, as well as some of their possible metabolites, and using this knowledge to develop appropriate analytical methods. (2) To apply the mass spectrometry and nuclear magnetic resonance (NMR) methods for the analysis and identification of metabolites in carcinogen activation and metabolism studies. (3) To study the basic chemistry of selected carcinogens as this might relate to their carcinogenic mode of action. (4) To elucidate the chemical structure of certain natural bioactive materials, or of their synthetic analogs, that may play a role in cancer causation and prevention mechanism. In particular, to develop the Fast Atom Bombardment (FAB) mass spectral method for sequencing of peptides and proteins with the aim of applying the method to structure studies on cell regulating peptides.

Methods Employed:

(1) Low and high resolution mass spectrometry, or gas chromatography interfaced with mass spectrometry using electron impact or fast atom bombardment ionization modes, (2) nuclear magnetic resonance spectrometry on samples in the solution state, (3) infrared spectroscopy of samples in the solution state or in salt pellet form, (4) synthesis of stable isotope labelled carcinogens using micro-scale preparative procedures, (5) chemical modification and derivatization of chemicals, (6) spectrophotometry, and (7) high pressure liquid chromatography.

Major Findings:

1. 2-Acetylaminofluorene (AAF) is a well recognized hepatocarcinogen, and its metabolism, DNA binding and biological activity has been widely studied. We initiated chemical and spectral studies to gain insight into the nature of chemical bonding in AAF and in its N-hydroxy, N-acetoxy, and other closely related derivatives. For example, the infrared spectrum indicate a polarized or hydrogen bonded amide functionality in N-hydroxy-AAF (KBr) on the basis of the observed low wave-number absorption of the amide I band found at 1615 cm^{-1}. The proton NMR spectrum of N-acetoxy-AAF exhibits an unusually broad amide methyl signal (8 Hz at half hight) relative to the much sharper acetoxymethyl signal (1 Hz at half hight), indicative of strengthened C-N bond in the molecule. Mass spectral results, published by others (Biomed. Mass Spectrom. 3: 21, 1976), were interpreted to show that the proximate carcinogen N-hydroxy-AAF could exist in two tautomeric forms and that this property would explain some of its reactivity and mode of rearrangements. In this connection we are investigating the methylation of AAF and of its N-hydroxy derivative in attempts to trap the tautomeric forms. Thus both diazomethane and dimethylsulfate treatment of N-hydroxy-AAF gave as a major

product the expected monomethylated derivative, N-methoxy-AAF. However, diazomethane also converts AAF into a monomethylated derivative, whose mass spectrum is identical to the published spectrum of the construed ring-chain tautomer derived from N-hydroxy-AAF, according to the above publication. Our results indicate this product to be a methyl acetimidate derivative of aminofluorene. A third mode of methylation by trimethylanilinium hydroxide on AAF produces mono-, di-, and trimethylated products, but none of these represent an O-methylated product. Plans are being made to chemically synthesize and observe the spectral behavior of 2-aminofluorene derivatives that would represent a stable form of nitrenium ion, the arylaminating intermediate postulated as the electrophilic species in the carcinogenic activating pathway of aromatic amines.

2. Electron ionization induced fragmentation pattern of dialkylnitrosamines provides a means of recognizing individual chemical structures of this class of carcinogens in synthetic, environmental or in biological matrices on the picomole scale. However, rational interpretation of these spectra are complicated by the large number of molecular rearrangements required before the ions are detectable in the mass spectrometer. The detailed fundamental study of nitrosamine mass spectrometry has been the subject of our research in the past several years and the publication at the end of this report deals with our earlier results. Recently we have labeled with deuterium the 2-position of N-nitrosomethyloctylamine and the 2- and 5-position of N-nitrosomethylhexylamine. Mass spectral examination of these compounds, taken together with our earlier results, indicate that the source of hydrogen for the hydroxyl radical loss from the molecular ion is not from carbon-2 of the hexyl or octyl side chain, as would be expected if a six membered transition state was favored, but is transferred most likely from carbon-3. The carbon-3 position remains to be labeled to confirm this interpretation. In a solution chemistry analogy to our gas phase studies it was found recently (Gann 74: 41, 1983) that both the microsomal metabolism and radical mediated oxidation of N-nitrosodibutylamine direct hydroxylation chiefly on carbon-3 of the side chain, allowing the speculation that even in the microsomal metabolism of N-nitrosamines the heteroatoms are actively involved in the metabolic transformation step.

3. It is generally accepted that the activated form of carcinogenic polycyclic aromatic hydrocarbons involve an epoxydiol functionality and a precursor to it is a dihydroxy compound. The aqueous solvolysis products of epoxydiols are tetrahydroxy compounds and the NADPH reduced forms and DNA bound forms in vivo and in vitro are triols. In these polyol derivatives the hydroxyl groups are vicinal to each other. We have been investigating the relative stereochemistry of these compounds by preparing their isopropylidene derivatives. The mass spectra of these derivatives are distinct and provide useful structural information. Recently we have studied the relative rate of isopropylidene derivative formation of compounds such as the vicinal cis and trans cyclohexanediols and found that with such flexible carbocyclic ring systems even the trans diols form derivatives, but at a much slower rate than the cis isomer.

4. Mass spectral studies contributed to the structural confirmation of synthetically and metabolically formed N-oxide of guanethidine, a drug used as a substrate to measure the level of flavin-containing monooxygenase activity in biological systems (Biochem. Biophys. Res. Commun. 112: 537, 1983). Fast atom bombardment ionization mass spectrum on the N-oxide demonstrated a good pseudomolecular

ion (m/z 215). The location of the oxygen atom on the tertiary heterocyclic nitrogen was confirmed by the presence of an ion at m/z 142 in the electron impact mass spectrum of the hexafluoroacetylacetone derivative of guanethidine N-oxide. This ion, with elemental composition of $C_8H_{16}NO$ corresponds to an N-oxyformaldiminium ion containing the heptamethyleneimine end of the molecule.

5. Several collaborative projects are concerned with chemical structural investigations of bioactive materials relevant to cancer prevention and causation mechanisms. In joint studies with Frolik and Sporn (LC) high resolution mass spectral and NMR studies were pivotal in confirming the cytostatic component, which was isolated from various organs, to be spermine. In joint studies with DeLuca (LCCTP) it was found that electron impact mass spectra of nonvolatile hydrogenated retinyl phosphate could become meaningful if ammonium iodide was added to the microsample where in situ displacement of phosphate with iodide took place. In attempts to increase ionization efficiency of nonvolatile compounds, such as metal salts of acids, and yet retain the rich information containing fragments provided by electron impact ionization mass spectrometry, we have found (in collaboration with Aszalos and Dinya of FDA) ammonium chloride and 4-cyanopyridinium chloride to be effective in producing spectra of salts of β-lactam antibiotics, disaccharides and vitamin C at low probe temperatures, and we plan to apply this approach to our inhouse projects.

Significance to Biomedical Research and the Program of the Institute:

The understanding of the mass spectral behavior of specific classes of organic compounds is a prerequisite to the interpretation of spectra and to the structural determinations of a number of biologically important compounds. Toward this goal, detailed spectral studies on known N-nitroso compounds, polycylic aromatic compounds and aromatic amines have been valuable. Establishing the exact molecular structure and conformation of relevant biomolecules by nuclear magnetic resonance and other techniques is necessary in a modern approach for ultimate understanding of the complex molecular transformations taking place in living systems, particularly in cancer causation mechanisms. Structural studies on metabolites of aromatic amines and of polycyclic aromatic hydrocarbons have given insight into the pathways of carcinogen degradation or activation. Identification of carcinogens, or of potential carcinogens, in the human environment or in food staples is important in developing prevention methods.

Proposed Course:

The studies described will be completed and published. A number of studies are continuing and their significance to cancer causation will be evaluated. Major emphasis will be put on development of sequencing methods for polypeptides using fast atom bombardment ionization mass spectrometry using standard polypeptides, and subsequently applying that technology to structure studies of cell cycle modulating proteins. The method is expected to be uniquely suited for structure analysis of modified polypeptides or of post-translationally altered polypeptides.

Publications

Roller, P. P., Slavin, B. W., Angeles, R. M. and Miller, J. R.: Mass spectral rearrangements of acyclic dialkylnitrosamines: N-nitrosomethyl-n-butylamine and analogs. In Bartsch, H., O'Neill, I. K., Castegnaro, M. and Okada, M. (Eds.): N-Nitroso Compounds Occurrence and Biological Effects. IARC Scientific Publications No. 41, Lyon, International Agency for Research on Cancer, 1982, pp. 199-208.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05260-02 LCM

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Gene Expression and Differentiation in Neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Carole A. Heilman Staff Fellow LCM, NCI

COOPERATING UNITS *(if any)*
I. T. Magrath, PO, DCT, NCI; T. H. Robbins, LMO, DCCP, NCI;
E. Westin, LTB, DCT, NCI

LAB/BRANCH
Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.5	1.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The object of this project is to examine the regulation of gene expression in neoplasms as well as during the normal growth and differentiation process. Our aim is to identify and characterize both cellular and genetic factors that are of critical importance in the oncogenic process. The experimental systems presently under study are: (1) a variety of undifferentiated human B cell lymphomas and human acute lymphocytic and myeloid leukemia; (2) the human promyelocytic leukemia cell line HL60; and (3) the rat hepatoma cell lines and primary hepatocytes. The results obtained and the experiments in progress in the human lymphoma/leukemia system includes (1) characterization of the transcriptional activity of c-mos and c-myc oncogenes in human B-cell lymphomas; (2) chemical transformation of lymphoblastoid cell lines; (3) further characterization of the role of EBV in human B-cell lymphomas; and (4) two-dimensional gel analysis of proteins associated with acute lymphocytic and myeloid leukemias using the NIH 3T3 transfection system. The results obtained and the experiments in progress in the HL60 cell line include: (1) delineation of c-myc RNA expression in growth or differentiation; (2) myristic acid regulation in the expression of differentiation specific proteins; and (3) two-dimensional gel analysis of HL60 whole cell proteins after chemically induced differentiation induced by 12-O-tetradecanoyl-phorbol-13-acetate (macrophage) or by dimethylformamide (granulocyte). The results obtained and the experiments in progress in the rat hepatoma and primary hepatocytes include: (1) partial construction of cDNA libraries using the expression vector system to trans-stilbene oxide induced rat liver, for the cloning and subsequent characterization of epoxide hydrolase as well as to partially hepatectomized rat livers for the cloning and subsequent characterization of genes associated with cellular proliferation in the liver; (2) characterization of changes in transcriptional levels of genes coding for differentiation specific mRNAs, specifically gamma-glutamyl-transpeptidase as well as alpha-fetoprotein and albumin genes after in vitro treatment of rat hepatoma cell lines with sodium butyrate and dexamethasone.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Snorri T. Thorgeirsson	Chief	LCM, NCI
Robert T. Maguire	Clinical Associate	LCM, NCI
Peter J. Wirth	Expert	LCM, NCI
Irene B. Glowinski	Staff Fellow	LCM, NCI

Objectives:

The objective of this project is to examine the regulation of gene expression and differentiation in neoplasia by employing both the technique of molecular biology and quantitative measurement of total cellular proteins. Our aim is to identify and characterize both cellular and genetic factors that are important in the neoplastic process. The experimental systems currently under study are: (1) a variety of undifferentiated human B-cell lymphoma lines of both American and African origin; (2) human promyelocytic leukemia cell line (HL60); and (3) the rat hepatoma cell lines or primary hepatocytes at different stages of differentiation and growth.

Methods Employed:

Methods used in these studies include: tissue culture techniques; radioimmunoassay; generation of monoclonal and polyclonal antibodies; differential centrifugation and chromatographic techniques; radioisotopic measurements using tritium, carbon-14, phosphorus-32, sulfur-35 and iodine-125; enzyme assays involving radiometric or spectrophotometric determination; and computer assisted two-dimensional gel electrophoresis; chemical synthesis; and recombinant DNA technology including generation of cDNA and genomic libraries.

Major Findings:

A. Studies on human B-cell lymphomas and human acute lymphocytic and myeloid leukemia. (1) Expression of oncogenes in human B-cell lymphomas. We have continued our studies of oncogene expression in Burkitt lymphoma cell lines using genomic clones of chicken c-myc and human c-mos to assay for the expression of c-myc or c-mos RNA in lymphoma and lymphoblastoid cell lines. Poly(A) RNA was isolated and fractionated in denaturing formaldehyde agarose gels. The RNA was blotted onto nitrocellulose paper and hybridized to in vitro nick translated c-myc or c-mos DNA. We have found a 2 to 5-fold increase in c-myc specific RNA in lymphoma lines with 8;14 or 8;22 translocations when compared to translocation negative lymphoblastoid cell lines. Tumor lines with an 8;14 or 8;22 translocation expressed similar amounts of myc specific RNA. Tumor cell lines of African origin contained slightly higher levels of myc-specific RNA than did those of American origin. However, level of expression does not appear to correlate with the presence or absence of Eptein-Barr virus. There was no evidence of mos-related transcripts in any of the cell lines tested.

(2) Chemical transformation of lymphoblastoid cell lines. Lymphoblastoid cell lines do not form colonies in soft agar when plated at 10^4-10^5 cells/60 mm dish. We are attempting to promote the growth of these cells in semisolid media by treating them with the direct acting carcinogens MNNG and N-acetoxyacetylaminofluorene (N-acetoxy-AAF). 10^7 Cells from each of 4 lymphoblastoid lines were treated overnight with MNNG (.1, .5, 1, 5 µg/ml) or N-acetoxy-AAF (1, 5, 10, 15 µg/ml). The cells were then suspended in fresh media and were subcultured 1:5 every 3-4 days for at least 20 population doublings (approximately 5 weeks) at which time they were seeded in soft agar at 10^5 cells/60 mm dish. Control cells were treated with DMSO and were then handled similarly. We will quantitate soft agar colonies for each cell line at various concentrations of carcinogen and compare this with background colony formation from the DMSO treated populations. Colonies will be picked and grown to sufficient numbers for injection into nude mice. Those that give rise to tumors will be assayed for increased myc-specific RNA expression, chromosomal abnormalities, and will be used in NIH 3T3 transfection assays in an attempt to identify altered genes responsible for tumor formation.

(3) Role of EBV in human B-cell lymphomas. The association of Epstein-Barr virus (EBV) with Burkitt lymphomas of African origin has been well documented. We are presently looking at the role this virus might play in the specific translocation events associated with this tumor. We have identified a small region within the virus that shares strong DNA sequence homology with the noncoding region of the myc gene, specifically that region which has recently been reported to be involved in the 8;14 rearrangement. Southern blot analysis of cellular DNAs from tumor cell lines of American or African origin containing EBV and infectious mononuclease cell lines strongly suggest that this fragment is integrated, at very low copy number, in these cell lines, as well as being present in free viral DNA episomes. Interestingly, this fragment is not found associated with 3/3 cellular DNAs from EBV infected cord blood lines, nor does it appear to be present in the free viral DNAs found within these lines although EBV DNA fragments adjacent to this myc-related EBV sequence show no variations in any of the B-cell lines tested. We are presently trying to identify the integration site(s) for this myc-related EBV fragment by generating a phage genomic library of one of the African Burkitt lymphoma cell lines.

(4) Two-dimensional protein gel analysis of NIH 3T3 cells transformed with DNA from ALL and AML cells. We are presently collaborating with Dr. Eric Westin in an attempt to identify new proteins in NIH 3T3 cells transformed with DNA from human acute lymphocytic and myeloid leukemias. In addition to the above, we have ^{14}C-labeled and prepared lysates from NIH 3T3 cells which were untreated, transformed with a clone of the Harvey-ras gene, or which were allowed to spontaneously transform. We have 3T3 cells after primary, secondary and tertiary rounds of transfection with DNA from two human acute lymphocytic leukemias. Two-dimensional gels have been prepared and are currently being analyzed.

B. Studies on the differentiation of the human promyelocytic leukemia cell line, HL60. (1) c-myc Specific RNA expression/association with growth or differentiation. It is well known that c-myc specific RNA levels are increased at least 10-fold in HL60 cells when compared to normal circulating white cells or lymphoblastoid cell lines. This increased level of expression can be negated if one artificially drives these cells to terminally differentiate to macrophages with

tetradecanoyl-phorbol-acetate ester (TPA). The resultant decrease in c-myc-RNA expression could be due to either the differentiation of the cells or to their lack of proliferation.

Difluoromethylornithine (DFMO) is an agent which allows one to separate differentiation related phenomena from growth related phenomena in this cell line. An ornithine analog, this chemical causes a marked decrease in polyamine synthesis and consequently HL60 cells cease to proliferate 2-3 days after being seeded in media containing 5 mM DFMO. Putrescine, if added simultaneously with DFMO, can overcome the block in proliferation. The HL60 cells cease growing but remain as healthy promyelocytes until approximately day 5 when they begin to die. We will be analyzing cells treated with either DFMO alone or DFMO plus putrescine for their level of c-myc RNA expression.

(2) Myristillation of differentiation specific proteins. It has recently been discovered that several proteins concerned with cell transformation, and therefore likely involved in growth or differentiation, have long chain fatty acids (especially myristate or palmitate) covalently bound to their N-terminus. The proteins seem to interact with cell membranes via these fatty acid additions. We are exploring whether proteins with covalently bound myristate groups might play a role in the differentiation of HL60 promyelocytic leukemia cells. We are therefore treating HL60 cells with TPA to induce macrophage differentiation. We then label these cells and untreated control cells with ^{14}C myristate. After approximately 6 hours the cells are thoroughly washed and lysed and lysates are prepared for analysis by two-dimensional gel electrophoresis.

(3) Two-dimensional gel analysis of HL60 cell proteins after chemically induced differentiation. TPA induces HL60 promyelocytic leukemia cells to differentiate terminally towards macrophages, while hexamethylene bisacetamide (HMBA) and dimethylformamide (DMF) cause HL60 cells to differentiate towards granulocytes. We have treated HL60 cells with these agents and prepared ^{14}C-labeled cell lysates for protein analysis on two-dimensional gels at 8, 24, 48, 72 and 96 hours post-treatment, using untreated HL60 cells as controls. Two-dimensional gels have been run and will be analyzed by a computer program designed in our laboratory by Mark Miller and Arthur Olson. Previously, we showed that in vitro translates of RNAs from HL60 cells could be analyzed in this fashion.

C. Studies on rat hepatomas and primary hepatocytes. (1) Rat liver cDNA libraries. We are presently constructing cDNA libraries using the expression vector system recently described by Okayama and Berg (Mol. Cell. Biol. 3, 1983) to a) trans-stilbene oxide (TSO) induced rat livers as well as to b) liver poly(A) RNA obtained from a rat after partial hepatectomy. The advantages of this technique compared to the classical methods include 1) the increased number of recombinant DNA clones, and 2) the direct expression of cDNAs following transfection into eukaryotic cells.

(a) Epoxide hydrolase. Epoxide hydrolase catalyzes the conversion of epoxides to dehydrodiols and at least two distinct forms of this enzyme have been identified. Moreover, various hepatocarcinogens have been shown to induce this enzyme in preneoplastic liver foci. To gain further insight into the regulation of this family of microsomal and cytosolic enzymes, a cDNA library made from a trans-stilbene oxide induced rat liver is presently under construction. Since

the mRNA(s) coding for this enzyme(s) is present in low amounts (0.02% of membrane bound polysomal mRNA) and is not appreciably increased in any of the hepatoma cell lines, TSO induced poly(A) RNAs from rat liver are being used (estimated 100-fold increase in epoxide hydrolase mRNA). Following identification of the cDNA clone(s) using differential screening, verification of highly positive cDNA clones will be done by hybrid selection, in vitro translation and immunoprecipitation. Dr. S. Park kindly made available to us various epoxide hydrolase monoclonal antibodies for purposes of this screening. Using this cloning protocol we hope to obtain and identify the various classes of epoxide hydrolase specific mRNAs and study their regulation following transfection into appropriate eukaryotic cells.

(b) Characterization of genes associated with rapid growth of differentiated tissue. The delineation between growth and differentiation is often difficult to define. The rat liver partial hepatectomy system provides us with an excellent model system to characterize growth specific genes which are expressed in rapidly proliferating, yet differentiated tissue. To explore the nature of these genes, poly(A) RNA from partial hepatectomized rat livers in rapid stage of growth was obtained. The cDNAs will be cloned in the expression vector system and growth associated genes identified by differential screening using fully differentiated and growth arrested poly(A) RNA from rat liver as a negative control. Genes will be functionally tested in the appropriate eukaryotic transfection system and initially characterized using computer-assisted two-dimensional protein gel electrophoretic pattern.

(2) Characterization of changes in transcriptional levels of genes coding for differentiation specific mRNA. (a) γ-Glutamyl transpeptidase (γGT) catalyzes the transfer of the γ-glutamyl moiety of glutathione (or artificial substrates) to amino acid or peptide acceptors. In rats or mice, γGT activity is high in fetal liver and is associated with the plasma membrane of the biliary tract. At birth, this activity begins to fall and by 6-10 days postnatal has reached the characteristically low levels of the adult hepatocyte. Certain non-carcinogenic manipulations such as portacaval anastomosis and phenobarbital pre-treatment can cause 2-8 fold elevations of hepatocyte γ-GT activity. However, in rats or mice maintained on diets of various carcinogens or after single high dose carcinogen treatment, liver γ-GT activity can increase up to approximately 50-fold. Most transplantable hepatomas are strongly γ-GT positive and exhibit activities approximately 50-100 times that of normal liver. Of the various biochemical markers used to identify preneoplastic foci, γ-GT identifies a very high proportion when compared with other markers.

We are presently cloning the γ-GT gene in order to study its regulation and expression. A cDNA library from rat hepatoma cell line 7777 has been made using mRNA isolated from this cell line which yielded approximately 30,000 recombinant-containing bacteria. 7777 Cells have γ-GT activity approximately 20-30 fold elevated when compared to the Reuber hepatoma (H-4-II-E) cell enzyme. In order to isolate the specific recombinant clone, we have used a differential gene expression method. 2000 Recombinant clones were plated onto Whatman 541 filter paper, grown and lysed. ^{32}P-cDNA was made from both 7777 and Reuber mRNAs and separately hybridized to the replicate filters. Following autoradiography, a comparison of the hybridization reactions yielded approximately 90 colonies possessing a higher degree of hybridization using ^{32}P-cDNA 7777 than with ^{32}P-cDNA-

Reuber. The DNAs from these colonies were grown, isolated, purified, immobilized on nitrocellulose filters and used for hybrid selection of specific mRNA and translation. Immunoprecipitation using goat anti-rat-kidney-γ-GT revealed three possible clones. The low availability of the γ-GT antibody as well as its non-specific binding has encouraged us to make antibodies to 7777 cell purified γ-GT for more specific characterization of the cDNA.

γ-GT from 7777 cells was purified by a procedure including detergent extraction, ammonium sulfate precipitation, and Sephadex and affinity column chromatography. Polyclonal antibodies are being made in both a sheep and a rabbit, and will be affinity purified. Monoclonal antibodies are being made in mice. Briefly, mice are injected with 100 μg purified γ-GT in Freunds complete adjuvant once per week for 4 weeks. They are bled and tested for the presence of antibodies by RIA using ^{35}S-mouse anti-IgG. When positive for γ-GT antibodies, the spleens will be removed, hybridized to mouse myeloma cells and grown in culture under selection conditions. The presence of specific γ-GT antibodies will again be tested by RIA. Positive hybrids will be single-cell cloned, tested by RIA and used for immunoprecipitation of in vitro translated γ-GT from hybrid selected mRNA.

(b) Chemical modulation of differentiation specific RNAs and proteins. We have previously demonstrated that the rat hepatoma cell line 7777, a poorly differentiated cell line, contains approximately 160-fold higher levels of α-fetoprotein (AFP)/specific RNA as compared to the highly differentiated hepatoma cell line, Reuber H-4-II-E, and approximately a 20-fold lower level of albumin specific RNA as compared to Reuber. These values closely parallel the relative values observed for AFP or albumin protein synthesis in these cell lines as measured by radioimmunoassay. We are presently comparing the levels of AFP and albumin RNA synthesis in sodium butyrate (BA) and dexamethasone (DEX) treated 7777 in an attempt to further define the mechanism governing the regulation of synthesis of differentiation specific proteins by chemicals, a mechanism previously shown to be dependent on the stage of differentiation of the target cell. Radioimmunoassay levels of AFP in 7777 cells grown in defined media in the presence of 10^{-6} M DEX showed a constant decrease in AFP levels. Following 120 hours of DEX treatment, the level was approximately 50% that of the control. Treatment of 7777 cells with 2 mM BA showed no change in the level of AFP as compared to controls up to 96 hours post-treatment. Preliminary evidence suggests that the level of AFP specific transcripts closely parallels the radioimmunological data. Treatment of 7777 cells with 10^{-6} M DEX also resulted in a decrease in albumin synthesis in these cells to a level approximately 50% that of the control. Interestingly, treatment of these undifferentiated cells with 2 mM BA resulted in a 3 to 4-fold increase in albumin synthesis following 120 hours of treatment. The relative changes in albumin specific RNA levels are presently being determined.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying and characterizing both the cellular and genetic factors important in chemically induced and spontaneous neoplasia. The information obtained from these studies could provide a basis for a better definition of the factors involved in cancer cause and may help in formulating an effective cancer prevention program.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications

Benjamin, D., Magrath, I. T., Maguire, R., Janus, C., Todd, M. D. and Parsons, R. G.: Immunoglobin secretion by cell lines derived from African and American undifferentiated lymphomas of Burkitt's and non-Burkitt's type. J. Immunol. 129: 1336-1342, 1982.

Engel, L., Heilman, C. A. and Howley, P. M.: The transcriptional organization of BPV type 1. J. Virol. (In Press)

Maguire, R. T., Robins, T. S., Thorgeirsson, S. S. and Heilman, C. A.: Expression of cellular myc and mos genes in undifferentiated B cell lymphomas of Burkitt and non-Burkitt type. Proc. Natl. Acad. Sci. USA 80: 1947-1950, 1983.

Osofsky, S., Portman, R., Maguire, R., Saul, R. A., Duncan, J. and Kredich, D.: Systemic lupus erythematosis and nephrotic syndrome in infancy. Pediatrics (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05261-02 LCM

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Metabolism and Mutagenicity of Chemical Carcinogens

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Snorri S. Thorgeirsson Chief, LCM, NCI

COOPERATING UNITS *(if any)*
E. Dybing, National Institute of Public Health, Oslo, Norway;
E. Johnson, Scripps Clinic and Research Foundation, La Jolla, CA.

LAB/BRANCH
Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	4.5	2.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to identify and characterize, in an intact cell system, the metabolic processes that determine genotoxicity of known or suspected chemical carcinogens. Emphasis is placed on carcinogenic aromatic amines and amides, and environmental contaminants such as nitrosamines. A sensitive in vitro test system, measuring bacterial mutation frequency and DNA damage in mammalian cells (Salmonella/hepatocyte system), is employed. The research is at present focused on the following areas: (1) the relative roles of metabolic activation and detoxification in determining both the mutagenic and carcinogenic potential of aromatic amines and amides; (2) the relationship between DNA damage, measured by alkaline elution technique, in host cells and the bacterial mutation frequency when known or suspected chemical carcinogens are tested in the Salmonella/hepatocyte system; (3) the regulation of cytochrome P-450 dependent monooxygenase(s) induction during chemical hepatocarcinogenesis; and (4) the modification of both epoxide hydrolase and flavin-dependent monooxygenase activity during chemical hepatocarcinogenesis and the relationship of these enzyme activities with cell growth and differentiation. Results so far obtained include: (1) kinetic analysis of oxidative metabolism of 2-acetylaminofluorene (AAF) in liver microsomes from rat, rabbit and human subjects demonstrated that C-hydroxylation (detoxification) is catalyzed by several cytochrome P-450 isoenzymes, whereas N-hydroxylation (toxification) is catalyzed by a single cytochrome P-450 isoenzyme in all three species; (2) metabolic activation of N-acetylarylhydroxamic acids such as N-hydroxy-2-acetylaminofluorene and N-hydroxy-phenacetin to genotoxic agents in isolated and intact liver cells from rats and mice occurs via deacetylation; and (3) studies on epoxide hydrolase in both intact rat liver, isolated primary hepatocytes and rat hepatoma cell lines indicate that both microsomal and cytosolic epoxide hydrolases are under different regulatory control and that multiple forms of the microsomal enzyme exist.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Michael E. McManus	Visiting Associate	LCM, NCI
Irene B. Glowinski	Staff Fellow	LCM, NCI
Satoru Hayashi	Visiting Fellow	LCM, NCI
Chehab Razzouk	Visiting Fellow	LCM, NCI
Ritva P. Evarts	Veterinary Medical Officer	LCM, NCI
Peter J. Wirth	Expert	LCM, NCI
Mona Moller	Guest Researcher	LCM, NCI
Preston H. Grantham	Chemist	LCM, NCI

Objectives:

The main objectives of the project are: (1) to define, in an intact cell system, the metabolic processing of chemical carcinogens, especially carcinogenic aromatic amines and amides, and to identify the metabolic pathways that are responsible for activation and detoxification of these compounds; (2) to study the mechanism whereby carcinogenic aromatic amines and amides cause mutations and other types of genotoxicity in both microbial and mammalian cell systems; and (3) to examine and characterize the regulation and expression of cytochrome P-450 dependent monooxygenase(s), the flavin-dependent monooxygenase and epoxide hydrolase activities in normal rat hepatocytes during chemical hepatocarcinogenesis.

Methods Employed:

The principal methods employed are: (1) bacterial and mammalian culture techniques, (2) differential centrifugation, (3) enzyme assays, (4) recording spectrophotometry, and (5) high pressure liquid chromatography.

Major Findings:

A. Cytochrome P-450 dependent metabolism. The cytochrome P-450 dependent metabolism of the model hepatocarcinogen 2-acetylaminofluorene (AAF) has been studied in rat, human and rabbit liver microsomes and by purified forms of rabbit cytochrome P-450 to: (a) elucidate the balance between metabolic activation (N-hydroxylation) and detoxification (C-hydroxylation) in the oxidative processing of this carcinogen, (b) assess what effects modulators of the cytochrome P-450 system may have on the hepatocarcinogenesis of AAF, and (c) examine the usefulness of AAF as a probe for isoenzymes of cytochrome P-450.

(1) Human Study. The kinetics of 2-acetylaminofluorene (AAF) metabolism was studied in liver microsomes from four human subjects over a substrate range of 0.02-200 μM. The N-hydroxylation of AAF was best described by a single enzyme system with a mean Km of 1.63 μM and a mean Vmax of 61 pmol mg^{-1} protein min^{-1}. Biphasic kinetics for the 7-hydroxylation of AAF in all four subjects were observed and a two enzyme system best described the data. The mean Km and Vmax for the high affinity, low capacity enzyme were 0.69 μM and 30 pmol mg^{-1} protein min^{-1}, respectively, while for the low affinity, high capacity

enzyme the mean Km was 75 μM and the mean Vmax was 286 pmol mg^{-1} min^{-1}. In one of the four subjects studied the 5-hydroxylation of AAF was similarly resolved into a two enzyme system. The 1-hydroxylation of AAF in human microsomes was a major reaction and was best described by a single enzyme system. The 3- and 5-hydroxylation of AAF were minor metabolic pathways. Non-classical Michaelis-Menten kinetics were observed for the 9-hydroxylation of AAF and at high substrate concentrations this was the major metabolite formed. These data demonstrate the involvement of human cytochrome P-450 in the metabolism of AAF.

(2) Rat study. The metabolism of 2-acetylaminofluorene (AAF) has been studied in male Sprague-Dawley rat liver microsomes over a concentration range of 0.02-300 μM, and kinetic parameters determined for five oxidative pathways. The N-hydroxylation of AAF was best described by a single enzyme system with a mean Km of 0.033 μM and a mean Vmax of 3.63 pmol mg^{-1} min^{-1}. Pretreatment of animals with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) caused a marked induction of N-hydroxylase activity while phenobarbital had no effect. Biphasic kinetics for the 7-hydroxylation of AAF were observed in both control, TCDD and phenobarbital induced microsomes. The high affinity Km (0.051 ± 0.015 μM; mean ± SEM, n = 3) in control microsomes was three orders of magnitude lower than the low affinity Km (103 ± 16 μM; mean ± SEM, n = 3) indicating that each isoenzyme predominated at vastly different substrate concentrations. The mean Vmax's for the low and high affinity enzymes were 3.5 and 1351 pmol mg^{-1} min^{-1}, respectively. TCDD pretreatment markedly induced the activity of the low-capacity enzyme and reduced the activity of the high-capacity enzyme. Phenobarbital caused a significant induction of both enzyme pathways. Biphasic kinetics were also observed for the 5-, 3- and 1-hydroxylations of AAF in control and phenobarbital induced microsomes, but in TCDD pretreated microsomes only 1-hydroxylation exhibited biphasic kinetics. TCDD caused a marked induction of these metabolic pathways while pheno-phenobarbital had no effect. Non-classical kinetics were observed for the 9-hydroxylation of AAF and at high substrate concentrations detoxification via this pathway and 7-hydroxylation predominated. However, at low concentrations metabolic activation of AAF via N-hydroxylation was a major pathway. These data indicate that multiple forms of cytochrome P-450 are involved in AAF metabolism and that the balance between metabolic activation and detoxification of this substrate is dependent on both concentration and previous exposure to inducers.

(3) Rabbit study. The kinetics of AAF metabolism was studied using two purified forms of rabbit cytochrome P-450, and in control and TCDD induced liver microsomes from the same species. Kinetic parameters were defined for the 7-, 9-, 5-, 1- and N-hydroxylations of AAF. Both the 7- and 9-hydroxylations were best defined by a two enzyme system and the others by a single enzyme component. In TCDD microsomes kinetic parameters could only be defined for the 7-, 1- and N-hydroxylations of AAF. The 7-hydroxylation of AAF was again best described by a two enzyme system and both components were differentially affected by this pretreatment. Form 6, the major isoenzyme of cytochrome P-450 induced in neonatal rabbits, hydroxylated AAF solely in the 7-position and the Km and Vm for this reaction were 0.37 μM and 1125 pmol/nmol P-450/min, respectively. Form 4, the major purified form of cytochrome P-450 induced in adult rabbits, preferentially N-hydroxylated AAF. The Km and Vmax for this reaction were 0.35 μM and 775 pmol/nmol P-450/min, respectively. In TCDD microsomes these values were 0.25 μM and 737 pmol/mg protein/min, respectively. Clearly, the microsomal N-hydroxylation of AAF allows for an accurate determination of the isoenzyme of P-450 involved in this reaction.

B. Flavin-containing monooxygense enzyme. The flavin-containing monooxygenase (FCMO) is present in both hepatic and extrahepatic tissues and can comprise up to 4% of total microsomal protein. This enzyme has been demonstrated in vitro to activate with high activity many nitrogen and sulfur-containing compounds to mutagenic, carcinogenic, and cytotoxic metabolites. However, as many of these compounds are also potential substrates for the cytochrome P-450 system, its in vivo role in their toxicity has not been fully defined. In addition, the physiological role of this enzyme has not been established. Knowledge concerning the physiological role of FCMO is limited partly due to the fact that attempts to study this monooxygenase at the cellular level have been hampered by the instability of its reaction products, or competing enzyme pathways for its substrates. We have currently established a method based on guanethidine N-oxidation to study this enzyme in cell culture.

The usefulness of guanethidine N-oxide formation as a measure of cellular flavin-containing monooxygensae activity was assessed using the purified hog liver enzyme, rat liver microsomes and hepatocytes. The apparent Km and Vmax for this reaction in hepatocytes were 0.30 + 0.20 mM and 0.81 + 0.36 nmole per 10^6 cells min^{-1}, respectively. The Km for the purified enzyme was 0.31 mM and the Vmax was 0.56 nmole per µg enzyme min^{-1}. 2-Diethylaminoethyl-2,2-diphenyl valerate (SFK-525A) at a concentration of 0.5 mM had no effect on guanethidine N-oxide formation by either rat liver microsomes or the purified enzyme. In contrast 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) at the same concentration caused greater than a 100% increase in the microsomal production of guanethidine N-oxide. The tertiary amines, imipramine, chlorpromazine and methylpyrilene, inhibited N-oxide formation by both hepatocytes and the purified enzyme. These data indicate that guanethidine N-oxide formation can be used as a measure of cellular flavin-containing monooxygenase activity.

C. Epoxide hydrolase. Epoxide hydrolase catalyzes the conversion of epoxides to dihydrodiols and at least two distinct forms of this enzyme have been identified. Epoxides of many xenobiotics are strong electrophiles capable of covalent interaction with critical cellular macromolecules. Epoxide hydrolase has been implicated in both the detoxification and metabolic activation of the carcinogen 3,4-benzpyrene. Moreover various hepatocarcinogens have been shown to induce this enzyme in preneoplastic liver foci. To gain further insight into the regulation of this enzyme, we have studied its expression in cultured hepatocytes and rat hepatoma cell lines. Styrene oxide and benzpyrene 4,5-oxide were used as probes for microsomal epoxide hydrolase(s) activity and trans-stilbene oxide for the cytosolic enzyme.

In freshly isolated hepatocytes microsomal epoxide hydrolase activity was 7.7 and 10.8 nmol/mg cell protein/min with benzpyrene-4,5-oxide and styrene-7,8-oxide as substrates, respectively. Cytosolic epoxide hydrolase activity in the same cells was 195 pmoles/mg cell protein/min. Microsomal epoxide hydrolase activities increased by greater than 50% at 24 hours in cultured hepatocytes and remained elevated over 96 hours. However, cytosolic epoxide hydrolase activity had decreased by half at 24 hours and was 3% of freshly isolated hepatocytes after 96 hours in culture. A similar trend for microsomal epoxide hydrolase(s) activity was seen in hepatocytes cultured from rats pretreated with 2-acetylaminofluorene, N-hydroxy-acetylaminofluorene and trans-stilbene

oxide, except activities were at least 100% higher. None of these xenobiotics affected the activity of the cytosolic enzyme and its activity declined in culture as in control hepatocytes.

No significant difference was seen in epoxide hydrolase activity with styrene oxide as substrate between freshly isolated hepatocytes and the hepatoma cell lines McA-RH 7777 and Reuber H-4-II-E. However, when benzpyrene-4,5-oxide was used the McA-RH 7777 and H-4-II-E cell lines had 40 and 6% of the activity of freshly isolated hepatocytes. The differential expression of microsomal epoxide hydrolase in the two hepatoma cell lines, when assayed by either benzpyrene-4,5-oxide or styrene-7,8-oxide, is suggestive of more than one form of this enzyme. This observation was corroborated by immunoprecipitation studies of epoxide hydrolase using an antibody raised against a purified rat liver microsomal form of the enzyme. Studies were conducted where whole cell protein from sonicated hepatocytes and the two hepatoma cell lines were solubilized and immunoprecipitated. Cytosolic epoxide hydrolase activity in these two hepatoma cell lines was approximately 50% of freshly isolated hepatocytes and was not affected by the antibody. These data indicate that both microsomal and cytosolic epoxide hydrolases are under different regulatory control and that multiple forms of the microsomal forms of the microsomal enzyme exist. Epoxide hydrolase activity determined using benzpyrene-4,5-oxide was immunoprecipitated in all three cells, whereas the antibody had no effect on activity determined using styrene-7,8-oxide as a substrate.

D. Genotoxicity of N-hydroxy-acetylaminofluorene (N-OH-AAF), N-hydroxy-phenacetin (N-OH-P) and their derivatives in Reuber cells. The highly differentiated rat hepatoma cell, Reuber H-4-II-E, was used as a target cell to examine the metabolic processing and genotoxic effects of N-hydroxy-acetylaminofluorene (N-OH-AAF), N-hydroxy-phenacetin (N-OH-P) and their derivatives. In alkaline elution assays, dose-dependent DNA damage was observed after incubation of Reuber cells with N-hydroxy-aminofluorene (20-100 μm), N-acetoxy-acetylaminofluorene (5-25 μM), and N-hydroxy-phenetidine (20-200 μM). N-OH-AAF (20200 μM) caused low levels of DNA damage which was inhibitable by the deacylase inhibitor, paraoxon. In contrast, N-OH-P, in doses up to 1000 μM caused no observable genotoxicity.

N-OH-AAF can be metabolically activated by deacylation and acyltransfer. Both these enzyme activities are non-detectable in Reuber cells. Thus, a reconstituted system containing Reuber cells, N-OH-AAF or N-OH-P, and partially purified fractions of either deacylase or acyltransferase was created in order to assess which pathway is important with respect to genotoxicity. Incubation of Reuber cells with guinea pig deacylase and N-OH-AAF caused large dose-dependent increases in DNA strand breaks, and this damage was completely inhibited by pretreatment with paraoxon. Substitution of deacylase with rabbit acyltransferase in this system also caused large dose dependent increases in DNA damage. However, both paraoxon pretreatment and addition of tRNA were only slightly effective in blocking the damage. By contrast, the combination of N-OH-P with either of these partially purified enzymes did not cause any genotoxicity, presumably due to low rate of deacetylation of this substrate.

Thus it is clear that the metabolites formed outside the cell are capable of passing both the cellular and nuclear membranes to cause genotoxicity. Additionally, both deacylase and acyltransferase appear to be acting via a deacetylation mechanism to cause DNA damage in the presence of N-OH-AAF.

E. Aromatic amine induced DNA damage in mouse hepatocytes. N-Hydroxylation of aromatic amines and amides has been shown to be the rate limiting step in the in vitro mutagenic activation of these compounds by subcellular fractions from mouse liver. In order to examine the relative roles of metabolic activation vs detoxification pathways in the potential genotoxicity of carcinogenic aromatic amines and amides, DNA damage was measured in primary mouse hepatocytes after exposure to 2-acetylaminofluorene (AAF), 2-aminofluorene (AF) and other AAF derivatives using the alkaline elution technique. The importance of N-hydroxylation in the genotoxicity caused by AF and AAF was studied in intact hepatocytes from aryl hydrocarbon responsive C57BL/6N (B6) and nonresponsive DBA/2N (D2) mice, after pretreatment with the cytochrome P-448 inducer 2,3,7,8-tetrachlorodibenzodioxin (TCDD). AAF metabolism was measured in hepatocytes from both control and TCDD-treated animals were quantitated using high pressure liquid chromatography (HPLC).

Low levels of DNA damage were observed after exposure of hepatocytes to either AF or AAF (50-200 μM) while both N-hydroxy-2-acetylaminofluorene (N-OH-AAF) and N-acetoxy-2-acetylaminofluorene (N-OAc-AAF) showed clear dose-dependent increases in DNA strand breaks (5-100 μM). Treatment of hepatocytes with paraoxon, an inhibitor of microsomal deacetylase activity, prior to exposure to either N-OH-AAF or N-OAc-AAF, inhibited the DNA damage caused by these agents, indicating that these compounds are causing genotoxic effects after deacetylation.

The deacetylated metabolite, N-OH-AF, caused only low levels of DNA damage. However, after reducing intracellular glutathione levels by more than 50% with diethylmaleate, the damage caused by N-OH-AF was increased in a dose-dependent manner. This suggests that in untreated hepatocytes, N-OH-AF is effectively detoxified (reduced) before it can reach the nucleus.

Pretreatment of B6 and D2 mice with TCDD prior to isolation of hepatocytes did not lead to any significant increase in the amount of damage caused by either AF or AAF. Furthermore, no difference was observed between the B6 and D2 mice. These results indicate that mouse hepatocytes are capable of either detoxification of the resulting genotoxic metabolites and/or that C-hydroxylation (detoxification) is preferentially induced in the responsive B6 mice.

In order to study this in more detail, AAF metabolites produced in hepatocytes isolated from B6 mice were quantitated using HPLC. In hepatocytes from control mice, 7-OH-AAF (detoxification) was the major metabolite (174 pmol/min/10^6), and the rate of N-hydroxylation was low (12 pmol/min/10^6 cells) corresponding to approximately 4% of the total AAF metabolism. Pretreatment of the mice with TCDD increased the rate of formation of all metabolites, and even though the rate of N-hydroxylation was increased 20-30 times, C-hydroxylation pathways were still responsible for approximately 90% of the metabolism.

The present results clearly show that mouse hepatocytes have a high capability of detoxifying aromatic amines, mainly through the C-hydroxylation pathways. Since the levels of N-OH-AAF sulfotransferase and N,O-acyltransferase are very low in mouse liver cells, our data suggest that the deacetylase is playing an important role in the genotoxicity of N-OH-AAF. However, even after induction of the N-hydroxylation pathway, the cells are able to detoxify N-OH-AAF, most likely through an increase in glucuronidation and/or an efficient reduction of N-OH-AF by glutathione before it reaches the nucleus.

Intact, isolated liver cells provide a system in which competing metabolic pathways, availability of cofactors, and cellular defense mechanisms are operating. It is therefore an appropriate model for studying the metabolic processes involved in the hepatic genotoxicity and possible carcinogenicity of arylamines in this species.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at providing a better understanding of the metabolic processes that determine activation and/or detoxification of procarcinogens. We are also studying the mechanism whereby chemical carcinogens exert their genotoxic effects in both microbial and mammalian cell systems. The information derived from these studies may provide a sounder basis for possible prevention (chemoprevention) of chemically induced tumors as well as identifying individuals at risk to develop cancer.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications

Everson, R. B., Gal-el-Mawla, N. M., Attia, M. A. M., Chevlen, E. M., Thorgeirsson, S. S., Alexander, L. A., Flack, P. M., Staiano, N. and Ziegler, J. L.: Analysis of human urine for mutagens associated with carcinoma of the bilharzial bladder by the Ames Salmonella plate assay: Interpretation employing quantitation of viable lawn bacteria. Cancer 51: 371-377, 1983.

Hongslo, J., Haug, L. T., Wirth, P. J., Moller, M., Dybing, E. and Thorgeirsson, S. S.: Influence of conjugation reactions on the mutagenicity of aromatic amines. Mutat. Res. 107: 239-247, 1983.

Mattison, D. R., Chang, L., Thorgeirsson, S. S. and Shiromizu, K.: The effects of cyclophosphamide, azathioprine, and 6-mercaptopurine on oocyte and follicle number in C57BL/6N mice. Fertil. Steril. (In Press)

McManus, M. E., Grantham, P. H., Cone, J. L., Roller, P. P., Wirth, P. J. and Thorgeirsson, S. S.: Guanethidine N-oxide formation as a measure of cellular flavin-containing monooxygenase activity. Biochem. Biophys. Res. Commun. 112: 437-443, 1983.

McManus, M. E., Minchin, R. F., Sanderson, N. D., Wirth, P. J. and Thorgeirsson, S. S.: Kinetic evidence for the involvement of multiple forms of human liver cytochrome P-450 in the metabolism of acetylaminofluorene. Carcinogenesis (In Press)

McManus, M. E., Minchin, R. F., Sanderson, N., Wirth, P. J. and Thorgeirsson, S. S.: Kinetics of N- and C-hydroxylations of 2-acetylaminofluorene in male Sprague-Dawley rat liver microsomes. Cancer Res. (In Press)

Moller, M., Glowinski, I. B. and Thorgeirsson, S. S.: Aromatic amine induced DNA damage in mouse hepatocytes. In: Rydström, J., Montelius, J. and Bengtsson, M.: Extrahepatic Drug Metabolism and Chemical Carcinogenesis. Amsterdam, Elsevier/North-Holland Biomedical Press. (In Press)

Shiromizu, K., Thorgeirsson, S. S. and Mattison, D. R.: The effect of cyclophosphamide on oocyte and follicle number in Sprague-Dawley rats, C57BL/6N and DBA/2N mice. Cancer Res. (In Press)

Staiano, N., Erickson, L. C., Smith, C. L., Marsden, E. and Thorgeirsson, S. S.: Mutagenicity and DNA damage induced by arylamines in the Salmonella/hepatocyte system. Carcinogenesis 4: 161-167, 1983.

Thorgeirsson, S. S.: Metabolic determinants in the carcinogenicity of aromatic amines. In Kramer, F. and Oesch, F. (Eds.): Hoechst Workshop Conference Series on Biochemical Basis for Chemical Carcinogenesis. New York, Raven Press. (In Press)

Thorgeirsson, S. S., Erickson, L. C., Smith, C. L. and Glowinski, I. B.: Genotoxicity of N-acetylarylamines in the Salmonella/hepatocyte system. Environ. Health Perspect. 49: 141-145, 1983.

Thorgeirsson, S. S., Glowinski, I. B. and McManus, M. E.: Metabolism, mutagenicity and carcinogenicity of aromatic amines. In Hodgson, E., Bend, J. and Philpot, R. M. (Eds.): Reviews in Biochemical Toxicology. New York, Elsevier/North-Holland, Inc., Vol. 5, 1983, pp. 349-386.

Thorgeirsson, S. S., McManus, M. E. and Glowinski, I. B.: Metabolic processing of aromatic amines. In Mitchell, J. and Horning, G. (Eds.): Drug Metabolism and Drug Toxicity. New York, Raven Press. (In Press)

Thorgeirsson, S. S., Sanderson, N., Park, S. S. and Gelboin, H. V.: Inhibition of 2-acetylaminofluorene oxidations by monoclonal antibodies specific to 3-methylcholanthrene-induced rat liver cytochrome P-450. Carcinogenesis 4: 639-641, 1983.

Thorgeirsson, S. S., Wirth, P. J. and Evarts, R. P.: Early changes in gene expression during hepatocarcinogenesis. In Rydstrom, J., Montelius, J. and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogenesis. Amsterdam, Elsevier/North-Holland Biomedical Press. (In Press)

Thorgeirsson, S. S., Wirth, P. J. and Smith, C. L.: Genetics and induction of cytochrome P-450 dependent monooxygenases. In Flamm, W. G., Lorentzen, R. J., and Andrews, L. S. (Eds.): Handbook of Experimental Pharmacology. New York, Springer-Verlag. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05262-02 LCM

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of Chemically Induced Murine Hepatomas

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Ritva P. Evarts Veterinary Medical Officer, LCM, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to study the mechanism of chemically induced murine hepatomas, and to identify and characterize endogenous and exogenous factors that may control initiation, promotion and progression of these tumors. Topics of present interest are: (1) isolation and characterization of preneoplastic liver cell populations; (2) the time course of chemically induced hepatoma formation, and the changes in gene expression during this process; (3) modulating effects of blood flow and oxygen tension on hepatoma formation and development; and (4) the role of genetic predisposition in hepatoma development. Results obtained so far include: (1) centrifugal elutriation method for isolation of different populations (based on size) of parenchymal liver cells from untreated and carcinogen treated rats has been established; (2) computer-assisted analysis of total cellular proteins measured by quantitative two-dimensional gel electrophoresis, in preneoplastic rat hepatocytes as compared to normal hepatocytes, revealed few (5-8) qualitative protein differences. In contrast 10-15% of the cellular proteins in the preneoplastic cells were undergoing quantitative changes of at least 50% during the initiation stage; (3) an early event in hepatocarcinogenesis is a significant reduction of surface receptors for asialoglycoproteins. Based on this observation a new separation method for normal and preneoplastic rat hepatocytes was developed by using asialofetuin as a ligand to selectively bind normal hepatocytes; (4) the anterior chamber of rat eye has been successfully used to grow both normal and preneoplastic rat hepatocytes; (5) a lack of heme induced feedback inhibition of delta-aminolevulenic acid synthetase activity in preneoplastic rat hepatocytes was demonstrated in vitro; and (6) histochemical changes in rat liver following portacaval shunt operation were similar to those observed after administration of hepatocarcinogens, namely increased expression of gamma-glutamyltranspeptidase activity and lack of glucose-6-phosphatase activity. The long-term effects of this operation are under study.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Snorri S. Thorgeirsson	Chief	LCM, NCI
Carole A. Heilman	Staff Fellow	LCM, NCI
Irene B. Glowinski	Staff Fellow	LCM, NCI
Peter J. Wirth	Expert	LCM, NCI
Mark J. Miller	Senior Staff Fellow	LCM, NCI

Objectives:

The objective of this project is to study the mechanism of chemically induced murine hepatomas, and to identify and characterize endogenous and exogenous factors that may control initiation, promotion and progression of these tumors. Topics of present interest are: (1) isolation and characterization of preneoplastic liver cell populations; (2) the time-course of chemically induced hepatoma formation, and changes in gene expression during this process; (3) modulating effects of blood flow and oxygen tension on hepatoma development; and (4) the role of genetic predisposition in hepatoma development.

Methods Employed:

(1) Cell separation techniques combined with (2) centrifugal elutriation to isolate different liver cell populations according to their size. (3) Differential attachment of the separated cells on tissue culture dishes coated with asialoproteins. Characterization of the cells using (4) histochemical, (5) radiochemical, (6) radioreceptors, (7) microsurgical and (8) recording spectrophotometric methods.

Major Findings:

1. Preneoplastic and neoplastic changes in rat liver has been produced by a single injection of diethylnitrosamine to newborn rats followed by administration of phenobarbital; a single injection of diethylnitrosamine 24 hours after partial hepatectomy followed by administration of phenobarbital; and by administration of a single large dose of diethylnitrosamine to adult rats combined with partial hepatectomy and feeding of acetylaminofluorene.

2. Centrifugal elutriation method for isolation of different sized liver parenchymal cells from untreated and carcinogen-treated rats has been established. Computer-assisted analysis of total cellular protein, measured by quantitative two-dimensional gel electrophoresis, in the preneoplastic cell populations as compared to normal hepatocytes, revealed few (5-8) qualitative protein differences. In contrast approximately 10-15% of the polypeptides in the preneoplastic cells undergo quantitative changes of at least 50% during the initiation stage. Similar comparisons of protein patterns from two rat hepatoma cell lines (Reuber H-4-II-E and McA 7777) with normal cultured rat hepatocytes revealed marked qualitative and quantitative differences between the protein patterns in normal and transformed cells, whereas the cellular protein patterns in the two hepatoma lines were very similar both qualitatively and quantitatively.

3. A significant reduction in the number of the surface receptors for asialo-orosomucoid in the transformed liver cells was observed. Based on this observation studies using different asialomucoproteins for coating the tissue culture dishes in an attempt to separate preneoplastic and neoplastic liver cells from normal liver cells are in process.

4. The attachment and growth of both normal and transformed liver cells in the anterior chamber of the eye in 70-80% of weanling rats was observed. Microscopic examination showed the attachment of these cells on the anterior surface of the iris. The growth was evident in two days, in contrast to transplantation to the spleen where the number of surviving cells was extremely low.

5. The radiochemical method for determination of delta-aminolevulinic acid synthetase in tissue culture samples has been established. The activity of this enzyme is increased several-fold under tissue culture conditions which points to an evident lack of heme. When heme was added to tissue culture medium, a feedback inhibition of this enzyme was obtained when normal liver cells were used, but not when cells from carcinogen treated animals were used.

6. Histochemical changes in rat liver after portacaval shunt operation were similar to those observed after administration of hepatocarcinogens; namely, expression of γ-glutamyltranspeptidase activity and lack of glucose-6-phosphate activity. The study of long-term effects of this operation are under study.

Significance to Biomedical Research and the Program of the Institute:

Our research projects are aimed at increasing the understanding of the multistep process involved in chemical carcinogenesis and thus providing means to possibly define both cancer cause and to establish effective cancer prevention.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications

Evarts, R. P., Marsden, E. and Thorgeirsson, S. S.: Regulation of heme metabolism and cytochrome P-450. Biochem. Pharmacol. (In Press)

Evarts, R. P., Raab, M. M., Haliday, E. and Brown, C.: Pyrazole effects on mutagenicity and toxicity of dimethylnitrosamine in Wistar rats. Cancer Res. 43: 496-499, 1983.

Thorgeirsson, S. S., Wirth, P. J. and Evarts, R. P.: Early changes in gene expression during hepatocarcinogenesis. In Rydström, J., Montelius, J. and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogenesis. Amsterdam, Elsevier/North-Holland Biomedical Press. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05263-02 LCM

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Computer Analysis of Carcinogenesis by Two-Dimensional Gel Electrophoresis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Mark J. Miller Senior Staff Fellow LCM, NCI

COOPERATING UNITS *(if any)*
Dr. Carl Merril and Dr. David Goldman, LGCB, NIMH;
Dr. Marshall Nirenberg and Dr. Karl E. Kruger, LBG, NHLBI

LAB/BRANCH
Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project was initiated to study the mechanism of chemical carcinogenesis by employing the technique of quantitative two-dimensional electrophoresis of total cellular proteins. This type of electrophoresis allows the simultaneous separation of hundreds of polypeptides in a single polyacrylamide gel. We have acquired, and have significantly expanded, a computer-based system to automatically analyze autoradiograms produced from these gels. This system automatically finds and measures the amount of radioactivity in any polypeptide resolved by these electrophoretograms. Programs were developed which automatically match together the spot patterns found in different gels. Still other programs link together a series of gels which may constitute an experiment, and allow the investigator to quantitatively follow the synthesis of any resolvable protein through that experiment, or series of experiments. The investigator may specify various parameters and ask the computer to list those spots whose pattern of synthesis may lie within those parameters. Finally, several sophisticated computer-graphics programs allows the investigator to visually compare and follow various polypeptides which may be matched on a virtually unlimited number of electrophoretograms.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professsional Personnel (other than the Principal Investigator) engaged on this Project:

Snorri S. Thorgeirsson	Chief	LCM, NCI
Peter J. Wirth	Expert	LCM, NCI

Objectives:

The main objective of this project is to study the mechanism of chemical carcinogenesis by employing the technique of quantitative two-dimensional gel electrophoresis of total cellular protein. Since this technique allows for the simultaneous separation of total cellular polypeptides on a single polyacrylamide gel, it is possible to follow changes in the rate of synthesis of individual proteins as well as qualitative changes in the protein patterns as the cell undergoes malignant transformation. Our aim is to identify and characterize those proteins that are highly associated with the transformed phenotype.

Methods Employed:

The principal methods employed are: (1) two-dimensional gel electrophoresis, (2) tissue culture technique, (3) computer-based quantitation of autoradiograms, and (4) radioisotope measurements.

Major Findings:

In the past year, the major effort within the Laboratory of Carcinogen Metabolism's Computer Facility has been to expand and further develop the computerized two-dimensional gel analysis system. In the past year, we have written, and documented, over 130 programs and subroutine packages. Most of this code has been written to aid in the matching and analysis of multiple gel experiments. Much of the remaining software has been created to interface new computer peripheral equipment to our Digital Equipment Corporation VAX 11/750 central processing computer (the VAX). A brief description of the major hardware and software developments follows:

A. Hardware: (1) Comtal: The Comtal Vision one/20 image processing system has been expanded from a one to a two user system. This allows two investigators to utilize the Comtal simultaneously from different user stations. This expansion required a significant rewrite of the Comtal software driver (a driver is a piece of software, installed as part of the operating system, which arbitrates the transfer of data and commands between a piece of peripheral equipment, such as the Comtal, and the central computer -- in our case, the VAX). One particularly vexing problem has been that the Comtal cannot easily handle two truly independent stations. If the different users attempt to simultaneously do certain, rather common, operations, the LSI-11 microcomputer in the Comtal will become overwhelmed and crash. Considerable effort has been spent in writing the driver so that these dangerous operations will be avoided or interlevered.

(2) Eikonix: In order to convert a two-dimensional image into a form a computer can manipulate, it is necessary to digitize that image. We have obtained an Eikonix Model 791 optical digitizer for this purpose. The Eikonix consists of a 2048 element photodiode array mounted behind a lens. The array scans across the focal plane of the lens, digitizing the incident light into one of 4096 discrete levels (gray levels) corresponding to from 0 to 1.6 optical density units. Each element of this array can be independently calibrated with respect to background subtraction and gain control so as to produce a uniform response. In addition, a lookup table within the Eikonix can be utilized to convert the data from linear transmission format to reverse transmission or optical density format. We have written a software driver to interface the Eikonix with our VAX. This driver allows for the transfer of commands, arrays, and data between the scanner and the VAX. Other software has been written to allow an investigator to initialize the scanner, calibrate the photodiode array and to scan autoradiograms, creating output that is suitable for analysis on our computer system. These programs will store the data on either disk or magnetic tape and work to ensure that the scanner is and remains in proper calibration.

B. Software: (1) Makespots. The spot generation program, makespots, has been rewritten to utilize a more generalized mechanism for finding spots. This program finds spots by collecting together all contiguous data elements (pixels) above a certain minimum intensity, or threshold. For each collection of pixels, the threshold is raised and elements whose intensity falls below the old threshold are dropped. If two or more spots appear where the old single one was, the old spot is replaced by the new ones. The old program merely raised the threshold one gray level at a time. The new one uses geometric tests to raise the threshold to a level where the spot might be split. This allows the program to take approximately the same amount of time to find the spots in a gel whether the data is in terms of 8-bit integers (256 possible gray levels), 16-bit integers (65,536 possible possible gray levels), or floating point (8,388,608 possible gray levels).

(2) Automatch. The automatch program matches spots of two films, in a completely automatic manner. The matching proceeds in two steps. First, for each spot in each film, the set of all surrounding spots within a certain radius of the central spot is found and listed. This set of surrounding spots is referred to here as the "cluster." The spots of each film are then sorted in order of intensity and, starting with the most intense, the spots are paired as a potential match, and their clusters are compared. If a fraction, minmtch of the spots in each cluster can be matched, and if, for each matched spot in the initial cluster, the same fraction of spots in the respective secondary clusters can also be matched, the first match is considered to be firm. The process is repeated for each of the surrounding matched spots until no new firm matches can be found. The second step is similar to the old makepairs program. The matched spots in the cluster about each unmatched spot are used to calculate a transform which maps the local coordinate space of gel A into gel B (i.e. $x' = aX + bY + c$, $y'' = dX + eY + f$) using the method of least squares. The coordinates of the unmatched spot are transformed into the space of the other gel and a search for a matching spot is made. The process is repeated, gradually relaxing the criteria for a good match until no new matches are found. If the program is unable to find a reasonable set of matched spots in a reasonable amount of time, or if it matches up the gel patterns incorrectly, it can be rerun with hand matched pairs as starting points (see viewseeds below). This program is a tremendous saver of investigator time

and effort. With high quality, similar gels, this program is very accurate and fast. On some gel sets it has matched as much as 99+% of the matchable spots. We feel that with some time and effort we can make the program more robust and accurate on poorer quality gel patterns.

(3) Listgroups and viewgroups. Listgroups reads gel spot pair files from the current working directory and forms groups of spots, where each spot in a group is paired with some other spot in the group and no spot in any group is paired with any spot in any other group. The output is printed in tabular format on either the terminal or line printer. A number of options and tests are available. It is, for example, possible to list all spots in a set of films that are present in one subset and absent in another subset and that contain more than a certain percentage of the total intensity in the gels. Viewgroups is very similar to listgroups, except that the spots in question are displayed successively on the Comtal, rather than listed. Each group and region surrounding it are displayed in a grid arrangement on the display screen. The computer displays two color-coded copies of this grid, one on top of the other. The investigator can use the data tablet to move the "top copy" around on the screen and compare gels.

(4) Viewseeds. Viewseeds is used to set up landmark matched spots for use by the automatch program when the program is unsuccessful at finding such spots on its own. The two images are displayed in such a way that one image can be overlaid over another in a manner similar to the comparison of two autoradiograms on a lightbox. A target can be placed over superimposed contours and those contours can be automatically found and marked as matched. These matched contours are used as starting points by the program automatch.

(5) Viewpairs. Viewpairs allows an investigator to view and edit the results of automatch. The two films are overlaid on top of one another in much the same way as in the viewseeds program outlined above. Matched and unmatched spots are displayed in four different colors in the two films. Automatched contours may be added or deleted interactively. The colors and outlining will be changed as the contours are matched or unmatched. Multiple matched spots (i.e., a spot matched to two or more spots in the other film) can be highlighted. Vectors can be drawn between spots matched in film 1 and its counterpart in film 2. These "matched vectors" greatly aid in determining exactly the spots which are matched.

(6) Library routines. We have created a series of subroutine packages that handle most of the common manipulation protocols used in our analysis system. Our plan is to isolate any set of subroutines that are used in two or more programs into an archived library. For portability reasons, we do the same with any set of subroutines that must utilize equipment specific to this installation. Thus, if in future years we should decide to change the format of any of the data files, or obtain new equipment, we will need only to modify the subroutine packages on which our programs depend, and not a large number of individual programs. Likewise, other installations which may wish to utilize our programs, but do not have the same equipment that we have, need to concentrate on rewriting the subroutines and not all of the programs. A brief description of some of the more significant packages is outlined below.

a) gel: These functions let the programmer work with the data associated with the gels. This package includes functions for dealing with whole gels, functions for dealing with spots, and functions for dealing with spot fragments.

b) filmio: These functions are used to transfer data from program memory to files. They are responsible for finding, opening, reading or writing, and closing requested files. They "know" the default names of the files and accept modifiers (suffixes) when searching for a specified file. All return pointers to dynamically-allocated areas of memory which may be manipulated in standard ways.

c) pairs: These functions operate on the files that list pairs of spots matched between two films, and are designed to allow a program to create, read, write, and edit the contents of any number of these files simultaneously.

d) slfind: Given a point, in terms of rows and columns, within the coordinate space of any film, these routines will find the contour to which that point belongs.

e) doflags: Handles the flags or arguments passed to a program. These functions are designed to: provide a standard way of handling flags, provide for liberal flag interpretation, ensure that usage messages reflect acceptable usage, and to simplify programming. Flags may be anywhere on the command line. Values that go with flags may be appended to the flag (' -Tvt100 ') or be in a following argument (' "-T vt100" '). Value-free flags may be strung together (' -ld ') or separate (' "-l -d" '). Flags may be upper case or lower case except where there are specific instructions that the different case flags do different things.

f) image: The functions in the image library let the programmer create and display images in a device-dependent way. (The device independence is of academic interest, since we have just one type of device to display images on -- our Comtal.) These functions treat an image as a number of rows and columns of picture elements, or "pixels." Low-numbered rows are at the top of an image; high-numbered rows are at the bottom. Low-numbered columns are at the left of an image; high-numbered columns are at the right. Some pixels in an image may be "transparent." Other pixels may be "fixed"--they may always be tied to a particular color. Still other pixels may be "stated"--the color they are tied to may be changed. And any pixel (even a transparent one) may be "overlaid"--temporarily tied to a particular color, with the possibility of later being returned to its previous color.

g) tranform: These functions compute transforms from given sets of input and output values, and apply the computed transforms to other values. This is used, for example, to transform the coordinate system of part of one film into the coordinate system of a second film. The constants and coefficients of the transformation matrix are computed using a "least squares" technique to minimize the sum of the squares of the differences between the given output values and the values that result from applying the transforms to the give inputs.

Significance to Biomedical Research and the Program of the Institute:

The technique of two-dimensional gel electrophoresis provides a virtual "snapshot" of the metabolic activity of a cell under a specific set of environmental conditions. Our laboratory's computer system gives us the capability of analyzing and cataloging the synthesis of any protein resolved by such gels. We are involved in studies which will analyze, catalog and compare the capability of a cell to synthesize its various proteins during both normal development and chemical transformation and to identify and characterize those proteins that are highly associated with the malignant phenotype. These studies should provide clues as to the biochemical nature of the malignant process and provide a means to identify the number of genes involved in this process.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications

Miller, M. J., Xuong, N.-H. and Geiduschek, E. P. Quantitative analysis of the heat shock response of Saccharomyces cerevisiae. J. Bacteriol. 151: 311-327, 1982.

L'rincz, A. T., Miller, M. J., Xuong, N.-H. and Geiduschek, E. P. Identification of proteins whose synthesis is modulated during the cell cycle of Saccharomyces cerevisiae. Mol. Cell Biol. 2: 1532-1549, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05264-02 LCM

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Conformational Changes in DNA and RNA Induced by Chemical Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
C.-H. Robert Lee Senior Staff Fellow LCM, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to acquire a better understanding of the mechanism of chemical carcinogenesis, we have initiated studies on the conformation of DNA and RNA in solution and their interactions with carcinogens and mutagens. The methods employed in these studies include nuclear magnetic resonance (NMR), circular dichroism (CD), electric linear dichroism, organic synthesis of DNA and potential energy calculations. Specific topics included in this project are: (1) studies on the interactions between the tryptophan pyrolysates Trp-P-1 and Trp-P-2, with dinucleoside phosphates CpG, GpC, UpA, ApU, dCpdG, dGpdC, dTpdA and dApdT. The capacity of these compounds to form nucleotide complexes differ in that Trp-P-1 was more effective than Trp-P-2. Also, the complex formation was more extensive with ribonucleotides than deoxyribonucleotides and with pyrimidine-purine sequence as compared to purine-pyrimidine sequences. (2) Studies on the effects of 5-methyl cytosine on the conformation of dinucleotides. It was found that the 5-methyl group in cytosine enhances base pair formation with guanine and stabilizes the base pair. (3) Studies on the solution conformation of trinucleotide diphosphates (trimers). We found the trimers assume the four stable conformations of dinucleoside phosphates. In addition, they also exhibit "bulged" structure with the bases of the two terminal residues stacking on each other and the middle residue sticking out of the stack. This bulged conformation is proposed as the structural model for frame-shift mutation. (4) Studies on the solution conformation of 1-nitroso-cis-2,6-piperidine dicarboxylic acid. It has rigid half-chair conformation in pyridine as well as in water solution, corresponding to the crystal structure of this compound. Change in pH induced distinct change of the 1-nitroso-cis-2,6-piperidine dicarboxylic acid structure from one rigid state to another rigid state.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Peter P. Roller	Chemist	LCM, NCI
Snorri S. Thorgeirsson	Chief	LCM, NCI
Shui-mei Wang	Visiting Fellow	LCM, NCI
Larry K. Keefer	Chief, Analytical Chemistry Section	LCM, NCI

Objectives:

The main objective of this project is to study the mechanism of chemical carcinogenesis at the molecular level by employing biochemical and biophysical methods to examine the conformation changes of DNA and RNA in solution when interactions with carcinogens and mutagens take place. These detailed studies on the interactions of carcinogens/mutagens with nucleic acids should provide important data towards understanding the mechanism of chemical carcinogenesis and mutagenesis at the molecular level.

Method Employed:

The principal methods employed are (1) nuclear magnetic resonance (NMR), (2) circular dichroism (CD), (3) electric linear dichroism (ELD), (4) gel electrophoresis, and (5) potential energy calculations.

Major Findings:

1. Studies on the interactions between tryptophan pyrolysates Trp-P-1 and Trp-P-2 with dinucleoside phosphates CpG, pC, UpA, ApU, dCpdG, dGpdC, dTpdA and dApdT. The capacity of these compounds to form nucleotide complexes is: (a) Trp-P-1 > Trp-P-2, (b) ribonucleotide > deoxyribonucleotide, (c) pyrimidine-purine sequence > purine-pyrimidine sequence, and (d) G,C nucleotides > A,T or U) nucleotides.

2. Studies on the effects of 5-methyl cytosine on dinucleotide conformation. It was found that the 5-methyl group in cytosine enhances base pair formation with guanine and stabilizes the base pair.

3. Studies on the solution conformation of trinucleotide diphosphates (trimers). We found the trimers assume the four stable conformations of dinucleoside phosphates. In addition, they also exhibit "bulged" structure with the bases of the two terminal residues stacking on each other and the middle residue sticking out of the stack. This bulged conformation is proposed as the structural model for frame-shift mutation.

4. Studies on the solution conformation of 1-nitroso-cis-2,6-piperidine dicarboxylic acid. It was found to have rigid half-chair conformation in pyridine as well as in water solution, corresponding to the crystal structure of this compound. Change in pH induced distinct change of the 1-nitroso-cis-2,6-piperidine dicarboxylic acid structure from one rigid state to another rigid state.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at providing a better understanding of the mechanism of chemical carcinogenesis and mutagenesis at the molecular level. The information obtained from these studies provide means toward the goal of both defining cancer cause and devising effective programs for cancer prevention.

Proposed Course:

This project will be terminated.

Publications

Lee, C.-H. and Charney, E.: Solution conformation of DNA. J. Mol. Biol. 161: 289-305, 1982.

Lee, C.-H., Sharpless, N. E. and Jennings, W.: Alternate g^-g^- conformation for dinucleoside phosphates in solution. Biophys. Chem. 16: 199-207, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05313-01 LCM

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Early Events in Aromatic Amine Induced Hepatocarcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Peter J. Wirth Expert LCM, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The project was initiated to study the regulation of growth and differentiation of normal and transformed cells using the hepatocyte model in combination with quantitative two-dimensional electrophoresis. Cultured hepatocytes were treated with non-toxic concentrations of the hepatocarcinogen N-hydroxy-2-acetylaminofluorene (N-OH-AAF) in order to obtain only "initiated" cell populations. Using these cells we have begun studies concerning: (1) early changes in gene expression as measured by quantitative two-dimensional gel electrophoresis of total cellular proteins; (2) the in vitro activities of sulfotransferase, deacetylase and acyltransferase, enzymes implicated in the in vivo activation of N-OH-AAF to its ultimate carcinogenic form; (3) identification and quantitation using P-32 postlabeling analysis of cellular carcinogen bound (fluorene) DNA adducts formed during initiation. Treatment of cultured hepatocytes with N-OH-AAF (0.1 µg/ml) resulted in a transient inhibition of protein synthesis as measured by incorporation of C-14 amino acids into cellular proteins which returned to control levels 72 hours after exposure. Treated cells, however, showed no morphological differences when compared to untreated hepatocytes. Two-dimensional electrophoretic analysis of total cellular proteins from treated and untreated cells revealed few (6-8) qualitative protein changes between the groups. In contrast 10-15% of the 600-800 readily detectable proteins were undergoing quantitative changes of at least 50% following treatment with N-OH-AAF. In order to examine the relationship between the covalent binding of carcinogenic (aromatic amines) to cellular DNA with gene expression in early initiated cells, we have synthesized the major known or proposed deoxyguanosine and deoxyadenosine DNA adducts of N-OH-AAF. In addition the respective 3-monophosphate and 3',5'-diphosphate adducts have also been prepared and we are currently developing a high pressure liquid chromatographic procedure for the separation and quantitation of the in vitro formed cellular DNA adducts.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Vanessa T. Vu	Staff Fellow	LCM, NCI
William Richards	Senior Staff Fellow	LCM, NCI
Snorri S. Thorgeirsson	Chief	LCM, NCI

Objectives:

The process of chemical carcinogenesis has traditionally been divided into three phases: initiation, promotion, and progression. Although the mouse skin was the first experimental model used to delineate these phases, the rodent liver is another tissue in which the initiation and promotion stages of chemical carcinogenesis can be clearly demonstrated in vivo. The initiation events are generally believed to be the covalent interaction of a carcinogen with critical cellular macromolecules (i.e., DNA, RNA, proteins) in the target tissues which results in alteration of the normal functions within the cell. These alterations may result in changes in both gene expression and phenotypic characteristics and under appropriate conditions, namely following promotion, develop into malignant tumor cells. Unfortunately very little is known concerning either the early cellular events in the initiation process or the gene products that control and maintain the initiated cells or how these products may change as a function of time during promotion and progression phases of the carcinogenic process. Therefore the main objective of this project is to characterize early biochemical events in the intiation process. Initial studies have focused on examining the relationship between covalent binding of carcinogens (aromatic amines) to cellular DNA and changes in gene expression as measured by quantitative two-dimensional gel electrophoresis of total cellular proteins.

Methods Employed:

The principal methods employed are: (1) tissue culture techniques; (2) cell separation techniques--elutriation; (3) histochemical staining; (4) enzyme assays involving radiometric (tritium, carbon-14, phosphorus-32, iodine-125) spectrophotometric, and spectrophotofluorometric determinations; (5) chemical and radiochemical synthesis; (6) high pressure liquid chromatography; (7) column and thin layer chromatography; (8) nuclear magnetic resonance and mass spectrometry; (9) two-dimensional gel electrophoresis; and (10) computer-assisted quantitation of autoradiograms.

Major Findings:

A. Cultured hepatocytes. Initial studies were performed to determine the toxicity of N-hydroxy-2-acetylaminofluorene (N-OH-AAF) to cultured hepatocytes. Hepatocytes from Sprague-Dawley rats were prepared and plated onto collagen-coated dishes and maintained in culture in defined (serum-free) medium. After 24 hours varying concentrations of N-OH-AAF (up to 10 µg/ml) were added and the hepatocytes maintained in the presence of carcinogen for 24 hours. After carcinogen treatment and at 24 hour intervals growth media was changed and the hepatocytes

examined for gross morphological changes and their ability to synthesize [C-14] labeled protein in the presence of radioactive amino acids (4 hour pulse). At higher concentrations of N-OH-AAF (> 1 µg/ml) hepatocytes within 24 hours of treatment began to detach from the surface of the plates and labeled very poorly (5% of untreated control) with [C-14] amino acids. At concentrations of 0.1 µg/ml of N-OH-AAF treated hepatocytes showed no morphological differences yet failed to synthesize proteins at the same rate as untreated controls until 72 hours after treatment. After 72 hours both treated and untreated cells exhibited no significant differences in protein synthesis for up to 168 hours in culture.

Two-dimensional electrophoretic separation (isoelectric focusing first dimension and molecular weight in the second dimension) of total cellular proteins from treated and untreated hepatocytes and quantitation of their respective autoradiograms revealed relatively few (6-8) qualitative protein differences between the groups. In a typical experiment we could readily detect and quantitate between 500-600 polypeptide spots. In contrast 10-15% of the proteins underwent quantitative changes of at least 50% following treatment with N-OH-AAF. One particular protein (MW 14000 and pI 6.4) which is a major protein (3-5% total radioactivity) and present in both untreated and treated hepatocytes decreased in a time-dependent manner greater than 100-fold as the hepatocytes were maintained in culture. This protein is now under current investigation.

Using the procedure of Randerath et al. we have begun, in parallel with the analysis of total cellular protein changes, to determine the extent of covalent binding of N-OH-AAF metabolites to hepatocyte DNA. Cultured hepatocytes with treated N-OH-AAF (0.1 µg/ml) for 24 hours and then cellular DNA was isolated at 24 hour intervals therafter. DNAs were enzymatically digested to deoxyribonucleoside 3'-monophosphates which were then converted to [5'-P-32] deoxyribonucleoside 3',5'-bisphosphates by T4 polynucleotide kinase-catalyzed [P-32] phosphate transfer from [P-32] ATP. The [P-32] labeled nucleotide adducts are currrently being resolved by both anion exchange thin layer chromatography (TLC) on polyethyleneimine cellulose and high pressure liquid chromatography (HPLC). As internal standards for both our TLC and HPLC separation procedures we have synthesized the major known or proposed DNA adducts of N-OH-AAF and their phosphorylated analogs: N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF); N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF); 3-(deoxyguanosin-N2-yl)-2-acetylaminofluorene (dG-N2-AAF); N-(deoxy-adenosin-8-yl)2-aminofluorene (dA-C8-AF); 3(deoxyadenosin-N6-yl)-2-acetylaminofluorene (dA-N6-AAF); N-(deoxyguanosin-8-yl)-2-acetylaminofluorene-3'-monophosphate (dGp-C8-AAF) and N-(deoxyguanosin-8-yl)-2-acetylaminofluorene-3',5'-diphosphate (dpGp-C8-AAF). The last two compounds will be converted (deacetylated) to their amino analogs via ammonia treatment to complete the series.

Also in parallel with the analysis of total cellular protein changes in hepatocytes following carcinogen treatment, we have begun preliminary studies concerning the activities of three enzymes implicated in the in vivo activation of N-OH-AAF to its ultimate carcinogenic form, namely sulfotransferase, deacetylase and acyltransferase. In suspension hepatocytes and in cultured hepatocytes acytransferase activity and adenosine 3'-phosphate 5'-phosphosulfate (PAPS) dependent sulfotransferase activity were readily determined. The covalent binding of generally labeled [H-3] N-OH-AAF to yeast tRNA in the presence of hepatocyte cytosolic fractions was used to estimate both acyltransferase and

sulfotransferase activities. Freshly isolated hepatocytes (100,000 x g supernatant) expressed much higher acyltransferase activity than cultured hepatocytes (10,000 x g supernatant) (140 pmol AF bound/mg protein/min vs. 40 pmol/mg/min), whereas both suspension hepatocytes and cultured hepatocytes expressed similar sulfotransferase activities: (430 vs 250 pmol bound/mg protein/min).

B. Partial hepatectomized hepatocytes. Since the initiating process in vivo appears to involve a proliferative event (Farber, Cancer Res., 1982), we have sought to identify the proteins that are possibly involved in "switching on" the normal stationary (G-phase) hepatocyte into proliferation mode. Sprague-Dawley rats were subjected to a 75% partial hepatectomy and then hepatocytes were isolated at 24 hour intervals and pulse labeled with [C-14] amino acids. Two-dimensional electrophoretic analysis of the total cellular proteins is currently in progress in an attempt to identify these "regulatory proteins."

Significance to Biomedical Research and the Program of the Institute:

The liver model offers many advantages over other systems used in the study of the mechanism(s) of chemical carcinogenesis. Included in these: broad responsiveness to both initiating and promoting agents; relative homogeneity of cell types within the organ; relative ease of identification and separation of early preneoplastic cell populations. Combined with our laboratory's computer system for the analysis of two-dimensional electrophoretograms of cellular proteins from normal and transformed cells, our studies should provide valuable information as to the biochemical mechanism(s) of chemical carcinogenesis.

Proposed Course:

Continue as outlined under Objectives and Major Findings.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05314-01 LCM

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Influence of Polypeptides on Neoplastic Cellular Development and Proliferation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Henry Krutzsch Expert LCM, NCI

COOPERATING UNITS *(if any)*

Lou Henderson, FCRF, Frederick, MD

LAB/BRANCH

Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to study the biochemical role of polypeptides in neoplastic cellular development and proliferation. The aim of this work is to identify and characterize polypeptides that are involved in oncogenesis and to relate the differences in these species as possessed by normal or transformed cell phenotypes. These polypeptides can have activity as growth inhibitors (chalones) or promoters or as regulators of the cell cycle. The primary emphasis of effort will initially be concerned with investigations on the liver cell system. Polypeptide products from normal cells and cells obtained from chemically induced or spontaneous hepatomas will be tested and compared. Putative active materials will be identified by computer assisted two-dimensional gel electrophoresis and by in vitro tissue culture bioassay techniques. After promising candidates have been identified, they will be isolated and purified by techniques such as high pressure liquid chromatography, in amounts sufficient to do further biochemical characterizations. Amino acid sequence analysis will also be carried out on these materials to relate their various activities to their primary molecular structures. In ongoing studies with several hydrophobic transforming proteins obtained from virally infected cells, preliminary data has indicated that fatty acid moieties are covalently bound to these species, possibly allowing these proteins to associate with the cell membrane and cause conversion to the transformed phenotype. Studies on the effect of differentiation of HL60 cells on the incorporation of one of these fatty acids, myristic acid, have also been initiated.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Snorri S. Thorgeirsson	Chief	LCM, NCI
Peter J. Wirth	Expert	LCM, NCI
Robert T. Maguire	Clinical Associate	LCM, NCI
Peter P. Roller	Chemist	LCM, NCI
Min-Kyung Song	Visiting Fellow	LCM, NCI

Objectives:

The objective of this project is to study the biochemical role of polypeptides and proteins in neoplastic cellular development and proliferation. The principle thrust of this work will be to identify and characterize polypeptides and proteins that are involved in oncogenesis and to relate the differences in these species as possessed by normal or transformed cell phenotypes. These polypeptides can have activity as inhibitors or promoters of cell growth or as regulators of the cell cycle.

Methods Employed:

The principle methods employed will be various tissue culture techniques, two-dimensional gel electrophoresis, ion exchange and reverse-phase chromatography, and protein primary structure determination procedures.

Major Findings:

The work being pursued will be initially concerned with the liver cell system. Polypeptide products from normal cells and cells obtained from chemically induced or spontaneous hepatomas will be tested and compared. Active materials will be identified by computer assisted two-dimensional gel electrophoresis and by in vitro tissue culture bioassay techniques. After promising candidates have been identified, they will be isolated and purified, by techniques such as high pressure liquid chromatography, in amounts sufficient to do further biochemical characterizations and for primary structure determinations.

In ongoing studies with several hydrophobic transforming proteins obtained from virally infected cells, preliminary data has indicated that fatty acid moieties are covalently bound to these species. In some cases, this fatty acid is myristic acid, and in other cases, it appears that two fatty acid molecules, one palmitic acid and one stearic acid, are both bound to the protein at the same site. Some studies on the relationship of terminal differentiation of HL60 cells to the incorporation of one of these fatty acids, myristic acid, have also been initiated.

Significance to Biomedical Research and the Program of the Institute:

This research project is aimed at increasing the knowledge of polypeptide and protein factors that influence normal and neoplastic cellular growth and development. This information should provide further understanding of the oncogenic process and give clues to its control.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications

Bluestone, J., Krutzsch, H. C., Auchincloss, H., Cazenave, P. A., Kindt, T. J., and Sachs, D.: Antiidiotypes against anti-H-2 monoclonal antibodies. IV. Structural analysis of the molecules induced by in vivo antiidiotype treatment. Proc. Natl. Acad. Sci. USA 79: 7947-7851, 1982

Dizdaroglu, M., Gajewski, E., Simic, M. G., and Krutzsch, H. C.: Identification of some OH radical induced products of lysozyme. Int. J. Radiat. Biol. 43: 185-193, 1983.

Dizdaroglu, M. and Krutzsch, H. C.: A comparison of weak ion exchange and reverse phase HPLC for peptide separation. J. Chromatogr. (In Press)

Dizdaroglu, M., Krutzsch, H. C. and Simic, M. G.: Separation of angiotensins by high performance liquid chromatography on a weak anion-exchange bonded phase. Anal. Biochem. 123: 190-193, 1982.

Guidotti, A., Konkel, D. R., Ebstein, B., Corda, M. G., Krutzsch, H. C., Meek, J. L. and Costa, E.: Isolation and characterization of GABA-modulin from rat brain. Proc. Natl. Acad. Sci. USA 79: 6084-6088, 1982.

Henderson, L. E., Krutzsch, H. C., and Oroszlan, S.: Myristyl amino terminal acylated murine retroviral proteins. Proc. Natl. Acad. Sci. USA 80: 339-343, 1983.

Krutzsch, H. C.: Polypeptide sequencing with dipeptidyl peptidases. Methods Enzymol. 93: 511-524, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05315-01 LCM

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Cell Surface Protein Expression in Normal and Neoplastic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

William L. Richards Senior Staff Fellow LCM, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to identify, primarily by two-dimensional gel electrophoresis, polypeptides that characterize the cell surface in normal and neoplastic cells. Studies on normal hepatocytes from rats at different stages of development and from regenerating livers will be compared to studies on preneoplastic and neoplastic liver cell populations in order to identify alterations in cell surface polypeptide patterns that distinguish the malignant cell from the normal cell. (1) We have compared three methods for cell surface protein radioiodination and have further refined one of these methods to yield high resolution, low background two-dimensional gel electrophoresis autoradiograms after a short exposure. Presumptive cell surface protein patterns of rat hepatocytes and of two rat hepatoma cell lines are currently being compared by computer analysis. (2) We are in the second phase of development of a medium designed to support the growth of rat hepatocytes under hormone-supplemented serum-free conditions.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Snorri S. Thorgeirsson	Chief	LCM, NCI
Ritva P. Evarts	Veterinary Medical Officer	LCM, NCI
Peter J. Wirth	Expert	LCM, NCI
Mark J. Miller	Senior Staff Fellow	LCM, NCI

Objectives:

The objective of this project is to compare, primarily by two-dimensional gel electrophoresis, the regulation of cell surface protein expression in normal, preneoplastic and neoplastic cells. Our aim is to identify alterations in cell cycle surface protein patterns that distinguish the malignant cell from the normal cell, and to examine the regulation of those surface proteins that are highly associated with the neoplastic process.

Methods Employed:

Methods used in these studies include: (1) examination of liver cells from rats at different stages of development; (2) examination of preneoplastic and neoplastic liver cell populations; (3) examination of hepatocytes from regenerating livers; (4) maintenance of primary cell cultures and cell lines in hormonally defined media; (5) use of synchronous cell cultures; (6) study of growth factor-mediated stimulation of DNA synthesis in density-inhibited cell cultures; (7) use of 3H-thymidine incorporation into DNA to measure DNA synthesis or percent cells entering S-phase. Use of percent 3H-thymidine-labeled mitoses to determine the phases of the DNA cycle. (8) Two-dimensional polyacrylamide gel electrophoresis of radiolabeled proteins with detection by autoradiography. The incorporation of 14C- or 3H-amino acids into proteins is used to examine the synthesis of individual proteins in the whole cell or in individual cell organelles. The same technique is adaptable to studying the synthesis of secreted proteins. Lactoperoxidase-catalyzed iodination by 125I is used to label plasma membrane proteins. The synthesis of plasma membrane proteins is examined by dual-label autoradiography or by parallel processing of duplicate samples each having one label. (9) Identification of cell surface polypeptide hormone receptors in two-dimensional patterns. (10) Use of cell mutants that are altered in the complement or concentration of hormones required to stimulate growth. (11) Determination of the expression of selected oncogenes during cell cycle initiation and traverse.

Major Findings:

A. Three methods for radioiodination of the hepatocyte cell surface have been tested. The methods yield comparable two-dimensional autoradiograms, each revealing about 100 major presumptive cell surface proteins. The method of direct addition of hydrogen peroxide to cell monolayers in a lactoperoxidase-PBS solution at 2-4° was chosen for further refinement. Experiments aimed at reducing the amount of radiolabel used per 55 mm diameter plate (currently 1 mCi) are in progress.

B. Surface proteins have been radioiodinated in log phase H-4-II-E-C3 and 7777 rat hepatoma cell lines and await two-dimensional gel electrophoresis. Triton X-100 extracts of these samples have been incubated with transferrin-sepharose beads and the adsorbed material, presumably the transferrin receptor, has been eluted into a denaturing buffer in preparation for two-dimensional gel electrophoresis and subsequent determination of its position in the overall pattern of protein spots.

C. We are in the second phase of development of a medium (TRMAB) that is designed to support the growth of normal hepatocytes under hormone-supplemented serum-free conditions. In the first phase, we found that cell attachment to collagen coated plates was similar in both the TRMAB and the traditional Waymouth's medium. The retention of protein per plate for 96 hours in medium $\pm$ insulin was greater in TRMAB than in Waymouth's medium.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying cell surface patterns that distinguish the malignant from the normal cells. The results of these studies could provide a basis for analyzing cancer cause and for selecting strategies designed to treat or prevent cancer.

Proposed Course:

Continue the course outlined under Objectives, Methods Employed and Major Findings.

Publications

Richards, W. L., Tsukada, Y. and Potter, V. R.: Phenotypic diversity of γ-glutamyltranspeptidase activity and protein secretion in hepatoma cell lines. Cancer Res. 42: 1374-1383, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05316-01 LCM

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Hepatic Asialoglycoprotein Receptor Mediated Gene Transfer

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation): Snorri S. Thorgeirsson Chief LCM, NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Carcinogen Metabolism

SECTION:

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.6	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to construct an effective in vivo and in vitro gene transfer system by utilizing the highly efficient endocytosis process whereby asialoglycoproteins are taken up by normal hepatocytes. This should enable us to examine the biological effects, particularly with respect to oncogenesis, of introducing specific genes, both under in vivo and in vitro conditions into a normal highly differentiated cell. This is accomplished by covalently coupling the asialoglycoprotein to the DNA by using two reagents, N-acetyl-N'-(p-glyoxylbenzoyl)cystamine and 2-iminothiolane. The former reacts specifically with nonpaired guanine residues and upon reduction generates a free sulfhydryl group. The latter reacts with the protein to provide another sulfhydryl group which is subsequently conjugated to DNA by an intermolecular disulfide interchange reaction. The experimental model currently under study is the rat liver. The initial coupling has been done using two transformation specific viral DNAs, namely the bovine papillomavirus DNA and the cDNA clone of the Harvey RNA tumor virus. Both tumor viruses have been well characterized in terms of transforming ability. The bovine papillomavirus DNA was prepared from pBR322 recombinants and tailed with approximately 50 residues of dGTP using terminal deoxytransferase. G-tailed viral DNA was coupled to modified asialofetuin by incubation under appropriate conditions. Harvey cDNA is presently being prepared and modified in a similar manner. Uptake and expression of viral DNAs coupled with asialofetuin into primary rat hepatocytes is currently being determined.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Carole A. Heilman	Staff Fellow	LCM, NCI
Peter J. Wirth	Expert	LCM, NCI
Ritva P. Evarts	Veterinary Medical Officer	LCM, NCI

Objectives:

The objective of this project is to construct an effective in vivo and in vitro gene transfer system by utilizing the highly efficient endocytosis process whereby asialoglycoproteins are taken up by normal hepatocytes. This should enable us to examine the biological effects of "transfecting" specific genes. This is accomplished by covalently coupling the asialoglycoprotein to the DNA by using two reagents, N-acetyl-N'-(p-glyoxylbenzoyl)cystamine and 2-iminothiolane. The former reacts specifically with nonpaired guanine residues and upon reduction generates a free sulfhydryl group. The latter reacts with a protein to provide another sulfhydryl group which is subsequently conjugated to DNA by an intermolecular disulfide interchange reaction. The experimental model currently under study is the rat liver both in vivo and in vitro.

Methods Employed:

Methods used in these studies include: chemical synthesis, recombinant DNA techniques, two-dimensional gel electrophoresis, radioisotope measurements using tritium, 32-P, carbon-14, and iodine-125, radioimmunoassays, phase contrast microscopy and tissue culture techniques.

Major Findings:

Cheng et al. (Nucleic Acids Res. 11: 659, 1983) have recently described a method which may prove quite useful in ligand-directed gene transfer studies. The procedure involves the attachment of DNA to a protein molecule that is selectively bound by a specific cell surface receptor. The receptor protein DNA complex is then transported into the cell via receptor mediated endocytosis. The method utilizes two reagents, N-acetyl-N'-(p-glyoxylbenzoyl)cystamine (Gbz-Cyn 2-Ac) and 2-iminothiolane. DNA of interest is tailed with homopolymer tracts of deoxyguanosine and then reacted with Gbz-Cyn 2-Ac. Gbz-Cyn 2-Ac reacts specifically with nonpaired guanine residues and is easily reduced to yield a free sulfhydryl group. Protein is similarly derivatized via reaction with iminothiolane to yield an intermediate which also contains a free sulfhydryl group. The derivatized protein is then conjugated to the glyoxylated DNA via an intermolecular disulfide exchange reaction.

Recently isolated rat hepatocytes have been shown to bind desialylated serum glycoproteins very rapidly via an asialoglycoprotein receptor which is efficiently internalized along with the asialoglycoprotein ligand during endocytosis. Utilizing this property of the hepatocytes we have attempted to introduce into the normal hepatocytes selected genes (BPV, ras, etc.) which have been implicated

in the oncogenic process. Gbz-Cyn 2-Ac was synthesized in a three step sequence starting from 4-acetylbenzoic. Acetylbenzoic acid was oxidized to p-carboxyphenylglyoxal with selenium dioxide in 50% yield. Condensation of p-carboxyphenylglyoxal with cystamine in the presence of 1-ethyl-3-(3-dimethylaminopropy)-carbodiimide yielded N-(p-glyoxylbenzoyl)cystamine (Gbz-Cyn 2). Gbz-Cyn 2, which is unstable, was then converted to the acetyl derivative, N-acetyl-N'-(p-glyoxylbenzoyl)cystamine (Gbz-Cyn 2-Ac) with acetic anhydride.

Preparation of derivatized asialofetuin. Dilute acid hydrolysis (0.1 N H_2SO_4-80°C-1 hr) of fetuin (Gibco) yielded asialofetuin (ASF). Asialofetuin was incubated with 2-iminothiolane at 0°C for 10 minutes. Excess 2-iminothiolane was separated from protein by a Pharmacia PD-10 column. The derivatized asialofetuin (free sulfhydryl group) was then converted to the mixed disulfide with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in phosphate buffer. The degree of derivatization of protein (mixed disulfide form) was determined by measuring absorbance at 412 nm. Excess DTNB and 2-nitro-5-thiobenzoic acid were separated from protein on a PD-10 column.

Addition of homopolymer tracts to DNA. Bovine papillomavirus DNA was tailed with dGTP using terminal transferase.

Conjugation of asialofetuin (ASF) to G-tailed DNA. Derivatized ASF was incubated with 32-P G-tailed DNA in triethanolamine-boric acid buffer at 23° for 2 hours. The DNA was then precipitated twice with ethanol, dried, and then reduced to the free thio with dithiothreitol in triethanolamine-boric acid buffer. The reduced DNA was precipitated twice with ethanol, dried, and then added to the derivatized ASF and incubated at 23° for 18 hours.

Initial coupling was done using two transformation specific viral DNAs, the bovine papillomavirus (BPV) DNA and the cDNA clone of the Harvey RNA tumor virus. Both tumor viruses have been well characterized in terms of transforming ability. The BPV viral DNA was prepared from pBR322 recombinants and tailed with approximately 50 residues of dGTP using terminal deoxytransferase. G-tailed viral DNA was linked to the modified ASF by incubation under appropriate conditions. Primary rat hepatocytes and the ASF-linked DNA were incubated for one hour in Hanks balanced salt solution and 5 mM $CaCl_2$ and then plated on collagen coated plates in the presence of hormone supplemented defined media. Cellular DNA was prepared at various times post-transfection and analyzed for the presence of BPV viral DNA using Southern blotting analysis and hybridization with in vitro 32-P labeled BPV probe. Initial analysis demonstrated the presence of incorporated but degraded BPV DNA in the hepatocyte cultures. Experiments designed to limit the endogenous nuclease activity are presently in progress. Harvey cDNA is presently being prepared and modified in a similar manner.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying critical and specific genetic factors that are important in the oncogenic process. The information obtained from these studies could provide a basis for a better definition of the factors involved in cancer cause and may help in formulating an effective cancer prevention program.

Proposed Course:

Continue as outlined under Objectives and Major Findings.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF CELLULAR CARCINOGENESIS AND TUMOR PROMOTION

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

The Laboratory of Cellular Carcinogenesis and Tumor Promotion plans, develops and implements a comprehensive research program to determine the molecular and biological changes which occur at the cellular and tissue levels during the process of carcinogenesis. Studies are designed to (1) define normal regulatory mechanisms for cellular growth and differentiation; (2) determine the mechanism by which carcinogens alter normal regulation and the biological nature of these alterations; (3) investigate the mechanism by which tumor promoters enhance the expression of carcinogen-induced alterations; (4) identify cellular determinants for enhanced susceptibility or resistance to carcinogens and tumor promoters; (5) elucidate the mechanism by which certain pharmacologic agents inhibit carcinogenesis.

The Laboratory is composed of three sections each of which is charged with a major responsibility for portions of the Laboratory goals. Because of the integrated approach toward an understanding of mechanisms of carcinogenesis, considerable interaction occurs among the sections. Areas of interaction are defined in the individual project reports.

IN VITRO PATHOGENESIS SECTION: The In Vitro Pathogenesis Section (1) develops relevant model systems for the study of all phases of the process of carcinogenesis; (2) defines regulatory mechanisms for the normal control of growth and differentiation and alterations in these controls induced by initiators and promoters; (3) produces, isolates and studies initiated cells; (4) studies functional alterations in gene expression produced by initiators and promoters and the mechanism by which these functional changes occur; (5) elucidates factors which determine susceptibility to carcinogenesis.

This section has directed its efforts toward both developing in vitro model systems to study chemical carcinogenesis in epithelial cells and to use these systems to study the mechanisms of tumor initiation and promotion. Mouse epidermis, the classic model for induction of squamous cancer by chemicals, has been adapted for in vitro study. Previous investigations had demonstrated that this model is a close in vitro analogue of the mouse skin carcinogenesis system in vivo. In vitro, epidermal cells proliferate and differentiate, metabolize carcinogens, repair DNA damage, and respond to tumor promoters like epidermis in vivo.

Regulation of Epidermal Growth and Differentiation: Previous results from this Laboratory have indicated that extracellular calcium concentration regulates epidermal proliferation and differentiation. Culture medium of 0.02 - 0.09 mM calcium concentration selects for proliferating cells which have morphological, immunological and biochemical characteristics of basal cells. Culture medium of >0.1 mM induces epidermal differentiation resulting in cessation of proliferation, vertical stratification, cornification and sloughing of mature squames. The regulation of differentiation by calcium is not associated with changes in

cyclic nucleotide levels but appears dependent on a functioning Na^+-K^+ ATPase pump as it is inhibited by ouabain. A number of other inhibitors of Ca^{++} induced terminal differentiation have been identified and their common actions together with studies of intracellular ion concentrations indicate that an increase in intracellular K^+ is required for the differentiation program to proceed. Epidermal proliferation and differentiation may also be modified via a specific cytosolic skin calcium binding protein which is synthesized only under conditions of high proliferation in normal cells. Retinoids alter epidermal differentiation and this may be mediated through an action on the cornification process by altering the function or subcellular distribution of the key enzyme in this pathway, epidermal transglutaminase.

Quantitative Assay for Carcinogen-induced Altered Differentiation: The capability to selectively grow basal cells in low calcium medium and induce differentiation in high calcium has provided an assay to select for cells with altered differentiative responses. Exposures of primary cultures of mouse keratinocytes to chemical carcinogens results in foci which resist the Ca^{++} signal to differentiate and continue to proliferate under high Ca^{++} conditions, producing countable colonies which stain red with rhodamine B. Cells obtained from mouse skin initiated in vivo show the same characteristics. A series of mouse skin initiators show similar potency for the production of altered foci in vitro and initiation of tumorigenesis in vivo. Cell lines derived from these experiments are initially non-tumorigenic, retain epidermal characteristics and all have in common the ability to proliferate in 1.2 mM Ca^{++}. They share these properties with cells isolated from chemically induced papillomas. Cells derived from altered foci are resistant to induced differentiation by phorbol ester tumor promoters but are stimulated to proliferate by these agents. Infection of epidermal cells with oncogenic retroviruses containing an activated *ras* gene indicate that expression of *ras* and subsequent synthesis of p21 provides a marked proliferative stimulus to basal cells. However, such cells respond to high Ca^{++} by cessation of proliferation. These cells do not terminally differentiate but appear to be blocked in some non-terminal but advanced state of differentiation.

Molecular Regulation of Epidermal Specific Differentiation Products: Our studies have indicated that initiation of carcinogenesis is associated with a change in normal differentiation. In order to understand this association at the molecular level, the regulation of specific differentiation products is being explored. In collaboration with Dr. John Stanley we have found that the basement membrane antigen, pemphigoid, is synthesized only by proliferating basal cells and this synthesis stops shortly after induction of differentiation. In contrast, the intercellular antigen, pemphigus, is synthesized only in the differentiating cell population. In an attempt to probe for functional changes in gene expression which may occur in carcinogen or promoter treated normal cells or in malignant cells, cloning of the genes for keratin peptides, the major differentiation proteins of the epidermis, has been accomplished. Clones corresponding to the 55, 59 and 67 kilodalton keratins synthesized by mouse epidermis in vivo and the 50, 54 and 60 kilodalton keratins synthesized by cultured mouse keratinocytes have been isolated and in some cases sequenced. Specific mRNA has been identified for each probe in newborn, adult and embryonic skin with the in vivo probes and in normal cultural epidermal cells for the in vitro probes. The expression of each set of keratin genes may represent a program characteristic of predominantly differentiating cells (in vivo) or

predominantly proliferating cells (in vitro). Malignantly transformed cells and preneoplastic cells show alterations in their programs of keratin gene expression suggesting that regulation of keratin genes in these cells differs from normal cells. It is anticipated that the identification of keratin profiles of individual cells or cell clones by in situ methods will be useful in characterizing their particular stage of differentiation.

Determinants for Susceptibility to Carcinogenesis: Epidemiological and medical genetic data have indicated major individual differences in cancer risk in humans. Increased risks are associated both with overall susceptibility to cancer or susceptibility in a particular target organ. In some cases specific genetic changes have been associated with increased risk, but in many examples, polygenic influences appear more likely. To date biochemical epidemiological studies have focused only on genetic differences in carcinogen metabolism. In the complex and multistage evolution of cancer, it seems unlikely that carcinogen metabolism is solely responsible for enhanced risks. In fact it seems likely that factors associated with the expression of neoplastic change would play an important role in host susceptibility. The development through selective breeding of animal strains with high susceptibility at a particular organ site provides an excellent model for the study of susceptibility determinants. This Laboratory has utilized the SENCAR mouse strain for susceptibility studies since this strain is especially sensitive to chemically induced skin carcinogenesis.

SENCAR mice are markedly susceptible to two-stage skin carcinogenesis compared to BALB/c mice. Grafting studies have shown that susceptibility is a property of the skin itself and other studies indicate that sensitivity is not due to differences in metabolism of polycyclic aromatic hydrocarbons. Yet by a variety of biological and biochemical parameters SENCAR epidermal cells behave identically to epidermal cells from less sensitive strains. These include in vitro growth kinetics, DNA repair, receptor binding of growth factors and phorbol esters, density and function of Langerhans cells, production of epidermal thymocyte activating factor, and induction of transglutaminase. SENCAR epidermis, however, appears to have a population of initiated cells which can be isolated from untreated cultures because they resist the terminal differentiation signal of 1.2 mM Ca^{++}. Thus, SENCAR may be constitutive for an alteration in gene expression associated with the earliest changes in transformation.

Immunological Techniques to Study the Interaction of Carcinogens With DNA: The interaction of carcinogens with DNA has been studied by a unique methodology pioneered by this Section. Antibodies have been developed in rabbits against guanosin-(8-yl)-2-acetylaminofluorene (G-8-AAF) and guanosin-(8-yl)-2-aminofluorene (G-8-AF) the major guanosine adducts formed in vivo and in vitro by the interaction of nucleic acids with the aromatic amine carcinogen 2-acetylaminofluorene (AAF). Antisera have also been developed against DNA substituted with the 7,8-diol 9,10 epoxide of benzo[a]pyrene (BPDE) yielding the trans (7R)-benzo[a]pyrene-N^2-deoxyguanosine (BPdG) as the major antigen. A third antiserum developed in this Section is to cis-dichlorodiammine platinum DNA (cis-DDP-DNA). These antisera, which are highly specific for adducts, have been used to develop sensitive quantitative immunoassays for monitoring carcinogen binding and for morphological localization of binding sites.

Currently these assays are able to detect one adduct per 10^7-10^8 nucleosides. Using immunological assays, the persistence and removal of AAF adducts has been monitored during liver carcinogenesis by AAF. These studies have shown that binding to liver DNA is saturable and that adduct removal reaches a steady state after several weeks. In these studies, carcinogen substitution of liver DNA during feeding protocols does not occur uniformly, and both interlobular and intralobular differences are observed. In cell culture, immunofluorescence studies with anti-BPdG antibodies have shown that bound benzo[a]pyrene (BP) is localized to the nucleus of all exposed cells and that RNase-sensitive "hot-spots" for binding are evident. Removal of BP adducts appears uniform in cell culture. Studies on BP painted mouse skin reveal saturation of adducts at extremely low levels of substitution and rapid removal. These results suggest extreme sensitivity to the initiating action of this carcinogen in skin. The BPdG antibody has also been used in immunoassays to screen DNA obtained from lung cancer patients and controls. Several positive samples were obtained in lung tissue from cancer patients and in DNA obtained from circulating lymphocytes. The production of a cis-DDP-DNA antibody has provided the first evidence that the cis-DDP-DNA adducts produced synthetically are structurally similar to those produced in vivo. The major adduct detected is the bidentate N-7 guanosine derivative. The cis-DDP-DNA immunoassay has been used to measure platinum bound to DNA of ascites tumor cells recovered from mice receiving cis-DDP therapy. DNA from buffy coats of patients receiving cis-DDP chemotherapy indicates that substitution by cisDDP is readily measurable. Current studies involve a prospective analysis comparing clinical course and DNA binding levels of patients receiving cis-DDP chemotherapy.

Mechanism of Action of Tumor Promoters and Antipromoters: Tumor promotion by phorbol esters has been an area of intense study in this Section. Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Our studies have indicated that basal cells are heterogenous in response to phorbol esters in that some cells are induced to differentiate while others are stimulated to proliferate. This could form the cellular basis for selection. The induction of terminal differentiation by phorbol esters, appear to be mediated by the phorbol ester receptor, and this action of phorbol esters is enhanced by Ca^{++}. The molecular basis for the pharmacological heterogeneity is suggested by studies of the phorbol ester receptor in cultured keratinocytes. Multiple receptor classes are found in differentiating cultures indicating that maturation state may modify receptor affinity. Putative initiated cells, isolated in our laboratory, cannot be stimulated to differentiate by phorbol esters and have only a single receptor class. The altered response pattern of initiated cells suggests a mechanism for their selective clonal expansion from a larger population of normal cells undergoing terminal differentiation in response to phorbol esters. Retinoids prevent the terminal differentiation induced by phorbol esters, and this is mediated by opposing actions on transglutaminase and cornification. We propose that this mechanism is related to the anti-promoting activity of retinoids. Studies on the progression of benign to malignant tumors in vivo indicate that promoters are incapable of accelerating the conversion process while genotoxic carcinogens have a marked enhancing and accelerating effect on malignant conversion. These results suggest a mechanism of multistage carcinogenesis involving three steps. A genetic change in the program of terminal differentiation characterizes the initiation step. This is a preneoplastic change. Tumor promotion involves cell selection and clonal

expansion of initiated cells but does not alter their preneoplastic character. A second genetic change is required in the third step to convert benign to malignant lesions.

DIFFERENTIATION CONTROL SECTION: The Differentiation Control Section (1) studies the biological and biochemical factors involved in normal differentiation of epithelial tissues; (2) uses pharmacological techniques to alter differentiation of normal, preneoplastic and neoplastic epithelial cells to determine the relevance of differentiation to carcinogenesis and to determine methods to intervene in preneoplastic progression; (3) studies the relationship between differentiation and growth control; (4) focuses on cell surface changes in differentiation and neoplasia.

Vitamin A and its derivatives, the retinoids, are of interest in cancer research because they play an essential role in the maintenance of normal differentiation in most epithelial tissues. Our laboratory has shown that retinol as well as a derivative of retinoic acid are found in membrane structures in the phosphorylated form and as such may play a role as specific carriers of the sugar mannose.

This mechanism of action may be related to specific glycosylation defects which have been demonstrated in vitamin A deficiency in at least three liver glycoproteins: the α_{2U} globulin, the α_1-macroglobulin and the very low density lipoprotein. Work from the Differentiation Control Section has evolved in the following areas during the past year.

Regulation of Cell Adhesion: Cell surface glycoproteins are believed to play a major role in cell substrate and cell-cell adhesion. Retinoic acid treated chick sternal chondrocytes were found to release into the culture medium fibronectin of a slightly higher apparent molecular weight than the fibronectin synthesized by control chondrocytes. Peptide mapping showed that this difference in MW was mainly localized in the carbohydrate-rich, collagen binding domain of fibronectin. Tunicamycin treatment suppressed the difference resulting from retinoic acid treatment. Endoglycosidase H digestion of thermolysin and chymotrypsin cleavage products of both forms of fibronectin revealed the prevalence of the high mannose type N-linked carbohydrate moiety in control chondrocyte fibronectin, whereas retinoic acid treated cells showed the prevalence of fibronectin containing mostly "complex type" oligosaccharide chains in the collagen binding domain. These results represent the first biochemical evidence of a direct interference of retinoic acid with the glycosylation pathway of a glycoprotein with known biological function. In related studies on mouse fibroblasts (3T6 and 3T12 cells) and JB6 cells, retinoic acid was shown to specifically enhance the incorporation of the monosaccharide mannose into a protein of apparent subunit molecular weight of 180,000. This protein was characterized in JB6 cells as procollagen. Glycosylation of procollagen was specifically enhanced without significant effect on amino acid incorporation. The tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), decreased the incorporation of radioactive mannose into procollagen in JB6 cells.

Subcellular Localization and Mechanisms of Action of Retinyl Phosphate: The endoplasmic reticulum from rat liver was shown to contain the substrate retinyl phosphate as well as the mannosyl transfer system that utilizes retinyl

phosphate and dolichyl phosphate as intermediates for protein glycosylation. A profound difference in the glycosylation systems mediated by the two carriers was found in that retinyl phosphate was shown to transfer mannose directly to protein, whereas dolichyl phosphate transferred it to lipid-linked oligosaccharides. Structural studies with perhydroretinyl phosphate, a compound without vitamin A activity, have demonstrated the absolute requirement for the conjugated system of double bonds for the mannosyl transfer activity of retinyl phosphate to endogenous acceptors of the membrane. The saturation by hydrogenation of the double bonds did not affect the mannosyl acceptor activity. In a related study the stimulatory activity of bovine serum albumin on retinyl phosphate mannose synthesis in vitro was shown to be due to the interactive complex that BSA forms with retinyl phosphate. In the absence of BSA the retinyl phosphate is found in a complex with Mn^{++}. Such Ret-P-Mn^{++} complex alters the UV absorption spectrum of Ret-P and the electron spin resonance spectrum of Mn^{++} and does not allow Ret-P to function as a substrate for the mannosyl transferase.

Functional Vitamin A Deficiency in Tumors: Transplanted Morris hepatomas whether minimally or maximally deviated from normal liver contained less than detectable amounts of retinyl palmitate. This study unequivocally demonstrated the vitamin A deficient status of microsomal membranes from hepatoma cells. Glycosylation pathways in the tumor may be defective because of lack of retinyl phosphate mannose in the presence of normal synthetic levels of dolichyl phosphate mannose. Inasmuch as minimally deviated hepatomas were found depleted of retinyl palmitate, the working hypothesis was proposed that vitamin A deficiency may function by itself as a tumor promoter by providing permissive conditions for initiated cells to express their tumorigenic potential.

MOLECULAR MECHANISMS OF TUMOR PROMOTION SECTION: The Molecular Mechanisms of Tumor Promotion Section, using relevant model systems, 1) studies the interaction of promoters with specific cellular receptor; 2) elucidates the functional importance of receptors in promoter action; 3) identifies endogenous ligands with specific affinity for receptors of exogenous promoters; and 4) characterizes the initial biochemical steps in the cascades associated with receptor occupancy. Understanding of the early events in promoter action should permit the analysis of their control, modulation, and function in human cells under normal and pathological conditions. Determination of the ability of less specific tumor promoters to perturb indirectly the same processes will shed light on the generality of mechanisms of promotion and will assist in the development of better assays for tumor promoters.

Identification of Phorbol Ester Receptors and Endogenous Ligands: Current work has characterized a specific, phorbol ester apo-receptor, present in brain cytosol, which requires addition of phospholipids to reconstitute activity. Structure-activity relations for the reconstituted apo-receptor closely resembled those for the membrane receptor, suggesting that they might be the same. The phorbol ester apo-receptor co-fractionated with protein kinase C activity, indicating a biochemical function for the receptor. Diacylglycerol is an endogenous activator for the protein kinase C, and the phorbol esters can substitute for diacylglycerol in this stimulation. Diacylglycerol therefore appeared to be a possible candidate for an endogenous phorbol ester analog. In confirmation of this prediction, diacylglycerol was shown to competitively inhibit phorbol ester

binding to the apo-receptor with a K_I of 0.4% (w/w) the concentration of the phospholipid. Structure-activity analysis of various diacylglycerol derivatives indicated that short or unsaturated side chains were required for activity.

A priori, the phorbol ester receptor could recognize either free phorbol ester or that dissolved in the membranes. The ability of insoluble diacylglycerols to mimic the phorbol esters strongly supports the latter possibility. A phorbol ester which was relatively insoluble in aqueous solution was synthesized. It was shown to be a potent inhibitor of phorbol ester binding if introduced into the phospholipids used for apo-receptor reconstitution, whereas it was substantially less potent if added to the aqueous incubation mixture instead. These findings further our interpretation of the structure-activity relations for highly lipophilic phorbol esters. They suggest, additionally, that relatively insoluble phorbol esters may provide a convenient reservoir for maintaining constant phorbol ester concentrations during systemic promotion experiments.

To better explore structure-activity relations for immobilized phorbol ester analogs as well as to prepare useful phorbol ester conjugates, a series of phorbol ester-biotin derivatives was prepared. All showed good binding potency in the absence of avidin and loss in the presence of avidin. The results indicate that little tolerance exists for introduction of bulky, hydrophilic groups at either the 12 or 13 positions of the phorbol esters molecule. In addition, the derivatives provide a mechanism for rapid, reversible inactivation of phorbol esters with an impermeant reagent, which may permit confirmation of the intracellular location of the phorbol ester receptor.

Heterogeneity of Phorbol Ester Receptors: The different structure-activity relations for the different stages of tumor promotion suggest the importance of phorbol ester receptor heterogeneity in phorbol ester action. Receptor heterogeneity has in fact been found for particulate preparations of mouse skin. To determine whether the heterogeneity reflects differences within a single cell type or differences between cell types, binding to intact keratinocytes was examined. A single binding site was observed in keratinocytes grown under low calcium conditions, whereas shift of the keratinocytes to high calcium medium rapidly led to receptor heterogeneity.

Phorbol Ester and Hormone Receptor Interactions: Coupling between receptor occupancy and biological response has been examined in greatest detail in two cell lines (GH_4C_1 and D16) which possess receptors for hormones thought to act through induction of phosphatidylinositol turnover and activation of protein kinase C. Analysis of receptor properties in cell lysates has shown that the receptor is in equilibrium between the cytosol and membrane fractions and that both fractions contribute to the total receptors measured in intact cells. Treatment with phorbol esters shifts the equilibrium to favor the membrane compartment. Hormone-mediated decrease in receptor number is associated with a decreased ability of phorbol esters to shift the equilibrium. Unlike the case in brain, phorbol ester binding to membranes in GH_4C_1 cells shows markedly lower affinity than that to the reconstituted cytosolic apo-receptor. This difference appears to reflect the receptor environment in membranes of these cells, since it it can be abolished by addition of an excess of the appropriate phospholipids to the assay.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04484-06 CCTP

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Modulation of Cell Surface and Growth Parameters by Retinoids and Tumor Promoters

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Luigi M. De Luca Research Chemist, LCCTP, NCI

COOPERATING UNITS *(if any)*

Howard University, Washington, D.C.; National Institute of Dental Research, Bethesda, Maryland; Laboratory of Viral Carcinogenesis, NCI, Frederick, Maryland; National Institute of Environmental Health Sciences, Chapel Hill, North Carolina

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

Differentiation Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Retinoids and tumor promoting agents are known to alter cell surface macromolecules. These groups of agents exerted opposing actions on the availability of epidermal growth factor receptors and on the biosynthesis and secretion of procollagen in JB-6 cells, a cell line derived from mouse epidermis, and in 3T12 cells, a cell line of spontaneously-transformed mouse fibroblasts. Generally, the cells responded to retinoid treatment by an increase in adhesion, in epidermal growth factor receptor number and in the biosynthesis of procollagen, whereas the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate caused the opposite effects. Moreover, retinoids usually had a growth inhibitory effect, whereas the tumor promotor stimulated colony formation in soft agar in JB-6 cells. Inasmuch as retinyl phosphate has been shown to act as an intermediate in glycosyl transfer reactions, changes in carbohydrate composition at the level of the cell surface were expected to occur as a result of retinoid treatment. Retinoid treated, more adhesive, fibroblasts were shown to contain relatively more of the complex type polysaccharide chains in glycoproteins at their cell surface, compared to control cells. A study of the cell surface glycoprotein fibronectin, released into the culture medium by chicken sternal chondrocytes after retinoic acid treatment, showed an increase in molecular weight compared to control. Analysis of the collagen binding domain of fibronectin from retinoid treated cultures showed a considerable increase in its molecular weight over control. The difference in molecular weight was abolished by tunicamycin, a known inhibitor of glycosylation. Moreover, β-endo-N-acetyl glucosaminidase H digestion of the 2-^{3}H-mannose-labeled collagen binding domain of fibronectin showed that retinoic acid caused a relative increase in complex oligosaccharide chains of this glycoprotein, in agreement with the findings on fibroblast cell surface. Thus, retinoids may control cell surface properties by modulating the glycosylation of crucial molecules such as fibronectin, thus possibly affecting their retention on the cell and/or biological activity.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Anton Jetten Expert LCCTP, NCI

Objectives:

To study the molecular mechanisms by which retinoids and tumor promoters modulate functional characteristics of the cell surface, such as growth factor reception, fibronectin and procollagen synthesis and the association of these molecules with the cell surface. The results are analyzed with respect to the way in which changes in these factors may control cell proliferation and differentiation.

Methods Employed:

Biosynthesis of cell surface molecules. The biosynthesis of fibronectin was studied in cultures of chicken sternal chondrocytes. Fibronectin was labelled with a variety of precursor molecules for the carbohydrate and protein portions of the molecule. The subunit molecular weight was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The fibronectin molecule was isolated from the media of cultured cells by immunoprecipitation. Susceptibility of the carbohydrate moiety of the collagen binding domain to β-endo-glucosaminidase H was followed by paper chromatography. The antibiotic tunicamycin was used to determine the specificity of the action of retinoic acid on the carbohydrate portion of the collagen binding domain. Chromatographic behavior on sepharose coupled lectins gave a useful index of change in the carbohydrate moiety of the molecule after retinoid treatment.

The biosynthesis of procollagen was studied in cultured fibroblasts or JB6 cells derived from mouse epidermis. The effect of retinoic acid and tumor promoters on the carbohydrate portion of the molecule was determined by specific precursor labeling.

Studies on sensitivity of the procollagen molecule to collagenase and pepsin treatment permitted the identification of the molecule as proα-1 collagen. The effect of retinoid treatment on exposure of fibronectin at the cell surface was studied by cell surface iodination. The availabilty of epidermal growth factor receptors at the cell surface was determined by radioiodinated epidermal growth factor.

Growth properties of cultured cells were studied under anchorage dependent and independent conditions.

Major findings:

1. Studies of the Mechanism of Retinoid-Induced Adhesion of Spontaneously Transformed Mouse Fibroblasts.

We have previously shown that transformed cells (Balb/c mouse,3T12-3) increased their adhesion to the substrate and displayed higher incorporation of (2-^{3}H)-mannose into both glycolipids and glycoproteins after treatment with retinoic acid. Stimulation of (2-^{3}H)-mannose incorporation into mannolipids was evident 8 hr after exposing the cells to retinoic acid, and stimulation of the incorporation into glycoproteins occurred slightly later. This correlated with the time course of increased adhesion. SDS-PAGE of (2-^{3}H)mannose glycoproteins indicated that both retinoic acid and retinol treatments stimulate the incorporation of the radiolabelled sugar into a glycoprotein with subunit MW 180,000 (Gp 180) and, to a lesser extent, into other glycoproteins. [^{3}H]Leucine incorporation into a protein banding at the same position as the ^{3}H-mannose labelled Gp 180 was not affected by retinoid treatment. A retinoic acid induced increase in the amount of Gp 180 could also be shown by lactoperoxidase catalyzed radioiodination of cultured 3T12 cells, and controlled trypsin digestion experiments indicate that Gp 180 is a component of the cell surface.

2. Inhibition by Tumor-Promoting Phorbol Esters of Procollagen Synthesis in JB6 Mouse Epidermal Cells

Treatment of JB6 cells with 12-O-tetradecanoylphorbol 13-acetate (TPA) or other tumor promoters results in the irreversible acquisition of tumorigenicity in nude mice and anchorage-independent growth in soft agar. One of the biochemical responses that occurs during TPA treatment is a greater than 75% reduction in a mannose-labeled glycoprotein with an apparent molecular weight of 180,000. This sensitive glycoprotein has now been identified on the basis of collagenase and pepsin sensitivity as procollagen pro-α(1) chain. [^{3}H]proline labeling also demonstrated a parallel decrease in the 150,000 procollagen pro-α2(1)component. Mezerein and epidermal growth factor, were also active in decreasing procollagen synthesis. Clonal derivatives of JB6, some resistant and others sensitive to growth in soft agar after TPA treatment, showed similar basal levels of procollagen synthesis as well as similar degrees of TPA-dependent procollagen loss indicating that the procollagen decrease may be necessary but is not sufficient to produce the anchorage independence. Comparison of chemically transformed cell lines derived from JB6 with their nontransformed precursor and with nontransformants of a different origin and their transformed derivatives suggests that reduced procollagen synthesis is a stably acquired phenotypic change and may therefore be involved in maintenance of the transformed phenotype as well as in its induction.

3. Action of Retinoids on the Anchorage-independent Growth of Rat Kidney Fibroblasts NRK 536 Induced by 12-O-Tetradecanoylphorbol-13-acetate or Sarcoma Growth Factor.

Retinoic acid dramatically enhances sarcoma growth factor induced soft agar colony formation of rat kidney fibroblast cells NRK 536-3 (SA6). Retinoic acid also stimulates colony formation in soft agar induced by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate although the phorbol ester alone is less effective in producing anchorage-independent growth than is the sarcoma growth factor. Retinoic acid not only enhances the number of colonies formed but also causes an increase in their average size. Retinoic acid by itself does not induce anchorage-independent growth but also acts synergistically with the

sarcoma growth factor and the phorbol ester as a mitogen on anchorage-independent growth of these NRK cells. In addition to retinoic acid, the 13-cis-, 4-oxo-, and trimethylmethoxyphenyl analogues of retinoic acid and retinol also stimulate colony formation in agar induced by sarcoma growth factor or the phorbol ester. Although the stimulation of growth in soft agar by various retinoids correlates with the specificity with which they bind to the cytosolic binding proteins in other systems, no binding proteins could be detected in the cytosol of these non-transformed rat kidney cells.

4. Retinoic acid causes a modification in the carbohydrate moiety of the collagen binding domain of chondrocyte fibronectin.

The mechanism of the retinoic acid induced increase in adhesion of chicken sternal chondrocytes was investigated. Fibronectins from control and from RA treated cultured sternal chondrocytes were obtained by immunoprecipitation after radioactive labeling with [2-^{3}H]mannose. The fibronectin derived from RA-treated cultures displayed a slightly higher molecular weight by SDS-PAGE.

The collagen binding domain was prepared by collagen-sepharose chromatography after thermolysin or chymothrypsin cleavage. Polyacrylamide gel electrophoresis of the domain showed an increase in MW caused by RA treatment over control. This increase in molecular weight was abolished when the cells were treated with tunicamycin an inhiitor of glycosylation. β-Endo N-acetyl glucosaminidase H treatment of the collagen-binding domains showed a relative prevalence of susceptible over resistant oligosaccharide chains in fibronectin from the control cells, thus permitting the conclusion that RA treatment caused a relative increase in the complex type oligosaccharide chains compared to control cultures.

Significance to Biomedical Research and the Program of the Institute:

Alteration in cell surface adhesion and in recognition phenomena appears to occur during neoplastic transformation. Therefore, agents which modify cell surface characteristics to resemble the normal phenotype are of obvious interest. Our studies are designed to characterize cell surface traits responsible for invasion, growth in soft agar and other peculiarities of transformed cells. It has been found that, in addition to their function as chemopreventive agents in chemical carcinogenesis, retinoids can alter cell surface morphology and adhesion of cultured transformed mouse fibroblasts, BALB/c 3T12-3 cells, to resemble the "normal phenotype." At the molecular level, it appears that retinoids play a role as carriers of mannosyl residues in the endoplasmic reticulum. The biosynthesis of specific cell surface glycoproteins such as procollagen and fibronectin has been shown to be profoundly affected by retinoids. Retinoids also seem to affect the availibility of receptors for certain growth factors, such as EGF-receptors on the cell surface.

Thus, it is reasonable to expect that this project will contribute to our understanding of the basis for the decreased adhesion and greater invasive potential of neoplastically transformed cells and that it will allow a better understanding of mechanisms of growth control as well as the biochemical targets for the opposing actions of retinoids and tumor promoters.

Proposed Course:

Our work on the cell surface glycoproteins of cultured spontaneously-transformed mouse fibroblasts has pointed to an effect of retinoic acid on the structure of the carbohydrate moieties of glycoproteins. Retinoic acid causes an increase in adhesion of 3T12 cells and a 58% increase in the amount of mannose incorporated into total cellular glycoproteins. Moreover the retinoid caused an increase in "complex type" oligosaccharide chains at the cell surface. During the past year we have found that RA also causes an increase in "complex type" oligosaccharide structures in fibronectin obtained from the medium of cultured chicken sternal condrocytes. This increase in the relative proportion of "complex type" oligosaccharides over "high mannose type" was found in the collagen binding domain of the fibronectin molecule which also displayed a higher molecular weight. Tunicamycin treatment equalized the MW of the collagen binding domain from secreted fibronectin of normal and RA-treated chondrocytes.

We propose to investigate whether the RA-induced change in carbohydrate structure of fibronectin is responsible for increased adhesion of the cells. First we will investigate whether the collagen binding domain from fibronectin from RA treated cells has a greater affinity for collagen than control fibronectin. This can be achieved by using increasing concentrations of urea in the elution of the collagen affinity column. It is expected that the collagen binding domain from RA-treated cells should display greater affinity to the column than the domain from control cells. The antibiotic tunicamycin, at concentrations which specifically block glycosylation, will be used in these studies to attempt to establish cause and effect correlation between glycosylation and cell adhesion.

Epidermal Growth Factor Receptor. The EGF-receptor is a glycoprotein of apparent molecular weight 150,000. We have found that retinoic acid at the level of the cell surface causes an increase in the number of EGF-receptor in a variety of cell lines, including cultured mouse keratinocytes. On the contrary TPA causes a decrease of EGF receptor number. In 3T6 fibroblasts RA did not affect significantly the rate of internalization and degradation of EGF. Moreover, when exposed to high concentrations of EGF, both RA-treated and control cells "down regulate" their EGF receptors and although the growth rate of RA-treated 3T6 cells is about one half that of control cells, the rate at which EGF binding capacity is restored after down-regulation is about 3 times as fast as in control cells. Post translational modification of the EGF-receptor molecule may be responsible for some of the observed phenomena: thus RA may regulate the availability of EGF receptor molecules at the cell surface by controlling receptor glycosylation. This hypothesis will be tested in cultured cells.

Radioactively-labelled EGF-receptor will be obtained from RA-treated and control cells by immunoprecipitation with a monoclonal antibody made available to us through the courtesy of Dr. Joseph Schlessinger of the Weizman Institute of Science. The EGF-receptor will then be purified by HPLC and the carbohydrate structure will be studied by cleavage with endo and exo glycosidase. The importance of the carbohydrate moiety for EGF binding to the receptor and/or to the cell surface will be studied.

Publications:

Adamo, S., Sasak, W., Dion, L. D. and De Luca, L. M.: Studies on the mechanism of retinoid induced adhesion of spontaneously transformed mouse fibroblasts. Acta Vitam. Enzymol. 5: 3-10, 1983.

Dion, D., Bear, J., Bateman, J., De Luca, L. M. and Colburn, N.: Inhibition by tumor-promoting phorbol esters of procollagen synthesis in promotable JB6 mouse epidermal cells. JNCI 69: 1147-1154, 1982.

Jetten, A. M.: Action of retinoids on the anchorage-independent growth of normal rat kidney fibroblasts induced by 12-O-tetradecanoylphorbol-13-acetate or sarcoma growth factor. Cancer Res. 43: 68-72, 1983.

Jetten, A. M. and Goldfarb, R. H.: Action of epidermal growth factor and retinoids on anchorage dependent and independent growth of non-transformed rat kidney cells. Cancer Res. 43: 2094-2099, 1983.

Lechner, J. F., Kaighn, M. E., Jetten, A. M., Groden, J. and German, J.: Bloom's syndrome cells have an abnormal serum growth response. Exp. Cell Res. (In Press)

Nagarajan, L., Jetten, A. M. and Anderson, W. B.: Characterization of a new cell line Dif 5 derived from retinoic acid treated F9 teratocarcinoma cells. In Teratocarcinoma Stem-Cells. New York, Cold Spring Harbor (In Press)

Nagarajan, L., Jetten, A. M. and Anderson, W. B.: Isolation and characterization of retinoic acid induced differentiated cell types from F9 teratocarcinoma cells. Exp. Cell Res. (In Press)

Strickland, J. E., Hennings, H., Allen, P. T., Jetten, A. M., Strickland, A. G., Hellman, K. B. and Yuspa, S. H.: Susceptibility determinants for mouse epidermal carcinogenesis. In B. Armstrong and H. Bartsch (Eds.): Host Factors In Human Carcinogenesis. Lyon, IARC Scient., 1982, Public. No. 39, pp. 259-268.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04504-11 CCTP

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Model Systems for the Study of Chemical Carcinogenesis at the Cellular Level

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. H. Yuspa Chief, LCCTP, NCI

COOPERATING UNITS *(if any)*

USUHS, Bethesda, MD; U. of Washington, Seattle, WA; Hopital des Enfants Malades, Paris, France; Merck, Sharpe and Dohme Research Institute, Philadelphia, PA

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.5	3.5	5.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mouse and human epidermal cell cultures and epidermal cell lines are utilized to study mechanisms of epithelial carcinogenesis. Proliferating basal cells are selected by growth in culture medium with reduced concentrations of Ca^{++}. Terminal differentiation is induced by increasing Ca^{++}. Exposure to a variety of initiating carcinogens yields cellular foci which resist Ca++-induced differentiation but are not tumorigenic. Cultured papilloma cells have similar properties. Exposure to retroviruses with an activated ras oncogene induces a marked proliferative-stimulus but only conditional effects on differentiation. To explore altered regulation of specific differentiation proteins during carcinogenesis, keratin genes have been cloned and sequenced and the amino acid sequence of several of the proteins deduced. Transformed cells have an altered program of keratin gene expression. Tumor promoters induce terminal differentiation in some basal cells through a mechanism similar to Ca^{++} and possibly requiring activation of the phorbol ester receptor. Carcinogen-altered preneoplastic cells are resistant to the differentiation-inducing effects of tumor promoters, but phorbol esters may stimulate DNA synthesis in these cells thereby providing a selective advantage during promotion. In vivo studies indicate that genotoxic carcinogens, but not tumor promoters, can accelerate malignant conversion of benign lesions.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

H. Hennings	Senior Chemist	LCCTP, NCI
M. Poirier	Research Chemist	LCCTP, NCI
D. Roop	Expert	LCCTP, NCI
J. Strickland	Research Chemist	LCCTP, NCI
U. Lichti	Expert	LCCTP, NCI

Objectives:

To study cellular and molecular changes during stages of chemical carcinogenesis through the use of unique in vitro model systems designed to simulate well-studied in vivo models. Studies are directed to give insight into general changes occurring in mammalian cells during malignant transformation and specific molecular events which may be causative to the transformation process. Specific markers of the transformed phenotype are also being sought and mechanisms to prevent or reverse transformation are being studied.

Methods Employed:

This laboratory has developed and utilized mouse epidermal cell culture as an appropriate model to approach the stated objectives. Previous studies have shown that this model functions biologically in a fashion highly analogous to mouse skin in vivo. Human epidermal cells obtained from neonatal foreskins have also been adapted to growth in vitro. In vivo studies utilizing the two-stage mouse skin carcinogenesis model and grafts of human or mouse skin onto nude mice are also employed. A number of laboratory techniques are required to pursue the objectives. Morphology is followed by light and electron microscopy and histochemical staining. Macromolecular synthesis and growth kinetics are studied by biochemical and autoradiographic procedures. Intracellular ion changes are assayed by atomic absorption spectrometry. Cellular metabolic functions, including the production of specific differentiation products, are monitored by enzyme assays, gel electrophoresis, amino acid analysis and radio-immunoassay. The progression to the malignant phenotype is monitored by growth rates, soft agar assay, karyotypic abnormalities, enzymatic changes, changes in gene expression at the level of mRNA and injection of cells into nude or newborn mice. A number of immunologic techniques including cell surface antibody production, fluorescent staining, immunoprecipitation and radioimmunoassay are being performed to recognize the normal or altered phenotype and to study specific molecules. Isolation of specific mammalian genes is performed through the preparation of epidermal mRNA, reverse transcription and cloning of transcripts in plasmid pBr322. Cloned genes are characterized by hybridization, selection-translation assays, blot analysis and sequencing.

Major Findings:

The pursuit of this project has led to major new findings in four pertinent areas: 1) factors controlling normal epithelial differentiation; 2) quantitation, selection and characterization of carcinogen altered cells; 3) biochemical

and molecular genetic characterization of specific marker molecules and assessment of their regulation in normal and transformed cells; and 4) understanding of the process of preneoplastic progression and the mechanisms of tumor promotion and anti-promotion.

Much of the progress in this project has developed from the discovery that ionic calcium is a critical regulator of epidermal growth and differentiation. At low ionic calcium concentrations in culture medium (0.02 - 0.09 mM), epidermal cells maintain a monolayer growth pattern with a high proliferation rate. Such cells do not form desmosomal attachments. Essentially 100% of the attached cells are in the proliferating cell pool. Our studies have shown that these cultures have low transglutaminase activity, virtually no cornified cells and synthesize the bullous pemphigoid antigen, all three characteristic of basal cells. The pemphigus vulgaris antigen, a marker for a cell type in a more advanced state of differentiation, is not expressed.

When cells maintained under low calcium growth conditions are switched to medium with calcium content above 0.1 mM (standard commercial culture media are 1.2 - 1.8 mM), differentiation is induced. Desmosomes form within minutes of exposure to high Ca^{++} medium.

These cells vertically stratify and cornify and slough from the culture dish. This program of differentiation is characterized by a high transglutaminase activity and the synthesis of the pemphigus vulgaris antigen. The synthesis of bullous pemphigoid antigen ceases during Ca^{++} induced differentiation.

The mechanism by which calcium induces differentiation in cultured keratinocytes has been studied in some detail. A variety of agents were examined as potential modifiers of the calcium response. Other multivalent cations which might be expected to substitute for Ca^{++} were inactive in inducing or preventing epidermal maturation. Modifiers of calcium or sodium fluxes, local anesthetics and protease inhibitors were also without effect. Modulators or analogues of cyclic nucleotides did not influence epidermal differentiation, and cyclic nucleotide levels did not change significantly in the first 10 minutes after increasing calcium.

Effective inhibition of calcium-induced differentiation, as estimated by morphology, ultrastructure and cornified envelope formation, was seen with the divalent cation ionophore A23187 and the $Na^{+}K^{+}$ATPase inhibitor ouabain. The well-known effects of ouabain on intracellular sodium and potassium suggested the possible involvement of these monovalent ions in the program of calcium-induced epidermal maturation. Direct intracellular ion measurements indicated an elevation of both sodium and potassium occurred within 12-24 hours of calcium-induced differentiation. The increase in intracellular potassium appears to be the more relevant of these changes since the increase was blocked by both ouabain and A23187. Other inhibitors of calcium-induced differentiation, including harmaline, 8(diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) and low (0.1 mM) potassium medium also blocked the rise of intracellular potassium. The 5 inhibitors had no consistent effect on intracellular sodium. Thus,

elevated intracellular potassium may be necessary for the later stages of epidermal differentiation. However, neither ouabain, A23187, nor harmaline affected the assembly of desmosomes, the earliest ultrastructural change noted after increasing medium calcium. This rapid change in cell-cell contact, beginning within minutes after calcium elevation, appears to be independent of changes in sodium and potassium but may instead be modulated by increased calcium at the cell surface.

The modulation of differentiation by retinoic acid and other retinoids has been examined. These agents are known to alter epidermal differentiation and to inhibit skin tumor promotion, but their mechanism of action is unknown. In collaboration with Peter Steinert of the Dermatology Branch of NCI we have shown that retinoic acid is a potent inducer of epidermal transglutaminase in basal cells. Paradoxically cells exposed to retinoic acid have a diminished number of isodipeptide crosslinked bonds and do not form cornified envelopes, whereas high transglutaminase activity in keratinocytes exposed to 1.2 mM Ca^{++} or phorbol ester tumor promoters is associated with increases in both of these products. In collaboration with Dr. Ulrike Lichti of the Differentiation Control Section of LCCTP, further studies have been performed.

The increase in transglutaminase activity by retinoids occurs gradually over 48 to 72 hours and requires the continued presence of retinoid to achieve maximum inductions of 30-50 fold. Withdrawal of retinoid causes a decrease in enzyme activity with a half life of about 24 hours. The decline in activity correlates with the metabolism of retinoids by cultured epidermal cells. The enzyme induced by retinoids is similar to that induced by phorbol esters and Ca^{++} on the basis of apparent Km for putrescine at pH 7.5, requirement for protein and RNA synthesis for induction, inhibition of induction by certain protease inhibitors (see project Z01 CM-05178-01) and positive reactions with antibody to rat epidermal TGase in immunofluorescent assays of induced cells. However the enzymes appear to be different with respect to subcellular distribution. Whereas only 20-30% of the Ca^{++} and TPA induced enzymes are in the soluble fraction after centrifugation at 12,000 x g, 80-90% of the RA induced enzyme is in the soluble fraction. This action of retinoic acid could provide a novel mechanism by which retinoids could modify differentiation and carcinogenesis (see below).

The regulation of epidermal differentiation by Ca^{++} in vitro suggested that a calcium binding protein might play a physiological role in epidermal differentiation in vivo. A unique 12.5 kd calcium-binding protein (SCaBP) in rat epidermis had been described by others. Immunofluorescent studies on rat skin indicated that SCaBP is limited to the basal layer of normal stratified squamous epithelia but may be also seen in cells in upper layers of metaplastic, dysplastic and neoplastic tissue. Using antisera to this rat protein, we found that SCaBP antigens of 12, 11 and 10 kd were immunoprecipitated from soluble extracts of mouse and rat keratinocyte cultures. The 11 kd antigen was phosphorylated. No antigen synthesis was detected in fibroblast cultures. The presence of mRNA for all three SCaBP antigens was detected in RNA isolated from newborn mouse epidermis and translated in a reticulocyte lysate.

Synthesis of SCaBP in cultured keratinocytes is high in basal cells cultured under low Ca^{++} conditions and ceases by 72 hours in cells switched to high Ca^{++} medium. However pulse-chase studies indicate that cells in high Ca^{++} retain previously synthesized SCaBP. Cultured basal cells transiently decrease SCaBP synthesis in response to phorbol ester exposure, and this correlates to the time when many cells are undergoing terminal differentiation. SCaBP synthesis resumes by 48 hours after phorbol ester exposure when cell proliferation increases. These results indicate a role for SCaBP in epidermal proliferation. Several tumorigenic and non-tumorigenic epidermal cell lines were analyzed for SCaBP synthesis. These lines showed variable synthesis and variable modulation by Ca^{++} of the 3 proteins immunoprecipitated by SCaBP antiserum.

The emphasis we have placed on understanding the regulation of normal epidermal differentiation evolves from our discovery that an early effect of carcinogen exposure to epidermal cells in vitro or mouse skin in vivo is an alteration in the response to differentiation signals such as high Ca^{++}. Altered cells are resistant to terminal differentiation and produce expanding foci while normal cells slough from the culture dish. We have proposed that this biological change is reflective of the initiating event in chemical carcinogenesis. A number of initiating agents have been studied for their potency in the induction of altered cell foci. The potency of each agent (7,12 dimethylbenz[a]anthracene > benzo[a]pyrene > N-methyl-N'-nitro-N-nitrosoguanidine > 4-nitroquinoline-N-oxide > N-acetoxyacetylaminofluorene) closely parallels its potency as an initiator in mouse skin. Where studied, colony number observed for each agent was proportional to carcinogen dose. The formation of DNA adducts does not necessarily correlate quantitatively with biological potency since N-acetoxyacetylaminofluorene forms many more adducts than equimolar concentrations of benzo[a]pyrene but produces far fewer colonies. Thus the qualitative nature of the DNA interaction appears to be crucial in specific target organs.

The properties of 8 keratinocyte cell lines which were resistant to Ca^{++} induced terminal differentiation were analyzed. Most lines have a stable epithelial morphology by light or electron microscopy, but each is distinctive in these parameters. When tested at low passage or within 4 months in vitro, only 2 were tumorigenic in vivo. Upon repeated passage or prolonged in vitro maintenance, most became tumorigenic. The tumors were carcinomas with varying degrees of differentiation. All but one line had a unimodal DNA content suggesting a clonal origin of each line, but some lines were near diploid while others were near tetraploid. The ability to grow in agar did not correlate with tumorigenicity. Activity of the enzyme gamma glutamyl transpeptidase did not correlate with tumorigenicity for these 8 lines. Epidermal transglutaminase activity was high in some lines and low in others and did not correlate with tumorigenic potential. Five of 8 lines synthesized keratin proteins as determined by immunoprecipitation of labeled cell extracts with antikeratin antiserum. Those lines not synthesizing keratins were the only lines which showed agar growth. These results suggest that mouse keratinocyte lines which are selectable by resistance to Ca^{++} induced terminal differentiation are preneoplastic, but the data failed to yield a characteristic cellular marker for this trait. Cell lines may be analogous to papilloma cells, and during the past year we have developed methods to culture papilloma cells. A variety of morphological cell types evolve from dissociated papillomas including characteristic epithelial cells. These grow well in 0.02 mM

Ca^{++} but are also capable of proliferation and subculture in 1.2 mM Ca^{++}. Thus a similar Ca^{++} regulation is seen in cells treated with carcinogens in vitro, cells obtained from skin initiated in vivo and from cells obtained from either benign or malignant tumors.

The availability of putative initiated (differentiation resistant) cells from carcinogen assays has provided an opportunity to search for unique pharmacological responses in these cell types. These lines proliferate at a high rate at all Ca^{++} levels in culture medium. Unlike normal epidermal cells, an increase in intracellular Na^{+} was not observed after switch to 1.2 mM Ca^{++} medium although the K^{+} increase was similar to that seen in normal keratinocytes. Exposure to retinoic acid induces a marked increase in transglutaminase activity, usually more pronounced than in normal keratinocytes. However transglutaminase activity was not modulated in these lines by high Ca^{++} medium or by exposure to phorbol esters. Several cell lines were stimulated to proliferate by exposure to the tumor promoter. Binding of epidermal growth factor (EGF) differed in these cell lines from that of normal keratinocytes. EGF binding decreased markedly when normal basal cells were switched to 1.2 mM Ca^{++} or when they were exposed to phorbol esters. EGF binding to putative initiated cells changed little upon exposure to phorbol esters. However the absolute response of each line to each condition was variable. The relationship of these findings to the mechanism of initiation remains to be determined.

The isolation of analogs of retroviral oncogenes from human tumors and the demonstration of their transforming activity in 3T3 cells has stimulated us to study the role of the activated ras oncogene in our culture model. Infection of basal cells with Kirsten or Harvey sarcoma virus causes a marked stimulation of basal cell proliferation. Challenge of infected basal cell cultures with 0.5 mM Ca^{++} medium indicates that terminal differentiation is altered since infected cultures persist in this medium for long periods without stratifying and sloughing from the culture dish. Infected cultures express much higher levels of p21 protein than controls, and studies with temperature sensitive mutants indicate that persistent p21 function is required for the observed effects of the viruses. In response to 0.5 mM Ca^{++}, the proliferation rate is markedly reduced in both control and virally infected cells. However, the proliferation rate of the sarcoma virus infected cultures is reduced less in response to elevated levels of calcium. Virus infected cells as well as controls also demonstrate a rise in the differentiation-associated enzymatic activity of epidermal transglutaminase and will terminally differentiate when the multiplicity of infection is below one. These results suggest that expression of the ras oncogene does not completely block epidermal cells in their program of terminal differentiation in response to 0.5 mM Ca^{++} and this differs from epidermal cells altered by exposure to chemical carcinogens and from malignant keratinocytes. Expression of p21 therefore is closely linked to epidermal proliferation and modifies the program of terminal differentiation. It appears that ras oncogene expression is not sufficient to be a complete transforming activity for a terminally differentiating tissue such as skin.

In an attempt to probe functional changes in gene expression in epidermis which may occur in carcinogen- or promoter-treated normal cells or in malignant cells, cDNA clones for keratin proteins, the major differentiation product of epidermal

cells, have been isolated. Initially, a cDNA library was constructed to total poly(A) RNA isolated from newborn mouse epidermis. From this library clones corresponding to the 55, 59 and 67 kd keratins, the major keratins synthesized in this tissue, were isolated. Using these cloned cDNAs as specific probes to detect transcripts of these keratin genes, we have been able to show that transcripts for these genes are abundant in RNA isolated from newborn epidermis but absent in RNA isolated from primary epidermal cell cultures. These results suggest that this subset of keratin genes is only expressed in differentiating cells within the epidermis. We have recently isolated cDNA clones corresponding to the keratins expressed in primary epidermal cell cultures, which are 50, 54 and 60 kilodaltons. Transcripts for these genes are present in RNA isolated from newborn mouse epidermis but at a concentration lower than that observed for the 55, 59 and 67 kd keratin genes. We assume that our primary epidermal cell cultures are representative of the basal (proliferating) cells found in intact epidermis and that the transcript levels observed for the 50, 54 and 60 kd keratin genes in newborn mouse epidermis reflect the relative contribution of basal cells, which consists of one cell layer, to total epidermis, which contains many cell layers at different stages of differentiation. Therefore, the cDNA clones which we have isolated provide probes for proliferating and differentiating epidermal cells.

The expression of keratin genes has been examined in two transformed epidermal cell lines (Pam 212 and Pam 321). Pam 212 cells express keratin genes normally active in proliferating epidermal cell cultures as well as the keratin genes active in differentiating epidermal cells in vivo. Pam 321 cells also express the keratin genes active in primary cultures. However, these cells do not express the keratin genes active in differentiating epidermal cells but instead express keratin genes coding for lower molecular weight keratins generally synthesized in other types of epithelia. Therefore, the program of keratin synthesis, which is highly regulated in normal proliferating and differentiating epidermal cells, is altered in transformed epidermal cells.

The expression of keratin genes has been studied in the epithelium of the vagina in collaboration with Jim Clark (Baylor College of Medicine). This tissue is of interest because the degree of keratinization in this epithelium is hormone dependent and it allows the induction of keratin gene expression to be studied in vivo in ovariectomized rats after exposure to estradiol. Two important observations have resulted from this study. First, the keratin genes expressed in epidermis are also expressed in the internal keratinizing epithelium of the vagina. Second, the temporal induction of these genes after exposure to estradiol correlates well with the observed morphological changes in this epithelium. There is a dramatic induction of the keratin genes expressed in proliferating cultured epidermal cells within 24 hr after exposure to estradiol and this correlates with the onset of proliferation in the vaginal epithelial cells. The expression of the keratin genes expressed in differentiating epidermal cells only becomes pronounced 48 hr after exposure to estradiol and this correlates with the observation of stratification and keratinization in this epithelium.

In collaboration with Peter Steinert (Dermatology Branch) the first complete amino acid sequence of an epidermal keratin subunit has been determined from the nucleotide sequence of the 59 kd keratin cDNA clone. This sequence data has

provided valuable information for formulating a general structural model for intermediate filament proteins. At the present time, the sequence for the 67 and 55 kd keratin cDNA clones is being completed. Sequencing information has been used to generate mono specific antiserum for individual keratin proteins which heretofore has been very difficult since these proteins contain common antigenic determinants. Preliminary data indicate that a monospecific antibody to the 67 kd keratin has been obtained using a 12 amino acid synthetic peptide corresponding to a unique region of this protein.

Finally, genomic sequences homologous to these keratin cDNA clones have been isolated and are being characterized and sequenced in an attempt to understand the molecular basis for the differential expression of keratin genes observed during normal differentiation of epidermal cells and for the altered expression of these genes in transformed epidermal cells.

In vivo and in vitro studies have indicated that phorbol ester tumor promoters markedly affect epidermal differentiation. Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Our studies have provided a cellular basis for selection in epidermis. Studies on the induction of the enzyme epidermal transglutaminase by the phorbol ester tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) in basal cell cultures have demonstrated a 2-4-fold increase in enzyme activity within 12 hours of exposure. This appears to be mediated by protease action (See Z01 CP 05178-02). The increase in transglutaminase activity parallels morphological differentiation in approximately 50% of the basal cell population, and differentiating cells slough from the culture dish within 24-48 hours as transglutaminase activity in attached cells returns to basal levels. The cells which remain are resistant to induced differentiation by 1.2 mM Ca^{++} medium in that they fail to demonstrate increased transglutaminase activity or decreased thymidine incorporation, both characteristics of control basal cells induced to differentiate by 1.2 mM Ca^{++}. Cells remaining after a single exposure to TPA do not respond to a second exposure with an induction of transglutaminase if the interval between exposures is 4 days. TPA pretreated cells do not undergo a transient decrease in thymidine incorporation (characteristic of control cells) when exposed to TPA a second time but instead are directly stimulated to proliferate by the phorbol ester indicating such cells are not refractory to the promoter. When the treatment-free interval after TPA is extended from 4 to 10 days, transglutaminase inducibility and inhibition of DNA synthesis are restored in basal cells to either TPA or 1.2 mM Ca^{++} as inducers. These results indicate that heterogeneity exists within the epidermal cell population and that exposure to phorbol esters induces differentiation in some cells while stimulating proliferation in others. Such heterogeneous responses would cause a selective redistribution of the epidermal cell population and could lead to clonal expansion of initiated cells. This explanation for the biological basis for tumor promotion is strengthened by the findings that putative initiated cells respond to TPA only in the proliferative pathway. During the past year studies have been conducted to determine the effect of promoting agents other than phorbol esters on the differentiation response of cultured basal cells and to explore the pharmacological basis for the heterogeneity in basal cells. The potent indole alkaloid skin tumor promoter, teleocidin, induces transglutaminase to the same extent or greater than TPA. The highly inflammatory and cytotoxic non-promoting agent resiniferotoxin

is not an inducer of transglutaminase. Incomplete skin tumor promoters, mezerein and retinyl phorbol acetate, were as potent as TPA as inducers of transglutaminase. Anthralin and benzoyl peroxide, skin tumor promoters which do not bind to the phorbol ester receptor, did not induce transglutaminase. TPA was used to study the influence of the state of epidermal maturation at the time of exposure on the differentiation response. Epidermal basal cells were induced to differentiate by elevating extracellular calcium to 1.2 mM. TPA markedly accelerated the differentiation program when given simultaneous with exposure to 1.2 mM Ca^{++} as indicated by measurements of DNA synthesis, transglutaminase activity and cornified cells. Furthermore, epidermal cells committed to differentiate by switching to 1.2 mM Ca^{++} medium remain responsive to the differentiative effects of TPA for at least 5 hours although they rapidly lose their capacity to increase ornithine decarboxylase activity in response to TPA. These results indicate that the induction of transglutaminase activity and cornification in epidermal basal cells is characteristic of phorbol ester promoters or other agents that bind to the phorbol ester receptor but is not characteristic of all skin tumor promoters. This result suggests that the phorbol ester receptor regulates epidermal differentiation. The state of differentiation of epidermal cells at the time of phorbol ester exposure may determine whether the cellular response will be in a proliferative or differentiative pathway.

The molecular basis for this pharmacological heterogeneity may be clarified in studies conducted in collaboration with the Molecular Mechanisms of Tumor Promotion Section of LCCTP. Measurements of [20-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) to mouse skin particulate preparations yielded curvilinear Scatchard plots which best fit a model of three phorbol ester binding components with affinities for PDBu of 0.7 nM (PBS-1), 10.3 nM (PBS-2) and 52.3 nM (PBS-3). Binding of [^{3}H]PDBu to intact cultured mouse primary keratinocytes was analyzed in low (0.07 mM) Ca^{++} and high (1.2 mM) Ca^{++}. [^{3}H]PDBu binding to proliferating keratinocytes in low Ca^{++} yielded a linear Scatchard plot consistent with a single binding component which had a Kd of 13.7 $\pm$ 1.6 nM and was present at 1.3 $\pm$ 0.3 pmol/mg protein (n = 4). The data span a range of receptor occupancies from 2% to 98% of the total bound. The binding parameters for the intact proliferating keratinocytes closely resemble those for PBS-2 in membrane preparations from epidermis. In contrast, specific binding of [^{3}H]PDBu to differentiating keratinocytes (cultured in the presence of 1.2 nM Ca^{++}) yielded a curvilinear Scatchard plot similar to that observed in skin particulate preparations. Thus the characteristics of binding of [^{3}H]PDBu to keratinocytes depend on their state of differentiation further supporting the idea that differentiation may modulate the pleiotropic response of this tissue to phorbol esters. Interestingly, putative initiated cell lines display only a single binding component most similar to PBS-2, in either high or low Ca^{++}

We have continued to explore the mechanism of action of antipromoter compounds in the mouse skin culture model. The discovery that retinoids and phorbol esters are active inducers of transglutaminase has led to studies on the interaction of these two agents in this pathway. A variety of biologically active retinoids induce transglutaminase activity and their effectiveness correlates with their reported antipromoter activity. Exposures to both retinoic acid and TPA are antagonistic, resulting in less than additive induction. Induction kinetics with both inducers are more like those of retinoic acid than of the phorbol ester.

Simultaneous exposure to retinoic acid and TPA protects the epidermal basal cell population from induced terminal differentiation and cell loss which is observed in response to the promoter alone. These results suggest that the antipromoting action of retinoids could be mediated by modification of phorbol ester accelerated terminal differentiation through an effect on transglutaminase and cornification. This action of retinoids would block a critical aspect of cell selection involving loss of cells and subsequent regenerative hyperplasia, although simple hyperplasia may still occur.

While pursuing mechanistic studies on epidermal carcinogenesis in vitro, this project is continually and vitally supported by in vivo carcinogenesis studies performed under contract CP 1-5744. In vivo studies are designed both to confirm results obtained under the artificial conditions of cell culture and to extend our knowledge of carcinogenesis to provide direction for new in vitro studies. These studies have revealed an important new component of skin carcinogenesis. Initiation-promotion protocols in mouse skin generally yield large numbers of papillomas and very few carcinomas. Our studies indicated that genotoxic agents can accelerate the conversion of benign lesions to malignant tumors. SENCAR mice were initiated once and promoted for 10 weeks until benign tumors developed. The conversion rate during 42 additional weeks with no further treatment or with continued promoter application was low and equal for both groups. However, if animals received either urethane, N-methyl-N'-nitro-N-nitrosoguanidine or 4-nitroquinoline-N-oxide weekly for 30 weeks, the conversion rates were much accelerated and enhanced. Animals not receiving prior initiation and promotion did not develop significant numbers of tumors. These studies suggest that a second genetic change is required to convert benign to malignant lesions.

Significance to Biomedical Research and the Program of the Institute:

The majority of human cancers are associated with environmental exposures and most of the tumors are of epithelial origin. Animal models have been extremely useful for bioassay and some mechanistic studies, but they are not as useful for investigations at the cellular level because of complicated interactions between host and environment as well as physiological variations within an experimental protocol. The use of cell culture systems, particularly those of epithelial origin, offers the opportunity to extend the present conceptual models of carcinogenesis mechanisms to a more basic and cellular level. In addition, cell cultures ultimately should be useful and inexpensive as bioassay screening procedures to detect carcinogens and cocarcinogens in the environment. Many of our present basic concepts concerning the pathogenesis of cancer were developed from studies utilizing carcinogen painting on mouse skin. The irreversibility of initiation, the phenomena of cocarcinogenesis and tumor promotion, the role of hyperplasia and metabolism are examples of such concepts. The development of a cell culture system for epidermal carcinogenesis has been a major advance toward extending our knowledge of mechanisms of carcinogenesis. Earlier efforts were directed to proving that epidermal cells in culture responded to carcinogens and promoting agents as in vivo; in almost every parameter studied, this was the case. Differentiation, metabolism, proliferation, metabolic activation and covalent binding of carcinogens, and promoter responses were highly analogous to the in

vivo situation. These findings enhanced the validity of any subsequent observations made in vitro. For the last several years the model system has been utilized to a much greater degree to ask questions about mechanisms of transformation, carcinogen and promoter interactions, and the role of anticarcinogenic agents such as retinoids. The routine isolation of cells selected for resistance to terminal differentiation, as well as the development of non-tumorigenic and tumorigenic cell strains, offers the opportunity to study the cancer phenotype and tumor progression. The role of differentiation in carcinogenesis and the mechanism by which promoting agents enhance the expression of the tumor phenotype have been studied. From these studies a clearer picture of the biology of initiation and promotion have evolved. Our studies have provided specific markers as tools to detect normal and abnormal states and to provide a rapid assay for differentiation. Greater understanding of mechanisms of tumor promotion and malignant conversion provides an opportunity to devise schemes for intervention in the process of carcinogenesis prior to the development of overt malignancy. Finally, while bridging the gap between relevant animal models and in vitro systems, this laboratory hopes to be able to continue its development of a human skin culture model which had to be abandoned during FY1983. Thus ultimately a chain of systems will be available to determine the relevance of findings in any one model for the entire spectrum of models including the human.

Proposed Course:

This project represents an integrated comprehensive approach to understanding the biological changes associated with initiation and promotion of carcinogenesis and their underlying molecular mechanisms. Future studies are a logical extension of each component of the overall approach. In order to understand the regulation of normal epidermal differentiation, the calcium-modulated culture model will be explored in depth. The role of ions will be investigated by direct measurement of intracellular ionic changes by flame photometry and by ion flux measurements. Assays for determination of Na-K ATPase activity will be developed to define the role of that enzyme in differentiation. Activation, subcellular distribution, and potential substrates of protein kinase C, the putative receptor for phorbol esters, will be studied under various Ca^{++} conditions since it is a likely candidate for a cell surface trigger enzyme for epidermal differentiation.

Further efforts to clarify the role of the calcium binding protein in epidermis will be made through studies at the protein level and through cloning of the gene to study expression at the molecular level. The results obtained with normal keratinocytes will be compared to those with preneoplastic and neoplastic keratinocytes in all of the studies on the regulation of differentiation.

Transformation studies utilizing resistance to induced differentiation will be expanded. Additional chemicals of varying initiating activity will be tested. Modification of the target cells at the time of carcinogen exposure will be utilized to attempt to enhance or inhibit the transforming event. Modifiers will be chosen which are known to alter initiation in mouse skin in vivo. Modifiers which can alter the extent or pattern of carcinogen binding to DNA will also be utilized to determine the effect on initiation. Immunological assays developed in this laboratory (see project Z01-CM-05177-01-CCTP) will be

used to monitor binding. Parallel studies will be performed in vivo and epidermal cells selected in vitro. Progression from the initiated cell to the malignant cell will be systematically studied to elucidate the temporal sequence of this change and to examine the capability of additional carcinogen or promoter treatment to accelerate progression. The development of clonal transformation assays will continue with the testing of clonal lines and dose-response studies. The mechanism by which ras gene expression and the p21 transforming protein can alter proliferation and differentiation will be explored at the molecular level. Virus altered cells will be characterized to determine the differentiation state these cells represent. Other viruses will be studied in an effort to find oncogenes which might block epidermal cells in the particular differentiation state characteristic of chemically altered cells. Combined treatments with chemicals and viruses will be performed. Parallel studies using labeled oncogene probes will be conducted to analyze for expression of genes in various states of altered differentiation and transformation. Transfection of DNA from altered cells into normal keratinocytes will be performed along with high Ca^{++} selection in order to isolate sequences which impart differentiation resistance.

The availability of cDNA clones for keratin genes provides unique markers for a gene family whose expression may characterize specific differentiation states. Individual keratin mRNAs will be localized in specific layers of the epidermis by in situ hybridization of biotinated keratin cDNA probes to histological sections of epidermis. If this is successful, this technique may provide a means of identifying cells with altered patterns of keratin gene expression which may result after exposure of cultured epidermal cells or mouse skin to carcinogens or promoters. Epidermal RNA will be isolated from mice which have been treated with promoters and analyzed for changes in the expression of keratins. Genomic keratin sequences will be isolated to better understand the structure, localization and regulation of this family. Sequencing will continue and for each keratin gene unique peptide will be synthesized to produce specific antibodies. Transfection studies will be performed in order to analyze the functional consequences of specific keratin protein expression. The discovery of the heterogeneity in responsiveness of basal cells to phorbol ester tumor promoters provides a theoretical basis for the cell selection apparent in promotion. Subpopulations will be isolated after promoter treatment and studied individually for specific markers. The number and character of TPA receptors on each population will be analyzed, and their response to various pharmacologic agents will be explored. Antibodies to each cell type will be prepared by hybridoma techniques or in rabbits, and localization of each cell type in the epidermis will be studied by indirect immunofluorescence. Reconstruction experiments with normal and initiated cells or papilloma cells will assess the ability of phorbol esters to select initiated cells from a mixed population. Similar studies will be performed with agents such as mezerein and teleocidin.

In vivo studies will assess the 3 stage carcinogenesis system with regard to mouse strain differences, requirements for third stage agents and inhibitor effects in malignant conversion.

Publications:

Dale, B. A., Scofield, J. A. H., Hennings, H. Stanley, J. R. and Yuspa, S. H.: Identification of filaggrin in cultured mouse keratinocytes and its regulation by calcium. J. Invest. Dermatol. (In Press)

Hennings, H.: Experimental skin carcinogenesis. In Baden, H. P. and Soter, N. A. (Eds.): Pathophysiology of Dermatologic Disease. New York, McGraw Hill, 1983 (In Press)

Hennings, H. and Holbrook, K. A.: Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. An ultrastructural study. Exp. Cell Res. 143: 127-142, 1983.

Hennings, H. and Holbrook, K.: Extracellular calcium regulates growth and terminal differentiation of cultured mouse epidermal cells. In Boynton, A., McKeehan, W. L. and Whitfield, J. F. (Eds.): Ions, Cell Proliferation and Cancer. New York, Academic Press, 1982, pp. 499-516.

Hennings, H., Holbrook, K. A. and Yuspa, S. H.: Factors influencing calcium-induced terminal differentiation in cultured mouse epidermal cells. J. Cell. Physiol. (In press)

Hennings, H., Holbrook, K. A. and Yuspa, S. H.: Potassium mediation of calcium induced terminal differentiation of epidermal cells in culture. J. Invest. Dermatol. (In Press)

Hennings, H., Shores, R., Wenk, M. L., Spangler, E. F., Tarone, R. and Yuspa, S. H.: Malignant conversion of mouse skin tumors is increased by tumor initiators and unaffected by tumor promoters. Nature (In Press)

Holbrook, K. A. and Hennings, H.: Phenotypic expression of epidermal cells in vitro: A Review. J. Invest. Dermatol. (In Press)

Jones, C. R., Goldman, A. E., Steinert, P. M., Yuspa, S. H. and Goldman, R. D.: Dynamic aspects of the supramolecular organization of intermediate filament networks in cultured epidermal cells. Cell Motility 2: 197-213, 1982.

Roop, D. R., Hawley-Nelson, P., Cheng, C. K. and Yuspa, S. H.: Expression of keratin genes in mouse epidermis and normal and malignantly transformed epidermal cells in culture. J. Invest. Dermatol. (In Press)

Roop, D. R., Hawley-Nelson, P., Cheng, C. K. and Yuspa, S. H.: Keratin gene expression in mouse epidermis and cultured epidermal cells. Proc. Natl. Acad. Sci. USA. 80: 716-720, 1983.

Stanley, J. R., Hawley-Nelson, P., Yaar, M., Martin, G. R. and Katz, S. I.: Laminin and bullous pemphigoid antigen are distinct basement membrane proteins synthesized by epidermal cells. J. Invest. Dermatol. 82: 456-459, 1982.

Stanley, J. R., Yaar, M., Hawley-Nelson, P. and Katz, S. I.: Pemphigus antibodies identify a cell surface glycoprotein synthesized by human and mouse keratinocytes. J. Clin. Invest. 70: 281-288, 1982.

Stanley, J. R. and Yuspa, S. H.: Specific epidermal protein markers are modulated during calcium-induced terminal differentiation. J. Cell Biol. 96: 1809-1814, 1983.

Steinert, P. M., Rice, R. H., Roop, D. R., Trus, B. L. and Steven, A. C.: Complete amino acid sequence of a mouse epidermal keratin subunit: Implications for the structure of intermediate filaments. Nature 302: 794-800.

Steinert, P. M., Steven, A. C. and Roop, D. R.: Structural features of epidermal keratin filaments reassembled in vitro. J. Invest. Dermatol. (In Press)

Yuspa, S. H.: Alterations in epidermal functions resulting from exposure to initiators and promoters of carcinogenesis. In Seiji, M. and Bernstein, I. A. (Eds.): Normal and Abnormal Epidermal Keratinization. Tokyo, Tokyo University Press, 1982 (In Press)

Yuspa, S. H.: Cutaneous carcinogenesis: Natural and experimental. In Goldsmith, L. A. (Ed.): Biochemistry and Physiology of the Skin. New York, Oxford University Press, 1983, Vol. II, pp. 1115-1138.

Yuspa, S. H: Retinoids and tumor promotion. In Roe, D. (Ed.): Current Topics in Nutrition and Disease: Diet, Nutrition and Cancer: From Basic Research to Policy Implications. New York, Alan R. Liss, Inc., 1983, pp. 95-109.

Yuspa, S. H.: Methods for the use of epidermal cell culture to study chemical carcinogenesis. In Skerrow, D. and Skerrow, C. (Eds.): Methods in Skin Research. Sussex, England, John Wiley and Sons, Limited, 1983 (In Press)

Yuspa, S. H.: Tumor promotion in epidermal cells in culture. In Slaga, T. (Ed.): Mechanisms of Tumor Promotion. Boca Raton, FL, CRC Press, 1983, Vol. 3, (In Press)

Yuspa, S. H., Ben, T. and Steinert, P.: Retinoic acid induces transglutaminase activity but inhibits cornification of cultured epidermal cells. J. Biol. Chem. 257: 9906-9908, 1982.

Yuspa, S. H., Hennings, H., Lichti, U. and Kulesz-Martin, M.: Organ specificity and tumor promotion. In Langenbach, R., Nesnow, S. and Rice, J. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Press, 1983, pp. 157-171.

Yuspa, S. H., Kulesz-Martin, M., Ben, T. and Hennings, H.: Transformation of epidermal cells in culture. J. Invest. Dermatol. (In Press)

CONTRACTS IN SUPPORT OF THIS PROJECT

MICROBIOLOGICAL ASSOCIATES INC. (N01-CP0-5637)

Title: Biochemistry and Cell Culture Resource

Current Annual Level: Terminated

Man Years: 1.0

Objectives:

To provide cell culture and biochemistry support services to LCCTP. Specialized media or cell cultures are prepared to support LCCTP intramural work. Experiments with human epidermal cell cultures are performed in parallel with the mouse epidermal cell culture experiments performed by LCCTP. The synthesis of phosphorylated retinol derivatives is performed via this contract in support of project Z01 CP 04798-12.

Major Findings:

Using new modifications of culture medium, human epidermal cells can be maintained in the absence of a feeder layer in a highly proliferative state. The growth and differentiation of human epidermal cells, as observed for mouse epidermal cells, is regulated by extracellular calcium. The tumor promoter, TPA, induces terminal differentiation in cultured human epidermal cells.

Retinylphosphate is a critical intermediate in glycosyl transfer reactions and thus is important in the retinoid regulation of glycoprotein synthesis. Under this contract, approximately 10-15 mg of retinyl phosphate is synthesized per week. This is the only known source for this intermediate and the compound has been shipped to laboratories throughout the world as well as for studies in LCCTP.

Significance to Biomedical Research and the Program of the Institute:

The use of rodent tissues has been extremely valuable in defining mechanisms of carcinogenesis. While it is generally believed that mechanisms of carcinogenesis in all mammalian cells will be similar, direct test of this idea is required. By studying parallel model systems for the same target tissue (skin) from rodents and humans, this idea can be validated. Thus, the establishment and use of the human system under this contract is a vital link in a series of model systems required to fully understand human carcinogenesis. Retinoids are potent inhibitors of carcinogenesis and this action may be mediated via effects on glycoprotein synthesis. The isolation and evaluation of the retinylphosphate intermediate is important to understanding the mechanism of retinoid action.

Proposed Course:

This contract was terminated on February 28, 1983 and because of new contracting guidelines in DCCP could not be renewed.

CONTRACTS IN SUPPORT OF THIS PROJECT

MICROBIOLOGICAL ASSOCIATES INC. (NO1-CP1-5744)

Title: Rodent and Rabbit Facility as a Resource to LCCTP

Current Annual Level: 322,000

Man Years: 3.0

Objectives:

To provide space, care and technical support for the conduct of in vivo experiments designed to correlate, validate and extend the in vitro findings developed in this project. In addition this contract supports the antibody production work of project Z01CP05177-03 and the genetic susceptibility studies of project Z01CP05178-03.

Major Contributions:

Project Z01CP04504-11 LCCTP: Factors influencing the progression of benign papillomas to carcinomas were studied in SENCAR mice. After initiation with 7,12-dimethylbenz[a]anthracene and promotion for 10 weeks with TPA, papillomas were the only neoplastic lesions. Continued TPA application for 30 weeks did not alter the carcinoma incidence from that of control animals in which promotion was terminated at 10 weeks. However, administration of a tumor initiator, either urethane (systemically), MNNG (topically) or 4NQO (topically), markedly accelerated and enhanced (4-fold) the conversion of papillomas to carcinomas. The papillomas stage appears to be required in the production of carcinomas since no carcinomas developed when treatment with acetone was substituted for TPA in the promotion stage of the protocol. Inhibitors of tumor promotion by TPA (fluocinolone acetonide and retinoic acid) are currently being tested as potential inhibitors of the progression of papillomas to carcinomas. In another experiment in progress, the conversion of papillomas to carcinomas by treatment of papilloma-bearing mice with a tumor initiator (urethane) has been confirmed in Charles River CD-1 mice.

Formaldehyde, a hazardous agent to which humans are exposed, has been reported to induce nasal cancer in rats. In mouse skin, however, repeated topical application caused only a few benign tumors. In SENCAR mice, formaldehyde was inactive as an initiator but demonstrated promoting activity. Repeated application for 80 weeks following DMBA initiation produced 37 papillomas and 6 suspected carcinomas on 30 mice.

Tumorigenicity of cell lines derived from in vitro transformation experiments are being conducted by injection into nude mice. More than 20 lines have been tested, some repeatedly, with the results showing that many lines which demonstrate altered response to differentiation signals after carcinogen exposure are initially not tumorigenic. However, progression to malignancy results with

prolonged passage of these cell lines in culture. Nude mice are also receiving skin grafts of human foreskins and these mice will be subjected to initiation-promotion protocols to assess the response to the human skin.

Project Z01-CP-05178-03 LCCTP: Grafting of SENCAR mouse skin to nude mice and performing carcinogenesis studies on grafted tissue have indicated that susceptibility resides in the target tissue. The possibility that other tissues are more susceptible in SENCARs was examined by administering an initiating dose of DMBA or urethane intraperitoneally, then promoting with topically applied TPA. Initiated and promoted mice developed the expected skin tumors, but no tumors of other tissues could be attributed to the initiators or the promoter, either separately or in combination.

Project Z01-CP-05177-03 LCCTP: Rabbits are maintained for antibody production. In previous years a number of carcinogen-DNA adduct antibodies were produced under this contract (see project description). During the past year antiserum has been raised against the deacetylated adduct of the acetyl aminofluorene-guanosine product (AF-dG) and recognizes femtomolar quantities of this adduct in DNA. New antibodies against specific epidermal differentiation products have also been produced. Specific antibodies to cornified envelope proteins are now available and provide a unique opportunity to isolate precursors to that important component of terminally differentiated cells. Of great significance has been the production of antibodies to the 67 kd and 59 kd keratins which were obtained by immunization of rabbits with a 12-amino acid peptide sequence of each protein. These sequences were determined to be unique from the keratin sequencing studies performed under Z01CP04504-10 CCTP. Characterization of each antiserum is currently underway. If successful these would be the first completely specific antisera available to individual keratin peptides.

Significance to Biomedical Research and the Program of the Institute:

Studies in epithelial target tissues are the most relevant with regard to the pathogenesis of human cancer. While information gained from in vitro experiments on epithelial cells have yielded major insights into mechanisms of carcinogenesis, correlative in vivo experiments are required for validation of results and confirmation of concepts using a tumor endpoint. These in vivo studies have also been invaluable to identify mechanisms of tumor progression and the genetic basis for cancer susceptibility, both extremely important and relevant research areas in carcinogenesis. The work contributed by this animal support project is an integral part of the overall research program of LCCTP.

Proposed Course:

This contract will be continued at the current or slightly increased level to pursue studies on the mechanism of conversion of benign to malignant lesions, the basis for susceptibility to carcinogenesis and to provide rabbit antibodies for various program needs. The nude mouse colony will be used to assess tumorigenicity of cells and for experiments involving heterografts or allografts.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04798-13 CCTP

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Metabolism and Mode of Action of Vitamin A

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Luigi M. De Luca Research Chemist, LCCTP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

Differentiation Control Section

INSTITUTE AND LOCATION

NIH, NCI Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.0	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The aim of this project is to understand biochemical mechanisms of action of vitamin A. The phosphorylated vitamin, retinyl phosphate, acts in biological membranes as a glycosyl carrier and appears to regulate the biosynthesis of glycoproteins. Subcellular fractions from rat liver were analyzed for the topological distribution of the mannosyl transferases which glycosylate the lipid intermediates retinyl phosphate and the polyisoprenoid derivative dolichyl phosphate. The glycosyl transferases as well as the substrates retinyl phosphate and dolichyl phosphate were found localized mainly in the endoplasmic reticulum of rat liver. The activity of retinyl phosphate as glycosylating agent was lost upon hydrogenation of the four double bonds in the side chain of the molecule. This procedure also abolished growth promoting activity, but it did not affect glycosyl acceptor activity. Retinyl phosphate was found to function in a direct glycosylation of endogenous glycoproteins, whereas dolichyl phosphate transferred mannose to oligosaccharide-lipids. The condition of nutritional vitamin A deficiency caused a depletion of liver retinyl palmitate and retinyl phosphate pools at the same time as dolichyl phosphate increased fourfold. Post nuclear membranes from transplanted and primary rat hepatoma tissue were in a status of retinyl palmitate and retinyl phosphate depletion, whereas their dolichyl phosphate content was not altered significantly from that of the host rat liver. Vitamin A depletion of the tumor tissue did not appear related to cell division rates, inasmuch as hepatectomy failed to have a considerable effect on retinyl palmitate and retinyl phosphate content of regenerating liver at 24 and 48 hours. It should be considered that the vitamin A deficiency condition of the tumor cell may be either the consequence of cell selection during carcinogenesis or a permissive condition for the development of the tumor from initiated cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Freesia Huang	Expert	LCCTP, NCI
Donata Rimoldi	Visiting Fellow	LCCTP, NCI
Ulrike Lichti	Expert	LCCTP, NCI

Objectives:

To study the mechanism of action of vitamin A at the level of its intervention in glycosylation reactions of the membrane and at the level of gene expression. To study the vitamin A status of hepatomas from the point of view of retinyl palmitate and retinyl phosphate contents. To explore the possibility that the mechanism of tumor promotion is based on depletion by tumor promoters of natural tumor antipromoting substances or of their functional manifestation.

Methods Employed:

The function of retinyl phosphate in glycosylation. This project utilizes a variety of chemical and biochemical techniques to study the involvement of retinyl phosphate in mannosylation of membrane glycoproteins. The main technical breakthrough has been the employment of bovine serum albumin as a carrier of exogenously added retinyl phosphate to the microsomal membranes of rat liver in "in vitro" studies of glycosylation utilizing guanosine diphosphate mannose as the glycosyl donor. The biosynthesis of retinyl phosphate mannose and the polyisoprenoid derivate dolichylphosphate mannose is then followed by thin layer chromatography and/or high pressure liquid chromotography on anion exchangers. Characterization of the membrane domain on which retinylphosphate mannose is synthesized and functions as a mannosyl donor was carried out in various subcellular fractions. The endogenous glycoprotein acceptors were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequent fluorography.

Chemical synthesis of the hydrogenated derivative of retinol, was carried out by catalytic hydrogenation. The resulting perhydromonoeneretinol was phosphorylated by the same techniques used for retinylphosphate synthesis.

Complex of retinyl phosphate and divalent cations. Interactive complexes between retinyl phosphate and divalent cations were monitored by specific spectral changes in the UV absorption maximum of retinyl phosphate from 325 nm to 280 nm and by electron spin resonance spectra of the cation.

Measurement of vitamin A in hepatomas. Hepatomas and host liver tissue were homogenized and the post nuclear membrane fraction used for determination of retinyl palmitate after extraction with cholorform/methanol (12/1). The extracts were analyzed by high pressure liquid chromatography on reverse phase resins.

Major Findings:

1. Synthesis of retinyl phosphate mannose and dolichyl phosphate mannose from endogenous and exogenous retinyl phosphate and dolichyl phosphate in microsomal fraction. Specific decrease in endogenous retinyl phosphate mannose synthesis in vitamin A deficiency.

Retinol and its derivatives (retinoids) are necessary in normal physiology to maintain differentiation of epithelial tissues, growth as well as the reproductive and visual functions. They also modify growth and adhesive properties of transformed cells in the direction of the normal phenotype. A biochemical correlate for these functions, is the regulations of the incorporation of the monosaccharide mannose into glycoproteins by retinoids. This incorporation is greatly reduced in vitamin A deficiency and enhanced by excess vitamin A. Recent studies have demonstrated that rat hepatocytes from deficient animals fail to incorporate mannose into a specific glycoprotein, the α_{2U}-globulin even though synthesis of the protein remains unaltered. These findings suggest a specific glcosylation defect in A deficiency.

Similar studies have been conducted in a variety of tissues in the past decade to support the concept that in normal physiology the vitamin is somehow involved in the biosynthesis of specific mannose-containing glycoproteins.

In 1970 we first reported that rat liver microsomal membranes can synthesize a lipid compound containing retinol, phosphate, and the sugar mannose. This was the first hint into a possible mannose carrier function for vitamin A in microsomal membranes. As our techniques improved, we found that we were really dealing with two mannolipids: the major compound was a derivative of the polyisoprenoid dolichol, and the minor product was the derivative of vitamin A.

Dolichyl phosphate has a more general role as a glycosyl carrier, whereas retinyl phosphate is specific for the sugar mannose and probably for certain glycoproteins. The development of the bovine serum albumin based technique for the synthesis of Ret-P-Man permitted an investigation of the endogenous amounts of these lipid intermediates. It was found that rat liver microsomal fraction synthesized Ret-P-Man (retinyl phosphate mannose) and Dol-P-Man (dolichyl phosphate mannose) from endogenous Ret-P (retinyl phosphate) and Dol-P (dolichyl phosphate). Ret-P-Man synthesis displayed an absolute requirement for a bivalent cation, and also Dol-P-Man synthesis was stimulated by bivalent metal ions. Under optimal conditions the amount of endogenous Ret-P mannosylated in incubation mixtures containing 5 μM-GDP-mannose in 15 min at 37°C was approximately 3 pmol/mg of protein. In the same assays about 7-10 pmol of endogenous Dol-P was mannosylated. In addition to Ret-P-Man and Dol-P-Man, a mannolipid co-chromatographing with undecaprenyl phosphate mannose was detected. Microsomal fraction from Syrian golden hamster livers was also active in Ret-P-Man and Dol-P-Man synthesis from endogenous Ret-P and Dol-P. At 5 mM-Mn^{2+} about 2.5 pmol of endogenous Ret-P and 3.7 pmol of endogenous Dol-P were mannosylated from GDP-mannose per mg of protein in 15 min at 37°C. On the other hand, in microsomal fraction from livers of vitamin A-deficient hamsters 1.2 pmol of Ret-P and 14.1 pmol of Dol-P were mannosylated in 15 minutes. Since GDP-mannose: Ret-P and

GDP-mannose: Dol-P mannosyltransferase activities were not affected, as measured in an assay containing exogenous Ret-P and Dol-P, depletion of vitamin A must affect Ret-P and Dol-P pools in opposite ways.

2. Interactions between retinol phosphate and divalent cations.

In the presence of Mn(II) ions, the U.V. absorption spectrum of retinyl phosphate (Ret-P) in Triton X-100 micelles, lecithin, or rat liver microsomes exhibited a shift from 330nm to 287nm. The effect of Mn (II) was reversed by EDTA or phosphate buffer. The same spectral change was found in the presence of poly-L-lysine in place of (Mn(II). Electron spin resonance spectrum of Mn(II) in the presence or absence of Ret-P showed that 75% of the initial concentration of Mn(II) ions is bound to Ret-P when the molar ratio of Ret-P to Mn(II) ions is four to one; no such binding occurred in the presence of retinol or retinoic acid. The appearance of two isosbestic points at 303 and 368 nm in the presence of Mn(II) ions suggests the existence of an equilibrium between a Mn(II)-bound monomer and a Mn(II) bound dimer of Ret-P in Triton X-100 micelles. The same effect on the U.V. absorption spectrum of Ret-P was also induced by Co(II), Cr(II), Zn(II), and Fe(II), but not by Mg++ or Cu(II). The formation of the complex between Ret-P and Mn(II) or Co(II) inhibited by over 70% the synthesis of Ret-P-Man from Ret-P and GDP-[^{14}C] mannose when BSA was added after the metal ion relative to addition of BSA before the metal ion. However, the order of addition did not influence Ret-P-Man synthesis in incubations containing $MgCl_2$, which does not form the metachromatic complex with Ret-P. These results suggest that the availability of proteins, polyamines and metal ions may control the extent to which Ret-P can be mannosylated in the intact membrane.

3. The polyenic system of retinyl phosphate is required for its mannosyl donor activity but not for acceptor activity.

We investigated whether the polyenic and allylic phosphate systems of retinyl phosphate are essential for its mannosyl acceptor and donor activities in rat liver postnuclear membranes. Perhydromonoeneretinyl phosphate, a compound without growth-promoting activity in vitamin A-deficient animals, was prepared by catalytic hydrogenation of retinol and phosphorylation. Perhydromonoeneretinyl phosphate mannose synthesis from GDP-mannose showed continued accumulation for at least 60 min, while retinyl phosphate mannose synthesis showed a maximum at 20-30 min and then declined. Moreover, only retinyl phosphate and not the perhydro derivative stimulated transfer of mannose from GDP-mannose to endogenous proteins, which were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Thus, hydrogenation of side-chain double bonds in retinyl phosphate impaired only slightly its mannosyl acceptor activity, but caused loss of mannosyl donor activity.

4. Mannosyl carrier functions of retinyl phosphate and dolichylphosphate in rat liver endoplasmic reticulum.

Of the subcellular fractions of rat liver the endoplasmic reticulum was found the most active in GDP-mannose:retinyl phosphate mannosyl-transfer activity. The synthesis of retinyl phosphate mannose reached a maximum at 20-30 min of incubation and declined at later times. Retinyl phosphate mannose and dolichyl

phosphate mannose from endogenous retinyl phosphate and dolichyl phosphate could also be assayed in the endoplasmic reticulum. About 1.8 ng (5 pmol) of endogenous retinyl phosphate was mannosylated per mg of endoplasmic reticulum protein and about 0.15 ng (0.41 pmol) of endogenous retinyl phosphate was mannosylated with Golgi-apparatus membranes. About 20 ng (13.4 pmol) of endogenous dolichyl phosphate was mannosylated in endoplasmic reticulum and 4.5 ng (3 pmol) in Golgi apparatus under these conditions. Endoplasmic reticulum, but not Golgi-apparatus membranes, catalyzed significant transfer of [^{14}C]mannose to endogenous acceptor proteins in the presence of exogenous retinyl phosphate. Mannosylation of endogenous acceptors in the presence of exogenous dolichyl phosphate required the presence of Triton X-100 and could not be detected when dolichyl phosphate was solubilized in liposomes. Dolichyl phosphate mainly stimulated the incorporation of mannose into the lipid-oligosaccharide-containing fraction, whereas retinyl phosphate transferred mannose directly to protein.

5. Vitamin A deficiency status of hepatoma tissue.

The retinyl palmitate content of the post nuclear membrane fraction from ten Morris hepatomas, their host rat livers, two acetylaminofluorene induced rat liver hepatomas and their host livers, and of regenerating rat liver was measured by reverse phase high pressure liquid chromatography of the chloroform/methanol extracts. Membranes from the hepatoma tissue contained less than detectable levels of retinyl acyl esters, whereas membranes from host liver tissue and regenerating liver contained normal levels of retinyl palmitate. The ratio of endogenous retinyl phosphate (Ret-P) to the polyisoprenoid dolichyl phosphate (Dol-P) available for mannosylation in the same assay containing postnuclear membranes and guanosine-dephospho[^{14}C]mannose was decreased by a factor of 3 to 10 in hepatoma tissue. Such change in ratio was not attributable to specific changes in Ret-P-Man synthesizing activity, but it appeared related to the vitamin A deficiency condition of the membrane from tumors. Post nuclear membranes from Vitamin A deficient hamster liver, rat cystic hepatocarcinoma, Morris 7777, 3924A1-1 and 5123D-1-2 transplantable rat hepatomas and guinea pig line 10 hepatoma all synthesized a mannolipid with intermediate hydrophobic properties between Ret-P-Man and Dol-P-Man and not normally found in normal liver tissue. Therefore hepatoma cell membrane is in a status of vitamin A and of retinyl phosphate depletion, while dolichyl phosphate content appears similar to host liver membrane.

Significance to Biomedical Research and the Program of the Institute:

It is the aim of this project to investigate the mechanism(s) by which vitamin A functions in the body. Inasmuch as vitamin A and its derivatives, the retinoids, are active as preventive agents of epithelial cancer, such investigation may yield useful information on mechanisms whereby normal tissue-specific phenotypic expression is maintained by retinoids.

A substantial body of work has shown a biochemical involvement of vitamin A at the level of the biosynthesis of glycoproteins. The phosphorylated vitamin appears to function in mammalian membranes as a carrier of mannosyl residues destined to specific glycoproteins. Some glycoproteins mediate cell to cell

recognition and adhesion (e.g., fibronectins); others have hormonal functions in tissue growth and development (e.g., the gonadotropins); others display epithelioprotective functions (e.g., secretory mucins); thus, it is reasonable to propose that the involvement of the phosphorylated vitamin in glycoprotein biosynthesis may be related to its effect on mucus secretion, adhesion and the maintenance of normal phenotypic expression.

The reported findings suggest a correlation between the Ret-P/Dol-P ratio and the degree of differentiation of hepatoma tissue. However, even in the most differentiated tumors the ratio drops from about 0.3 in the host liver to about 0.1, demonstrating a specific deficiency in the amount of endogenous Ret-P available for mannosylation in microsomes from hepatoma tissue. Interestingly, the tumor tissue was also found deficient in vitamin A and its cellular binding protein. Such specific deficiency in Ret-P in the tumor could relate to altered functional cell surface characteristics of the tumor cells if one considers that a variety of cell surface molecules, including fibronectin, procollagen and epidermal growth factor receptors are glycoprotein in nature.

The antagonistic actions of retinoids and certain tumor promoters at the biological and biochemical levels are consistent with the concept that tumor promoting substances may interfere with essential functions at the target site, causing a deficiency of such functions. Therefore we have put forward the concept that under conditions of depletion of the essential element (e.g., vitamin A) or of its functions as caused by the tumor promoter (e.g., TPA), the mutation present in the initiated cell (and maintained latent by the vitamin or the expression of the vitamin's function within that cell or surrounding cells) is now expressed as a result of essential function deficiency and permits the establishment of a cell population that can survive in a more self-sufficient state, i.e., in a state in which the particular substance is no longer essential for survival.

This concept, if proven correct, may suggest novel approaches to "chemoprevention" and it may prove useful in the chemotherapeutic management of the tumor.

Proposed Course:

Involvement of Retinyl phosphate mannose in glycosylation. The specific mannosylation step in which Ret-P-Man is involved will be investigated using in vivo and in vitro systems. Oligosaccharide chains from glycoproteins and from lipid-oligosaccharides of liver from normal and vitamin A deficient hamsters will be studied for chain size and sugar composition. It is expected that if Ret-P-Man functions in the elongation of the oligosaccharide on the lipid intermediate dolichylpyrophosphate-oligosaccharide, prior to the transfer to protein, a shorter oligosaccharide should be found in vitamin A deficiency. If, on the other hand, Ret-P-Man transfers directly to protein these should contain modified and incomplete structures in vitamin A deficient tissues. If the defect resulting from vitamin A deficiency is a shorter oligosaccharide structure, an attempt will be made at correcting such defect by elongating the structure present in the endoplasmic reticulum of vitamin A deficient hamster liver through added Ret-P-Man, which should glycosylate the incomplete chain and restore its normal size.

However, a more complex situation may arise, if deficiency also causes a change in the pattern of processing of the oligosaccharide chains. This aspect will be investigated by specific endoglycosidase treatment of in vivo and in vitro synthesized glycoproteins to study the relative proportion of the endo-N-acetyl glucosaminidase-H susceptible and resistant chains. All the above studies will be conducted on in vivo and in vitro synthesized structures, using either [2-^{3}H]-D-mannose or its sugar nucleotide guanosine diphosphate-[^{14}C]mannose. Sizing of oligosaccharide chains will be performed on either Biogel-P-4 or by high pressure liquid chromatography.

Metabolism of retinoic acid. The function of vitamin A in growth and differentiation was clearly distinguished from the visual and reproductive functions, when it was found that retinoic acid can support growth, but not vision and reproduction. Therefore, to those of us interested in the growth function of the vitamin, two possibilities were open for consideration: either that retinoic acid might be the truly active metabolite of retinol or that one of its metabolites might mimic retinol in its growth function. In either case, molecular manifestations of vitamin A deficiency would have to be reversed, if either compound was administered to the vitamin A-deficient animals.

Both retinol and retinoic acid are active in restoring the normal appearance of the mucociliary epithelium, growth in the whole animal and in the induction of adhesive phenomena in transformed mouse fibroblasts.

Therefore, we are asking the question as to whether a metabolite of retinoic acid may act on the same mannosylation pathway as Ret-P. Several years ago we have reported that the injection of retinoic acid into vitamin A-depleted hamsters restores the incorporation of mannose into liver glycolipids and glycoproteins within a few hours to normal levels. In a similar experiment, rats were kept on a vitamin A-deficient diet supplemented with retinoic acid, and deficiency was caused by removal of the acid. Under these conditions the administration of retinoic acid caused an immediate rise within 15 minutes in the amount of labeled mannose incorporated into a mannolipid with the same chromatographic properties as Ret-P-Man. The shape of the induction curve was slightly different between the retinol-injected and the retinoic acid-injected rats, the retinoic acid showing a slightly (5 minutes) slower response than the injection of retinol. On the basis of these in vivo studies, we propose that retinoic acid mimics retinol in the mannosylation pathway after being metabolized to a retinoid, which can then "fool" the enzymes involved in phosphorylation and mannosylation of retinol. Preliminary results have suggested a retinoid phosphatemannose compound derived from retinoic acid in spontaneously transformed mouse fibroblasts (Bhat, P.V. and De Luca, L.M. 1981, Annals N.Y. Acad. Sci. 359: 135-149). We propose here to investigate the possibility that such compound is also synthesized under normal conditions in vivo. Radioactively labeled retinoic acid will be fed to hamsters which are marginal but not totally depleted in vitamin A. The amounts of RA will be in physiological ranges (1-2 µg/day) and the animals will be kept free of other sources of the vitamins. Liver and testes will then be analyzed for the presence of the presumptive retinoid phosphate mannose compound. This compound will be purified and tested for biological activity in prevention and reversal of squamous metaplasia caused by vitamin A deficiency in the respiratory epithelium of Syrian golden hamsters in organ

culture. In a related project the metabolic fate of retinoic acid in cultures of mouse epidermal cells will be pursued. S. H. Yuspa and U. Lichti have recently reported an effect of RA on the enzyme transglutaminase which may be a key enzyme in the formation of cornified envelopes and perhaps other structures of keratinocytes. The retinoid may alter the activity, synthesis or subcellular distribution of epidermal transglutaminase. To maintain high enzyme activity, frequent additions of RA are necessary, suggesting high rate of metabolism of the retinoid. The metabolism of radioactively labeled retinoic acid will be pursued in cultures of primary mouse epidermal cells. Our reverse phase high pressure chromatography system will be used in addition to thin layer chromatographic techniques to study the rate of disappearance of RA and its conversion to other products.

Gene expression and its control by retinoids. A main action of retinol, retinoic acid and derivatives is at the level of the maintenance of normal function in epithelial tissues. Under conditions of vitamin A deficiency mucociliary epithelia, such as the respiratory tract, undergo profound focal changes which include squamoid type of differentiation with production of a keratinizing epithelium.

We will characterize the keratin molecules in the respiratory tract from hamster tracheas maintained in culture for various periods of time in a vitamin A depleted medium. A variety of techniques will be employed in collaboration with the "In vitro Pathogenesis Section" where cloning of the genes for keratin peptide has been accomplished. The technique of in situ hybridization will also be used to permit investigations with small amounts of tissue.

In addition to respiratory tract, keratin profiles will be probed in mouse epidermal cells cultured in vitamin A depleted medium, which should permit the production of the 67,000 Mr Keratin, which is a normal differentiation product in vivo, but is not usually made in cultured keretinocytes.

Evaluation of vitamin A deficiency as a promoter of 7,12 dimethylbenzanthracene (DMBA) initiated skin papillomas in female SENCAR mice. The hypothesis that vitamin A deficiency may function as a promoting stimulus in skin tumorigenesis will be tested. Previously employed procedures involving two-stage skin carcinogenesis in SENCAR mice will be used. Female mice will be treated with a single topical dose of 20 µg DMBA as the initiator at 8 weeks of age and promoted by weekly topical applications of 2 µg TPA for 10 weeks. This schedule should produce approximately 30-35 papillomas per animal in 100% of the animals. Previous experience with feeding vitamin A depleted diets to newborn Balb/c mice has indicated that they can survive for at least 12 weeks when kept on a vitamin A deficient diet. At this time weight loss and mortality occur. It will be assumed that a similar minimum period of 12 weeks will also be necessary for manifest signs of vitamin A deficiency to occur in SENCAR mice. By that time it should be possible to compare the incidence and per animal burden of papillomas arising in various experimental groups. There will be four experimental groups, two on purina and two on Vitamin A deficient diet. All will be initiated with DMBA. One group of 30 mice will serve as a negative promotion control; one as a positive promotion control; a third group as test group for vitamin A deficiency to function by itself as a tumor promoting agent and a fourth group to study the combined effect of TPA and vitamin A deficiency on tumor promotion.

Publications

Creek, K. E., Morre, D. J., Silverman-Jones, C. S., Shidoji, Y. and De Luca, L. M.: Mannosyl carrier functions of retinyl phosphate and dolichylphosphate in rat liver endoplasmic reticulum. Biochem. J. 210: 541-547, 1983.

De Luca, L. M.: Deficiency of vitamin A in hepatocellular carcinoma tissue: Considerations on its establishment. In Campbell, T. C. (Ed.): Diet and Cancer. Alan Liss. (In Press)

De Luca, L. M.: Essential function deficiency as the result of tumor promotion and the establishment of biological antarchy. JNCI 70: 405-407, 1983.

De Luca, L. M., Brugh, M. R., Silverman-Jones, C. S. and Shidogji, Y.: Synthesis of retinyl phosphate mannose and dolichylphosphate mannose from endogenous and exogenous retinylphosphate and dolichylphosphate in microsomes: Specific reduction in endogenous retinylphosphatemannose synthesis in vitamin A deficiency. Biochem. J. 208: 159-170, 1982.

De Luca, L. M., Creek, K., Morre, D. J. and Shidoji, Y.: Retinyl phosphate as a carrier of mannose in the endoplasmic reticulum of liver tissue. Falk Symposium No. 33. Structural Carbohydrates In Liver. M.T.P. Press Limited. (In Press)

Jetten, A. M. and De Luca, L. M.: Induction of differentiation of embryonal carcinoma cells by retinol: Possible mechanisms. Biochem. Biophys. Res. Commun. (In press).

Shidoji, Y., Silverman-Jones, C. S. and De Luca, L. M.: The polyenic system of retinylphosphate is required for its mannosyl donor activity but not for acceptor activity. Biochem. J. 208: 865-868, 1982.

Shidoji, Y., Silverman-Jones, C. S., Noji, S. and De Luca, L. M.: Interactions between retinyl phosphate and divalent cations. Biochem. J. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05177-03 CCTP

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Use of Immunological Techniques to Study the Interaction of Carcinogens with DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

M. C. Poirier Research Chemist, LCCTP, NCI

COOPERATING UNITS *(If any)*

B. C. Cancer Center, Vancouver, British Columbia; LMPH, NCI, Bethesda, Maryland; USUHS, Bethesda, Maryland; MIT, Boston, Massachusetts; Columbia University, New York City, New York; McArdle Laboratories, Madison, Wisconsin, University of North Carolina, Chapel Hill.

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.75	2.25	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Antibodies specific for carcinogen-DNA adducts have probed the nature, extent and consequences of in vitro and in vivo DNA modification. Biological samples of DNA substituted with 2-acetylaminofluorene (2-AAF), benzo(a)pyrene (BP) or cis-dichlorodiammineplatinum (II) (cis-DDP) were analyzed by immunofluorescence and quantitative immunoassays able to detect 1 adduct in one hundred million nucleotides. Continuous AAF feeding resulted in a rapid accumulation of adducts (3-4 weeks) in rat liver DNA followed by a plateau (4-8 weeks.) When 2-AAF fed animals were placed on control diet, adduct removal was biphasic with a rapid initial Phase I (up to 2 wk) followed by a slow Phase II (2-4 wk). Persistent adduct accumulated slowly due to a loss of phase II removal after 2 wk of carcinogen feeding. In contrast to adduct levels observed in 2-AAF-fed liver, binding of BP to DNA of mouse skin and mouse epidermal cells in culture after exposure to either initiating or transforming doses was more than 50 fold lower. The kinetics of repair for BPdG in vivo and in vitro were biphasic (as in liver) but much more rapid, with 50% removal by 1-3 days. Thus adduct accumulation and removal seem to be specific for a particular target tissue and an individual carcinogen, and may not be quantitatively related to efficiency of tumorigenesis or transformation. Antisera specific for BPdG-DNA and cis-DDP-DNA (bidentate N7 dideoxyguanosine adduct) have been successful in measuring specific adducts as a result of polycyclic aromatic hydrocarbon exposure in lung tissue and buccal smears from lung cancer patients and smokers, and platinum drug chemotherapy in nucleated blood cell DNA from cancer patients.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this project:

S.H. Yuspa	Chief	LCCTP, NCI
J. Nakayama	Visiting Fellow	LCCTP, NCI

Objectives:

To develop and utilize specific and sensitive immunological methods to monitor the interaction of carcinogens with DNA. Studies are directed toward quantification of the extent of in vivo covalent binding and removal under experimental conditions and toward determining the structural and functional consequences of the formation of specific carcinogen-DNA adducts.

Methods Employed:

Both in vivo carcinogen exposure to experimental animals and carcinogen treatment of cultured cells are employed to pursue the objectives. Tissues and cells obtained from individuals environmentally exposed to carcinogens or patients given cancer chemotherapeutic agents are also utilized. The chemical synthesis of radiolabeled and unlabeled DNA-carcinogen adducts and their purification by column chromatography are currently performed. Isolation of macromolecules for carcinogen binding and repair studies utilize density gradient centrifugation. Antibodies are produced by injection of purified antigens into rabbits. A variety of immunological techniques are employed including the qualitative procedures of immunofluorescence and immunochemical electron microscopy and the quantitative radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). High performance liquid chromatography (HPLC) is being established for characterization of specific adducts.

Major Findings:

The interaction of carcinogens with DNA has been studied by a unique methodology pioneered by this Section. Rabbit antibodies have been developed against guanosin-(8-yl)-2-acetylaminofluorene (G-8-AAF) and guanosin(8-yl)-2-aminofluorene (G-8-AF), the major guanosine adducts formed in vivo and in vitro by the interaction of nucleic acids with the aromatic amine carcinogen 2-acetylaminofluorene (AAF). The antisera obtained have been employed to detect picomoles of each C-8 adduct in a competitive radioimmunoassay utilizing [^{3}H]G-8-AAF or [^{3}H]G-8-AF as tracer, with lower limits of sensitivity in the range of one adduct per 3.5×10^5 nucleotides. Sensitivity for detection of C-8 adducts has been increased with the development of the enzyme-linked immunosorbent assay (ELISA) for use with this antibody. By ELISA, using a p-nitrophenol end point, it is possible to measure less than one adduct in 10^7 nucleotides.

A variety of cultured cells from different species have been exposed to N-acetoxyacetylaminofluorene (N-Ac-AAF) and the deacetylated C-8 adduct was found to predominate in all cells except primary rat hepatocytes. The data indicated that specific patterns of AAF-DNA binding are determined at the cellular level

and that manipulation of the quantity and type of adduct formed can be achieved through selective pretreatments. Studies have been initiated to assess the effect of such modifications on the induction of differentiation-altered-cell foci in the BALB/c primary epidermal cell culture system. Recent data suggest that this carcinogen-induced trait occurs with low frequency after moderately-toxic N-Ac-AAF exposures which produce significant adducts. In contrast, concentrations of benzo(a)pyrene giving much lower adduct levels and similar toxicity induce transformation at significant frequency in this system. These findings suggest that specific adducts may be critical in determining a carcinogenic effect in a particular target tissue.

Quantitative immunoassays have also been utilized to study modification of liver and kidney DNA in rats fed a chronic, carcinogenic regimen. In collaboration with Drs. Brian Laishes and John Hunt at the McArdle Laboratory for Cancer Research, RIA has been used to detect C-8 adducts in male Wistar-Furth rats fed 0.02% 2-AAF either continuously up to 8 weeks or for a specific time followed by an interval for repair. In the past year a thorough investigation of the kinetics of formation and removal of C-8 adducts during the first month of 2-AAF feeding has been achieved through a combined application of immunoassay and radiolabeling techniques. Newly-formed adducts could be monitored using a [^{3}H]-2-AAF pulse, fed at the end of 25 days of unlabeled 2-AAF diet. The results show that the rates of newly-formed adduct formation and removal were similar in animals never fed 2-AAF and animals previously fed 2-AAF for 4 weeks. When animals were given control diet after 2-AAF feeding, adduct removal was biphasic with a rapid Phase I occurring during the first 7-14 days on control diet, and a much slower Phase II in effect between 14 and 28 days on control diet. The persistent fraction of adducts, consisting only of dG-8-AF, appeared to accumulate as the result of a very low Phase II removal rate in animals fed 2-AAF for more than 2 weeks. Since the kinetic data suggested that accumulation of 2-AAF adducts in liver DNA might not be uniform, experiments were performed with immunofluorescence and quantitative immunoassays to survey adducts in various liver lobes. Using both techniques, high levels of adducts were observed in the left lateral lobe and low levels were seen in the triangular lobe after 3 days of 2-AAF feeding, indicating that adduct accumulation was not uniform. In addition immunofluorescence revealed a lack of adduct accumulation in the periportal areas of liver lobules. Kinetics of adduct formation and removal in kidney DNAs from animals fed 2-AAF were very similar to liver suggesting that similar mechanisms for formation and removal may be functioning in the non-target organ. However, overall binding levels were always several-fold lower in the kidney.

Antisera developed against DNA substituted with the 7,8-diol, 9,10 epoxide of benzo[a]pyrene (BPDE-I, the anti-isomer) such that the only adduct was trans-(7R)-benzo[a]pyrene-N^2-deoxyguanosine (BPdG), have a higher affinity for BP-substituted DNA than for the isolated BPdG adduct (suggesting antibody recognition of the DNA backbone). The ability of this antiserum to recognize BPdG in intact (non-hydrolyzed) DNA makes it particularly useful for morphological studies. In collaboration with Dr. David Kaufman of the University of North Carolina, electron microscopic visualization of BPdG adducts on DNA fibers has been achieved using the specific antiserum, calf thymus DNA modified with BPDE-I in vitro and ferritin-conjugated goat anti-rabbit IgG. The most recent investigation, using monovalent Fab fragments, has yielded quantitative detection by EM

when results were compared to those obtained by ELISA. Immunofluorescent localization of BPdG adducts has been achieved at the cellular level by examining BPDE-I-exposed cultured mouse epidermal cells. Generalized nuclear fluorescence punctuated with discrete bright spots was observed in BPDE-I-exposed cells with specific antiserum, but normal serum, absorbed specific serum, and non-exposed cells yielded no fluorescence. RNAase treatment removed the particularly intense nuclear staining believed to be localized in the nucleolus. Analysis of buccal smears from smokers in the Philippines were conducted by indirect immunofluorescence with BPdG antiserum. Smears were obtained from individuals who are at risk for cancer of the upper palate, since they smoke with the lighted end of the cigarette in their mouths. Bright nuclear and some cytoplasmic fluorescence was observed in stained smears from several smokers, with the most intensely-stained samples from smokers who also consumed alcohol. Smears of buccal cells from an equal number of non-smokers did not fluoresce and fluorescence was obliterated in smears of smokers if antiserum was first absorbed with BP-DNA. RNase treatment changed neither the intensity nor the pattern of observed brightness. The results of this study are being evaluated coordinately with an analysis of micronuclei, which indicate chromosome damage, in the same cells. This study is being performed in collaboration with Dr. Hans Stich at the British Columbia Cancer Center.

Quantitative analysis of BPdG adducts is currently achieved by a highly sensitive ELISA similar to that already described for anti-G-8-AAF. In studies designed to compare mechanisms of BPdG adduct formation and removal in mouse skin and cultured mouse epidermal cells, Dr. J. Nakayama has used ELISA to monitor BPdG after topical application of initiating quantities of BP in vivo and after transforming doses of BPDE-I or BP in vitro. A remarkable feature both in vivo and in vitro is that low levels of DNA binding are sufficient for initiation in mouse skin (2.5 fmol/µg DNA) and for induction of differentiation-altered foci in cultured mouse keratinocytes (0.4 - 2.8 fmol/µg DNA). This effect is even more striking since the half life for BPdG in mouse skin is 3-4 days and in cultured cells can be as short as 12-24 hr. In the cultured cells very high binding levels (500 fmol/µg DNA) could be obtained by exposure to toxic doses of BPDE-I. However in mouse skin several attempts to elevate the binding levels, including multiple doses of BP, benz(a)anthracene pretreatment, and exposure to BPDE-I or the 7,8 diol did not increase the adducts above those obtained with the highest dose of BP (10 fmol/µg DNA).

Samples of DNA from human lung, lung tumor and white blood cells from lung cancer patients and individuals with other diseases have been screened by BP-DNA ELISA for evidence of adducts. Collection of the clinical material was coordinated by Dr. I. B. Weinstein of Columbia University. Lung tissue DNA from five lung cancer patients gave positive results in the ELISA assay using p-nitrophenol as substrate, however, most of the values were not easily-measurable in this assay and attempts have been made to achieve greater sensitivity. During the last few months the commercial availability of an automatic reader for a fluorescent ELISA substrate (methyl-umbelliferone) has allowed us to increase the sensitivity of this assay another 20-fold. Our continuing collaboration will involve assay of combined DNA and RNA from nucleated blood cells of heavy smokers using the more sensitive fluorescent ELISA. Preliminary data suggest that this is a much more satisfactory assay procedure.

To expand the use of the immunological technology for monitoring human exposures, rabbit antibodies have been elicited against DNA modified with the chemotherapeutic drug cis-dichlorodiammineplatinum (II) (cis-DDP) in collaboration with Dr. Stephen Lippard of MIT. The p-nitrophenol ELISA allows for detectability of 0.1 femtomole/μg DNA. Previous studies in collaboration with Dr. Leonard Zwelling of NCI constituted the first evidence that some cis-DDP adducts formed in vivo are recognized by the antibody elicited against DNA modified in vitro. Adducts were quantitated in DNA from cultured L1210 cells exposed to cis-DDP and from tumor ascites extracted from cis-DDP-exposed mice carrying L1210 tumors. Recent studies have focused upon elucidation of the chemical nature of the cis-DDP-DNA adduct recognized by the antibody and formed in vivo. Competitive ELISAs were performed with DNAs and deoxyribonucleotide polymers modified by a variety of platinum drugs and the results indicate that primary antigenic specificity is directed towards a bidentate adduct on adjacent deoxyguanosines (dG) formed with the dG-N7 position by displacement of the cis chloride groups. In collaboration with members of the Laboratory of Molecular Pharmacology we have found that this adduct is formed in human patients since DNA extracted from circulating nucleated cells in individuals receiving cis-DDP chemotherapy competes in the ELISA, whereas DNA from normal volunteers and individuals on other chemotherapy did not. Of the cis-DDP patients, 9 out of 12 had measurable levels of adducts, while all 8 of the controls were negative.

Significance to Biomedical Research and the Program of the Institute.

The development of immunological procedures for the investigation of carcinogen-DNA interactions has provided a powerful tool for the study of this phenomenon. Experimentally, antibodies are more specific and sensitive, and less costly than the conventional radiolabeled probes used for such studies. Standard carcinogenesis protocols need not be modified for DNA-binding studies, and prolonged sequential administration can be monitored. Morphological approaches can be employed to determine inter- and intracellular distribution of adducts, and even intramolecular localization is possible. Our most recent results suggest that antibodies may be useful to probe for adducts in humans exposed to DNA-damaging agents both in prospective epidemiological studies and in assisting the clinician to judge dosage on individuals exposed to DNA-interacting chemotherapeutic agents.

Proposed Course:

As an applied methodology, this technology allows for quantitation and localization of adducts in tissues from some human populations exposed to the polycyclic aromatic hydrocarbons and platinum-DNA binding in nucleated blood cells of cancer patients given cis-DDP chemotherapy. Both of these aspects are under intensive investigation. In cell culture studies both binding and removal of 2-AAF and BP adducts will be correlated to carcinogen-induced biological changes, such as altered epidermal foci, to discriminate the quantitative effects of binding from the qualitative requirements for biological activity. In vivo studies will be extended to explore the fate of adducts during initiation-promotion protocols, and in preneoplastic nodules induced by 2-AAF feeding. In addition we will continue to investigate localization of adducts in the liver by immunofluorescence since it should be possible to compare adduct accumulation

in preneoplastic foci with that in surrounding tissue. Collaborative studies utilizing electron microscopic immunohistochemistry (ferritin labeling) to detect specific localization of BP adducts on DNA will focus on 10 T 1/2 cells exposed in vivo and on a search for binding to initiation sites in replicons in SV40 and regenerating rat liver nuclei. We would like to utilize this approach to determine if gaps in nascent DNA are localized opposite adducts after exposure of cells to BPDE-I. In this laboratory immunofluorescence techniques will be applied to study distribution of bound BPDE-I in cultured cells and animal tissues during carcinogenesis protocols while quantitation of BPdG adducts will be carried out on parallel samples by ELISA. Other studies are designed to elucidate the mechanism of action of cis-DDP in biological systems and to explore the conformational aspects of various types of platinum modification on DNA. In collaboration with other members of the In Vitro Pathogenesis Section, antisera will be used to localize binding in particular DNA sequences (cloned genes) and to determine the functional consequences of the adduct on gene regulation.

Publications:

Lippard, S. J., Ushay, H. M., Merkel, C. M., and Poirier, M. C.: Use of antibodies to probe the stereochemistry of antitumor platinum drugs. Biochemistry (In Press)

Paules, R. S., Poirier, M. C., Mass, M. J., Yuspa, S. H. and Kaufman, D. G.: Quantitation by electron microscopy of the binding of highly-specific antibodies to benzo[a]pyrene-DNA adducts. Carcinogenesis (In Press)

Perera, F. P., Poirier, M. C., Yuspa, S. H., Nakayama, J., Jaretzski, A., Curnen, M. M., Knowles, D. M. and Weinstein, I. B.: A pilot project in cancer epidemiology: Determination of benzo[a]pyrene-DNA adducts in animal and human tissues by immunoassays. Carcinogenesis 3: 1405-1410, 1982.

Poirier, M. C.: The use of antibodies to detect carcinogen-DNA adducts in vivo and in vitro. In 13th International Cancer Congress. Seattle, 1983 (In Press)

Poirier, M. C., Lippard, S. J., Zwelling, L. A., Ushay, H. M., Kerrigan, D., Thill, C. C., Santella, R. M., Grunberger, D. and Yuspa, S. H.: Antibodies elicited against cis-diamminedichloroplatinum (II)-modified DNA are specific for cis-diamminedichloroplatinum (II)-DNA adducts formed in vivo and in vitro. Proc. Natl. Acad. Sci. USA 79: 6443-6447, 1982.

Poirier, M. C., Nakayama, J. N., Perera, F. P., Weinstein, I. B. and Yuspa, S. H.: Identification of carcinogen-DNA adducts by immunoassays. In Millman, H. and Sell, S. (Eds.): Applications of Biological Markers to Carcinogen Testing. New York, Plenum Press, 1983 (In Press)

Poirier, M. C., True, B. and Laishes, B. A.: Determination of 2-AAF adducts by immunoassay. Environ. Health Perspect. 49: 93-99, 1982.

Poirier, M. C., Yuspa, S. H., True, B. and Laishes, B. A.: DNA adduct formation and removal in N-acetoxy-2-acetylaminofluorene-exposed cultured cells and in organs from rats fed 2-acetylaminofluorene. In Langenbach, R, Nesnow, S. and Rice, J. M. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Press, 1982, pp. 619-635.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05178-03 CCTP

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

James Strickland Research Chemist, LCCTP, NCI

COOPERATING UNITS *(if any)*

Laboratory of Pulmonary Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	2.25	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

SENCAR mice are markedly more susceptible to skin carcinogenesis than other mouse strains. Susceptibility, a property of the skin itself, is not due to differences in carcinogen metabolism. Cultures of both basal and differentiating epidermal cells of newborn SENCAR and BALB/c (a resistant strain) mice responded similarly to modulation of epidermal growth factor (EGF) binding by treatment with the tumor promoter TPA or with retinoic acid, but when basal cells were induced to differentiate, SENCAR lost EGF binding ability to a greater extent. TPA treatment of the basal cells caused a rapid and profound decrease in EGF binding while retinoic acid treatment resulted in increased binding and was partially protective against TPA effect. Differentiating cells responded much less to TPA, and retinoic acid was suppressive rather than stimulatory. Differentiating cells, in contrast to basal cells, bind but do not metabolize EGF. EGF stimulated epidermal basal cell growth in the absence of other cell types or conditioned media but only if cell proliferation was already occurring. TPA and retinoic acid treatment induced transglutaminase activity in epidermal cells of both mouse strains similarly by a mechanism involving a protease. Treatment of mouse skin of either strain in vivo or of primary epidermal cells in vitro with tumor promoters led to increased numbers of Langerhans cells. Methods were devised to enhance survival and enrich cultures for this cell type. Culture conditions for adult mouse epidermal cells have been developed and studies have begun to develop and characterize carcinogen-initiated cells selected for resistance to calcium-induced terminal differentiation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Stuart H. Yuspa	Chief	LCCTP, NCI
Henry Hennings	Research Chemist	LCCTP, NCI
Dennis Roop	Expert	LCCTP, NCI
Hideki Kawamura	Visiting Fellow	LCCTP, NCI

Objectives:

To elucidate the cellular and molecular mechanisms of enhanced sensitivity to carcinogenesis in genetically-derived susceptible mouse strains.

Methods Employed:

The SENCAR mouse was developed by a selective breeding protocol for enhanced susceptibility to skin tumors produced by initiation-promotion protocols. In order to elucidate the basis for this susceptibility, skin of SENCAR mice or SENCAR epidermal cells in culture are exposed to carcinogens and tumor promoters. Comparisons are made with BALB/c as a representative resistant strain. For culture of adult mouse epidermal cells for in vitro carcinogenesis experiments, the epidermis can easily be separated from the dermis after flotation of the skin, dermis side down, on a solution of 1 g trypsin per 100 ml phosphate-buffered saline (without calcium and magnesium) for 1 hr at 37°. Epidermal cells are plated on dishes coated with fibronectin and collagen and are cultured in low-calcium medium conditioned by dermal fibroblasts. After treatment with initiating doses of carcinogens, selection for cells resistant to calcium-induced terminal differentiation can be made. Receptor binding studies with cells in culture have used radioactively-labeled epidermal growth factor or phorbol dibutyrate. Langerhans cells have been detected by rosette formation using sensitized red blood cells, by ATPase histochemical staining, by anti-Ia immunofluorescence, and by electron microscopy. Functional tests for Langerhans cells include allergic contact sensitization and allogeneic T-cell stimulation. Effects of various agents on epidermal cell growth kinetics have been determined in culture by cell counts and thymidine incorporation.

Major Findings:

There are a number of suggestions of an important immune system component in skin carcinogenesis. We have previously compared density and function of epidermal Langerhans cells in SENCAR and BALB/c and found them similar in all respects except that SENCAR Langerhans cells have markedly reduced plating efficiencies compared to those of BALB/c mice. Though this implies cell surface differences, we were unable to identify any altered Langerhans cell functions in SENCAR. One problem with the study of Langerhans cells in culture has been their very short half-life - approximately half the number of rosetting cells remains after 24 hr. We have not determined whether this represents in vitro

of chymostatin by $NaBH_4$, which abolishes anti-proteolytic activity, also eliminated the inhibitory effect of chymostatin on TPA-induction of transglutaminase. A number of other protease inhibitors was also tested. Based upon the response pattern observed at 0.5 mM inhibitor, these can be divided into 4 groups: 1) Chymostatin, antipain, and leupeptin inhibit the TPA effect without inhibition of protein synthesis. 2) Pepstatin, soybean trypsin inhibitor, and aprotinin inhibit both protein synthesis and TPA induction of transglutaminase activity. 3) Elastatinal was inhibitory to neither. 4) TPCK did not inhibit enzyme induction but was inhibitory to protein synthesis. Retinoic acid treatment also induced transglutaminase activity in SENCAR epidermal cells as had previously been shown for BALB/c. Pretreatment of cells with chymostatin was strongly antagonistic to the retinoic acid effect as it was for TPA, thereby suggesting involvement of a protease in the mechanism of induction in both cases. Chymostatin treatment did not affect phorbol ester binding to mouse epidermal cells, nor did it affect TPA-mediated reduction in EGF binding. The results of mixing experiments suggested the presence of a transglutaminase inhibitor, and preliminary antibody studies indicate that more transglutaminase protein is present after TPA treatment. Previous work demonstrated a requirement for protein synthesis, implying a true induction. It appears, therefore, that TPA may induce synthesis of both enzyme protein and protease. The enzyme may exist in an inactive form, either as a proenzyme or complexed with an inhibitor, and the protease activates the transglutaminase. The particular protease has not been identified, but the protease inhibitor pattern we have found is most consistent with cathepsin B, a lysosomal enzyme.

The binding and metabolism of epidermal growth factor (EGF) by basal and differentiating epidermal cells as well as by differentiation-resistant cells derived from in vitro carcinogenesis studies has been of interest because of the modulation of epidermal cell interactions with EGF by tumor promoters and retinoids. SENCAR and BALB/c epidermal cells responded similarly in a variety of experiments with EGF and had similar levels of EGF receptors per cell. Thus, there is little evidence that modulation of epidermal cell interactions with EGF or elaboration of growth factors which compete at the EGF receptor contributes to susceptibility of SENCAR to skin carcinogenesis. We utilized the low calcium (<0.1 mM) culture system (Hennings et al., Cell, 19: 245-254, 1980) to study basal cell responses to EGF, and by raising calcium levels to 1.4 mM, we induced terminal differentiation and observed how that process affected interactions with EGF. In both mouse strains EGF binding per cell increased with increasing cell density as basal cell cultures approached confluency (10^4 to 10^5 cells/cm^2). TPA treatment strongly decreased EGF binding within less than four hours. Maximum effect (more than 80% reduction) occurred at 50-100 ng/ml TPA, though a 50% reduction was seen at 1 ng/ml. Retinoic acid treatment (10^{-5} M, 36-48 hr) resulted in a 70-100% increase in EGF binding by basal cells and partially prevented the TPA-mediated suppression of binding. Calcium-induced terminal differentiation was accompanied by loss of ability to bind EGF. This was the one case in which SENCAR and BALB/c clearly responded differently. Whereas, the early losses (4 hr after calcium switch) were similar in the 2 strains, SENCAR continued to decrease to 15-20% of basal cell values by 48 hr, BALB/c leveled off to about 50% at the same time. The difference was found whether binding assays were done at 0° or 37°, suggesting that the effect is primarily binding rather than metabolism. We do not know whether the difference results

loss of rosetting ability or actual death of cells, but use in culture media of fetal calf serum which has been incubated 30 min at 56° greatly extends the life of rosetting cells in culture. Increasing serum concentration from 8 to 15% had no effect nor did varying calcium concentration in media from 0.05 to 1.4 mM.

A single treatment of newborn (3-4 day) mouse skin with 25 µg TPA in 25 µl acetone was followed the next day by removal of epidermis and culture of epidermal cells. After 24 hours in vitro, rosette assay indicated an increase of about 50% in the number of rosetting cells, compared to solvent controls, in both BALB/c and SENCAR mice. This represents one of the earliest known effects of tumor promoters on newborn mouse skin since epidermal cells are generally insensitive to phorbol esters until day 5 after birth. In media with normal fetal calf serum these rosetting cells from both strains had the expected short half-life compared to medium with heat-treated serum, while peritoneal macrophage controls survived equally well in normal and heat-treated sera. In parallel experiments with primary epidermal cell and macrophage cultures, TPA treatment had no effect on the number of rosetting macrophages compared to solvent control but TPA, teleocidin B, and mezerein treatments increased the number rosetting cells in epidermal cell cultures similarly to the in vivo treatments. The results of in vitro survival experiments with and without heat-treated serum were similar to those for tumor promoter treatment in vivo. TPA treatment did not affect survival of rosetting cells in either media. Similar results for BALB/c and SENCAR mice in these experiments do not suggest a role for Langerhans cells in the unusual sensitivity of SENCAR to epidermal carcinogenesis.

An additional discovery we have made regarding Langerhans cells is that they, in contrast to keratinocytes, resist attachment to culture dishes coated with fibronectin and collagen but attach strongly and rapidly to non-coated dishes. This property allows enrichment of epidermal cell cultures for Langerhans cells by absorption of primary cell suspensions first to coated dishes to deplete keratinocytes, followed by replating non-adherent cells, which includes most rosetting cells, on non-coated dishes. Most keratinocytes which do not attach to the coated dish will also not attach to the non-coated dish. After attachment, Langerhans cells are very resistant to trypsin, making it possible to remove contaminating keratinocytes by a brief trypsin treatment. Thus, by combining TPA treatment, selective absorption, and trypsin treatment, one can obtain highly enriched cultures of rosette-forming epidermal cells.

Since accelerated differentiation of normal epidermal cells by tumor promoters may be an important aspect of the promotion process, we have investigated the induction of transglutaminase by TPA in newborn SENCAR primary epidermal cell cultures. Transglutaminase, a critical regulatory enzyme in epidermal differentiation, catalyzes the formation of the protein cross-links in the cornified envelope. TPA induction of transglutaminase in SENCAR occurs in a manner similar to that previously shown for the BALB/c strain, which is less sensitive to skin carcinogenesis. A single dose of 100 ng/ml TPA, the optimal dose, resulted in maximal enzyme activity 8-12 hr after treatment. Pre-treatment of cells with chymostatin, a protease inhibitor, resulted in increasing inhibition of the TPA effect with time of pre-treatment, up to 80% for 24 hr. The effect was dependent upon chymostatin concentration up to 1 mM chymostatin. Reduction

from more synchronously differentiating SENCAR cells or greater loss of binding by each cell. The possible elaboration of a growth factor which competes at the EGF receptor by differentiating SENCAR cells has not been ruled out and will be explored in future studies. Binding studies done at 37° suggest that 36 hr after induction of terminal differentiation EGF is not metabolized in differentiated cells. Furthermore, the effects of TPA and retinoic acid are markedly different in basal and differentiated cells. In BALB/c or SENCAR primary newborn epidermal cells 36 hr after switch to medium with 1.4 mM calcium, TPA only slightly suppressed EGF binding. Retinoic acid added at the time of the calcium switch no longer led to enhanced EGF binding but was inhibitory to about the same extent as TPA. The combined retinoic acid-TPA treatments were suppressive to about the same level seen in basal cells.

In growth response studies with EGF, BALB/c and SENCAR primary epidermal basal cells proliferated at increased rates in the presence of 1 ng/ml or 10 ng/ml EGF, but only if cell proliferation was already occurring in the absence of EGF. If cells were plated at low density ($<10^3$ cells/cm^2) or at somewhat higher density but in low serum ($\leq$2%), such that cell number was not increasing with time, EGF had no effect. This, along with the cell density results, probably explains the reported requirement for feeder layers or conditioned media for EGF effects on epidermal cell cultures, especially at low plating density. Recent success with culturing adult mouse epidermal cells has confirmed and extended the use of selection for resistance to calcium-induced terminal differentiation as an early event after carcinogen exposure. Differentiation-resistant colonies occur spontaneously in untreated SENCAR cultures using adult keratinocytes just as we previously reported for in vivo initiation, in vitro selection studies. These colonies may indicate the existence of endogenously initiated cells in the SENCAR mouse epidermis. Such colonies were not seen in untreated BALB/c cultures but colonies are induced in a dose-dependent manner in dishes treated in vitro with the initiators DMBA or MNNG. Interestingly, in SENCAR cultures treated in vitro with MNNG, a morphologically altered colony type appears with less stratification and a more basal cell morphology. It appears from these experiments that the differentiation-resistant colonies obtained by carcinogen treatment of adult BALB/c epidermal cells in culture are similar to the spontaneous colonies obtained from SENCAR mice, but additional carcinogen exposure in SENCAR cultures may alter the colony type. This could indicate a progressive change associated with multiple genetic alterations induced by carcinogens.

Significance to Biomedical Research and the Program of the Institute:

Epidemiological and medical genetic data have indicated major individual differences in cancer risk in humans. Increased risks are associated both with overall susceptibility to cancer or susceptibility in a particular target organ. In some cases, specific genetic changes have been associated with increased risk, but in many examples, polygenic influences appear more likely. To date biochemical epidemiological studies have focused only on genetic differences in carcinogen metabolism. In the complex and multistage evolution of cancer, it seems unlikely that carcinogen metabolism is solely responsible for enhanced risks. In fact it seems likely that factors associated with the expression of neoplastic change would play an important role in host susceptibility. The

development of animal strains through selective breeding with high susceptibility at a particular organ site provides an excellent model for the study of susceptibility determinants. In vivo studies have indicated that carcinogen metabolism is unlikely to explain the sensitivity of SENCAR, and grafting experiments indicate the target tissue itself is somehow more susceptible. This validates the use of in vitro techniques to explore susceptibility. In vitro studies have suggested that certain biological alterations associated with carcinogen exposure are constitutive in the susceptible strains and that immunological differences could also be involved in the expression of neoplastic change. If this model reflects susceptibility determinants in human cancer, it will provide important insights and potential assays for studies in human populations. Furthermore, understanding determinants of susceptibility is likely to yield information concerning the molecular mechanisms of carcinogenesis.

Proposed Course:

Additional studies with adult mouse epidermal cells in culture will be quite useful because most of the in vivo biological data for murine skin carcinogenesis is from adult animals while most in vitro studies have been done with the easier to culture newborn epidermal cells. Special effort will be given to strengthening the evidence that cells resistant to calcium-induced terminal differentiation are initiated. These will include in vivo initiation and time course studies for in vitro selection to minimize the time required to obtain altered foci. Comparative studies of in vitro initiation and selection will be made with epidermal cell cultures of adult and newborn animals to define differences and compare with adult and newborn in vivo skin carcinogenesis studies previously done by our laboratory with BALB/c and SENCAR animals. Several additional mouse strains of varying susceptibility to skin carcinogenesis will be studied in initiator dose-response experiments to compare frequency of differentiation-resistant colonies. Most important is the transplantation of cells from resistant colonies into recipient animals (athymic nude mice) as a skin graft for experiments with tumor promoter treatment of the transplanted cells. Convincing evidence that differentiation-resistant cells are initiated must include their ability to form benign tumors in vivo either alone or under the influence of tumor promoter treatment. New efforts will be made to obtain subculturable cell lines arising from a single differentiation-resistant colony in different mouse strains and from different treatment conditions, to freeze them in a viable state at very early passage, to characterize their responses to a variety of known tumor promoter effects, and to obtain solid evidence for their epidermal nature. More molecular studies will investigate the expression in mouse epidermal cells of genes associated with oncogenic viruses. Expression of these genes seems to be related to defects in regulation of differentiation and proliferation. This study will be a follow-up of our earlier studies of expression of viruses and viral genes by BALB/c and SENCAR epidermal cells (see last year's annual report). The new work will utilize molecular probes not previously tested but which are much more likely to be helpful in understanding the mechanism of carcinogenesis. We will also attempt to obtain expression of such genes by introducing them into epidermal cells by viruses or by transfection to observe the biological effects.

Publications:

Kawamura, H., Strickland, J. E. and Yuspa, S. H.: Inhibition of 12-O-tetradecanoylphorbol-13-acetate induction of epidermal transglutaminase activity by protease inhibitors. Cancer Res. (In Press)

Strickland, J. E., Hennings, H., Allen, P. T., Jetten, A. M., Strickland, A. G., Hellman, K. B. and Yuspa, S. H.: Studies to elucidate susceptibility determinants for mouse epidermal carcinogenesis. In Armstrong, B.K. and Bartsch, H. (Eds.): Host Factors in Human Carcinogenesis. Lyon, France, IARC Publ. No. 39, 1982, pp. 259-268.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05270-02 CCTP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Mechanism of Action of Phorbol Ester Tumor Promoters

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Peter M. Blumberg Research Chemist, LCCTP, NCI

COOPERATING UNITS *(if any)*
Harvard Medical School, Boston, Massachusetts; Department of Biology, Flinders University, Adelaide, Australia; IARC, Lyon, France

LAB/BRANCH
Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION
Molecular Mechanisms of Tumor Promotion Section

INSTITUTE AND LOCATION
NIH, NCI Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	4.4	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The efforts of the MMTP Section are directed at understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. In addition to the previously demonstrated membrane receptors for the phorbol esters, specific phorbol ester binding activity has been characterized in cytosol in the form of a phorbol ester apo-receptor which has an absolute phospholipid requirement. Different phospholipids vary in their ability to reconstitute binding activity. Phosphatidylserine is most effective and phosphatidylcholine is least effective. The structure-activity requirements for binding by the reconstituted apo-receptor closely resemble those for the membrane receptor, suggesting they may be different forms of the same receptor. The apo-receptor co-fractionates with protein kinase C, thus identifying a biochemical function for the receptor. Diacylglycerol, an activator of protein kinase C, competitively inhibits phorbol ester binding, consistent with its being an (the) endogenous phorbol ester analog. The 50% inhibitory concentration for the diacylglycerol diolein is 0.4% of the concentration of phospholipid. Evaluation of different diacylglycerol analogs indicates that short or unsaturated side chains are required for activity. The ability of diacylglycerols to act as phorbol ester analogs suggests that the receptor recognizes that fraction of the phorbol esters which is dissolved in the lipid phase rather than that fraction in aqueous solution. Results with the highly lipophilic phorbol 12,13-didodecanoate confirm this prediction. Techniques for isolation of the receptor in good yield from membranes and cytosol are being developed. The role of the lipid environment in binding and response is being characterized, both by reconstitution and by photoaffinity labeling. Multiple phorbol ester receptors have been implicated in the heterogeneity of phorbol ester responses. The binding characteristics of intact, cultured keratinocytes change from homogeneous to heterogeneous as differentiation proceeds.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Arco Jeng	Expert	LCCTP, NCI
Huei-Mei Yeh	Staff Fellow	LCCTP, NCI
Karen Leach	Postdoctoral Fellow	LCCTP, NCI
Joseph A. Dunn	Staff Fellow	LCCTP, NCI
Bernard Konig	Visiting Fellow	LCCTP, NCI

Objectives:

The early events in the interaction of phorbol esters with cells and tissues are being characterized. Specific aims are as follows: 1) Biochemical characterization of the phorbol ester receptors. 2) Analysis of receptor localization and its role in receptor function. 3) Analysis of competitive inhibitors of phorbol ester binding. 4) Analysis of receptor function in genetic variants unresponsive to the phorbol esters. 5) Analysis of receptor heterogeneity and determination of its significance. Phorbol esters exert profound effects on cell growth and gene expression. Understanding of the mechanisms of endogenous modulation of the phorbol ester receptors may both provide insights into the process of human carcinogenesis as well as identify biochemical steps suitable for intervention.

Methods Employed:

This Section uses a wide range of techniques to pursue the above aims. Phorbol and derivatives are isolated from natural sources. Semisynthetic derivatives for affinity labeling, structure-activity analysis and binding studies are prepared and radioactively labeled as necessary. Binding studies are carried out using the ligands and methodology developed by us. Analysis of receptors utilizes both photoaffinity labeling and standard biochemical membrane methodology. The systems analyzed are chosen as optimal for the specific questions being examined. Brain homogenates, because of their richness in receptors, are being used for receptor purification and biochemical analysis. Mouse skin, cultured keratinocytes, and NRK cells are being used to dissect subclasses of receptors. Intact cells are being utilized to determine the relationship between receptor occupancy and biological responses. Importance is placed upon the ability to relate the answers obtained to the biological system of mouse skin promotion and to coordinate effectively in exploiting the systems being studied by the other Sections of the Laboratory.

Major Findings:

An important advance over the past year has been the identification of protein kinase C as at least a major sub-class of the phorbol ester receptors. The tissue distribution, evolutionary conservation, and phospholipid requirements for the phorbol ester receptor had been shown to resemble closely those for protein kinase C. Nishizuka and co-workers, moreover, had reported recently that

the phorbol esters at nanomolar concentrations stimulated protein kinase C activity directly. A striking inconsistency, however, was that protein kinase C activity was largely cytosolic, whereas phorbol ester binding was almost entirely to membranes.

Over the past year, we have demonstrated the presence in brain cytosol of a specific, phorbol ester apo-receptor, which requires addition of phospholipids to reconstitute activity. Phosphatidylserine was most effective at reconstitution. Phosphatidic acid and phosphatidylinositol were less effective, and phosphatidylcholine was relatively ineffective. Upon reconstitution, the specific binding activity of cytosol was similar to that for brain membranes. In terms of total binding activity in brain, cytosol accounted for 30-60%, depending on the conditions of homogenization. Comparison of structure-activity relations for phorbol ester binding to membranes and to the reconstituted cytosolic apo-receptor showed good quantitative agreement, differing by no more than 2.5-fold over a 10,000-fold range in binding potency.

Fractionation of the phorbol ester apo-receptor by ion exchange chromatography and by gel filtration demonstrated co-elution of the apo-receptor with protein kinase C activity. These findings, together with the observations on stimulation of protein kinase C by phorbol esters, strongly argue that protein kinase C is the major phorbol ester receptor, at least in brain.

Protein kinase C purification has been reported by Nishizuka and by Kuo, but in both cases yields have been very low (1-5%). To obtain larger amounts of protein for biochemical analysis, we have therefore been evaluating a variety of alternative purification procedures. As one approach, we synthesized an affinity column consisting of phorbol 12-ε-aminohexanoate 13-hexanoate coupled to Affi-gel. Although the ligand before coupling showed good affinity for the receptor, and although the ligand retained its affinity when its ε-amino group was blocked, little retention by the column of phorbol ester binding activity or of protein kinase C activity was found either for the cytosolic apo-receptor or for the solubilized membrane receptor. Further structure-activity analysis (see below) indicates that the problem is probably one of steric interference on the column. Among other approaches, selective absorption of the apo-receptor to phosphatidylserine vesicles and subsequent desorption appears promising. For direct comparison of the cytosolic apo-receptor with the membrane receptor, conditions for detergent solubilization of the membrane receptor have been developed.

Since protein kinase C activity is stimulated by unsaturated diacylglycerol and the phorbol esters can substitute for diacylglycerol in this stimulation, we examined the effect of diacylglycerols on phorbol ester binding to the reconstituted apo-receptor. Diolein inhibited specific [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu)binding in a dose dependent fashion to <10% of control levels. The inhibition curves fit that expected for a competitive inhibitor. Scatchard analysis was likewise consistent with competitive inhibition. At 0.96 mg/ml of phosphatidylserine, the K_I for diolein was 3.6 mg/ml (0.38% w/w the concentration of phosphatidylserine). At constant phospholipid concentration, the K_I determined for diolein was independent of the diolein concentrations over the range of 0.16-8.0%, suggesting that the inhibition did not arise simply by perturbation of the phospholipid bilayers. The K_I of diolein was approximately proportional

to the absolute phospholipid concentration. At 4.8 μg/ml of phosphatidylserine, for example, the K_I was 52 ng/ml (1.1% of phosphatidylserine). In addition to diolein, the shortchain saturated diacyglycerol derivatives dicaprylin and dicaproin also inhibited [^{3}H]PDBu binding, whereas the long-chain saturated derivatives dipalmitin and distearin were much less active. Our results suggest 1) that variation in lipid composition provides a mechanism for modulating phorbol ester receptor affinity and 2) that diacylglycerol may act as an endogenous ligand for the phorbol ester receptor. The existence of an endogenous ligand binding at the phorbol ester binding site had been predicted previously based on the high evolutionary conservation of the receptor.

Testable predictions from our findings are that the diacylglycerol derivatives should show stoichiometric binding to the receptor and that photoaffinity analogs of diacylglycerol and of the phorbol esters should bind to the same site on the receptor.

A long-standing issue in understanding phorbol ester structure-activity relations had been whether the phorbol ester receptor recognized the phorbol ester which was free in solution or that which was dissolved in the membranes. Since the long-chain-substituted-diacylglycerols are insoluble, their ability to act as phorbol ester analogs strongly supports the latter alternative. The ability to separately manipulate the lipid environment of the apo-receptor has made it possible to test the alternatives directly. A relatively insoluble phorbol ester, phorbol 12,13-didodecanoate (PDDoD), was synthesized. Inhibition of [^{3}H]PDBu binding by PDDoD was measured in one of two ways. In the one case PDDoD was added in organic solvent to the phosphatidylserine, the solvent was removed, and the liposomes used for apo-receptor reconstitution were then prepared. In the other case, PDDoD was added directly to the aqueous receptor assay mixture. As predicted, the PDDoD had high affinity when added directly to the phospholipid, whereas its affinity if added to the aqueous phase was very low. An implication of the results is that relatively insoluble phorbol esters may provide a convenient reservoir for maintaining constant phorbol ester concentrations, for example during systemic promotion experiments.

To better explore structure-activity relations for immobilized phorbol ester analogs (see above) as well as to prepare useful phorbol ester conjugates, a series of phorbol ester-biotin derivatives was prepared. The rationale behind preparation of these analogs was that avidin has high affinity for biotin. The phorbol derivatives could thus be conjugated to avidin or to modified avidins to yield high molecular weight phorbol ester conjugates. The four derivatives prepared were phorbol 12-hexanoate 13-biotinylaminohexanoate, phorbol 12-hexanoate 13-biotinylaminododecanoate, phorbol 12-tetradecanoate 13-biotinylaminohexanoate, and phorbol 12-biotinylaminohexanoate 13-decanoate. The derivatives differed in the position of the biotin moiety, the length of the spacer arm, and the length of the unsubstituted fatty acid. All showed good binding potency in the absence of avidin and loss in the presence of avidin. These results indicate that little tolerance exists for introduction of bulky, hydrophilic groups at either the 12 or 13 positions of the phorbol ester molecule. In addition, the derivatives provide a mechanism for rapid, reversible inactivation of phorbol esters with an impermeable reagent. A prediction of the identification of protein kinase C as a major phorbol ester receptor is that the receptor should be on the inner rather

than on the outer face of the membrane. The biotin-phorbol ester derivatives permit a test of this prediction. Experiments underway in collaboration with Dr. Yamasaki and Dr. Murray involve the micro-injection into cells of avidin followed by analysis of response to externally added biotin-phorbol ester derivatives.

We had previously demonstrated that [^{3}H]PDBu binding to particulate preparations from whole mouse skin was heterogeneous, consistent with three binding sites present at 0.14 (site 1), 1.6 (site 2), and 1.9 (site 3) pmol/mg protein and possessing affinities of 0.7, 10, and 50 nM, respectively. The structure-activity relations for sites 2 and 3 differed, suggesting that they might mediate different biological responses. To further explore the nature and significance of the receptor heterogeneity, [^{3}H]PDBu binding to intact keratinocytes is being characterized in collaboration with the In Vitro Pathogenesis Section. A single binding site of nM affinity was observed in keratinocytes grown under low calcium conditions. In contrast, shift of the keratinocytes to high calcium medium rapidly led to receptor heterogeneity, with the appearance of an additional low affinity binding site. Two epidermal cell lines (D and 308) resistant to calcium-induced differentiation continued to show a single high binding affinity when grown in the presence of the higher calcium medium. Current efforts are directed at determining whether the structure-activity relations at the two sites in the intact keratinocytes correspond to those found for sites 2 and 3 in the skin particulate preparations.

A second intact cell system showing marked heterogeneity of binding is that of NRK cells. Analyzed for two binding sites, the binding data yields affinities of 2.0 and 68 nM; at saturation, 0.50 and 4.0 pmol [^{3}H]PDBu per mg of protein is bound, respectively. The correspondence between binding and biological responses is being explored.

The relationship of protein kinase C to receptor heterogeneity is a critical issue. The competitive inhibition of [^{3}H]PDBU binding by diacylglycerol suggests that variation in lipid composition may afford one mechanism for generating heterogeneity of phorbol ester binding affinity from a single protein receptor. In fact, reconstitution of the brain apo-receptor into mixtures of different liposomes varying in their amounts of diolein yielded curved Scatchard plots having the shape predicted from the composition of the mixture of liposomes. Since diacylglycerol, being a competitive inhibitor, can only shift absolute but not relative affinities, the ability of different phospholipids to affect structure activity relations of binding is being examined. The photoaffinity phorbol ester derivatives phorbol 12-p-azidobenzoate 13-benzoate and phorbol 12,13-dihexadienoate are being used to probe the lipid environment for receptors of different affinity.

Significance to Biomedical Research and the Program of the Institute:

Much of human cancer is thought to result from a combination of carcinogenic and tumor promoting activities. Although considerable progress had been made in elucidating the mechanisms of carcinogens, much less is understood about the mode of action of tumor promoters. One of the most accessible model systems for analyzing this process is that of phorbol ester tumor promotion in mouse skin.

The unique value of the phorbol esters in this system lies in their very high potency, which facilitates the distinction between specific and non-specific effects. The Laboratory of Cellular Carcinogenesis and Tumor Promotion is conducting an integrated study of the skin tumor promotion system at multiple levels of analysis--whole animal, cellular, and biochemical. The current focus of the Molecular Mechanisms of Tumor Promotion Section on phorbol ester receptors should identify, unambiguously, the initial biochemical steps which are both neccesary and sufficient for tumor promotion by these agents. Identification of such biochemical steps should permit the analysis of their control, modulation, and function in human cells under normal and pathological conditions. Determination of the ability of other less specific tumor promoters to perturb, indirectly, the same processes will shed light on the generality of mechanisms of promotion. Such information is of central importance in attempting to develop better means of detecting tumor promoters and evaluating their potential hazard. In addition, the biochemical analysis should provide both a new avenue for the rational development of inhibitors of promotion as well as shed light on the mechanism of current classes on inhibitors.

The subdivision of tumor promotion in vivo into multiple stages implies that cellular or biochemical mechanisms (or indeed in vitro assays) will also be stage-specific. The analysis of receptor subclasses provides an essential basis for determining which processes belong to which subclass of response. Moreover, emerging evidence suggests that subclasses of receptors may have antagonistic effects. For example, the tumor yield for the complete tumor promoter, PMA, is reduced by co-administration of first or second stage tumor promoters. Understanding of the interaction between the processes belonging to each subclass may be essential for predicting biological outcome and may provide an additional means of intervention.

Proposed Course:

The overall aims of the section will remain the same over the coming year. Objectives will be to define the nature of the phorbol ester receptors, to clarify their function, and to understand their modulation. Particular emphasis will be directed at the protein kinase C, where an important issue will be to determine whether it modulates all or only some of the phorbol ester responses.

Purification of receptors should yield adequate material for biochemical characterization and antibody preparation. Characterization will focus on the phorbol ester binding domain and on receptor-lipid interactions. Microinjection experiments with receptors and antibodies will be used to clarify the functional role of the kinase.

To verify that diacylglycerol and phorbol esters in fact bind to the same site on the apo-receptor, photoaffinity labeling with photoaffinity diacylglycerol and phorbol ester analogs will be compared. Biological responses to exogenous diacylglycerol will be examined, with the effects on binding measured in parallel. Structure-activity analysis of diacylglycerol analogs will be expanded in an effort to identify possible pharmacological antagonists.

To obtain genetic evidence for the role of the protein kinase C, collaborative studies will be carried out with Dr. Huberman using an HL-60 variant isolated by

him which is unresponsive to the phorbol esters. Since Dr. Huberman has already shown that the variant is not altered in the major binding site, subsequent steps in the information transduction pathway, e.g., activation of kinase in response to binding, will be examined. This analysis will be complemented by attempts to identify minor binding sites in these cells, and, if such sites can be found, to compare them in the variant and the controls.

Analysis of protein kinase C in intact cells will be extended from the GH_4C_1 cell system to mouse keratinocytes. Questions of importance will include lipid environment, shifts between cytosol and membranes, and profile of substrates. The effects of phorbol esters will be compared with other agents such as diacylglycerol or calcium.

Publications:

Blumberg, P. M., Delclos, K. B., Dunn, J. A., Jaken, S., Leach, K. L. and Yeh, E.: Phorbol ester receptors and the in vitro effects of tumor promoters. Ann. NY Acad. Sci. (In press).

Blumberg, P. M., Dunn, J. A., Jaken, S., Jeng, A. Y., Leach, K. L., Sharkey, N. A. and Yeh, E.: Specific receptors for phorbol ester tumor promoters and their involvement in biological responses. In Slaga, T. J. (Ed): Tumor Promotion and Cocarcinogenesis in Vitro. (In press).

Delclos, K. B., Yeh, E. and Blumberg, P. M.: Specific labeling of mouse brain membrane phospholipids with [20-^{3}H]phorbol 12-p-azidobenzoate 13-benzoate, a photolabile phorbol ester. Proc. Natl. Acad. Sci. USA 80: 3054-3058, 1983.

Dunn, J. A. and Blumberg, P. M.: Specific binding of [20-^{3}H]12-deoxyphorbol 13-isobutyrate to subclasses of phorbol ester receptors in mouse skin particulate preparations. Cancer Res. (In press).

Leach, K. L., James, M. L. and Blumberg, P. M.: Charaterization of a specific phorbol ester apo-receptor in mouse brain cytosol. Proc. Natl. Acad. Sci. U.S.A. (In press).

Nagle, D. S. and Blumberg, P. M.: Regional localization by light microscopic autoradiography of receptors in mouse brain for phorbol ester tumor promoters. Cancer Lett. 18: 35-40, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05356-01 CCTP

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Phorbol Ester Receptor Occupancy-Response Coupling in Hormone Responsive Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Susan Jaken IPA Fellow, LCCTP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION
Molecular Mechanisms of Tumor Promotion Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to define events associated with phorbol ester action, beginning with occupancy of the phorbol ester receptor in hormone-responsive cells. Studies have focused on three types of receptor modulation (receptor number, affinity, and coupling to activation of protein kinase C) each of which may contribute to regulation of cellular sensitivity to this class of tumor promoters. First, analysis of receptor properties in cell lysates of control cultures has shown that the receptor is in equilibrium between the cytosol and membrane fractions and that both fractions contribute to the total receptors measured in intact cells. Treatment with phorbol esters shifts the equilibrium to favor the membrane compartment. In cultures pretreated with hormones that cause a decrease in phorbol ester receptor number, there is a decreased ability of phorbol esters to shift this equilibrium. Thus, regulation of receptor number is associated with a decrease in phorbol ester-directed stabilization of the membrane association of the cytoplasmic receptor. Secondly, in intact cells, receptor affinity is temperature-dependent. Studies with the cytoplasmic and membrane receptor in vitro demonstrate the importance of the phospholipid environment of the receptor in regulation of the apparent receptor affinity. Thirdly, certain biological systems show a decrease in responsiveness to phorbol esters in the absence of measurable changes in binding. Investigations of the phospholipid requirements for phorbol ester binding and activation of protein kinase C indicate that in vitro, both activities depend on the lipids used in the assays. Therefore, changes in the phospholipid composition of the membrane may play a role in regulation of the coupling between receptor occupancy and protein kinase C activation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Peter M. Blumberg Research Chemist LCCTP, NCI

Objectives:

The overall objective of this project is to characterize the events involved in the coupling of phorbol ester receptor occupancy with biological responses. The specific goals include 1) characterizing the role of the recently identified cytosolic receptor in phorbol ester binding to intact cells; 2) defining the mechanism of phorbol ester receptor down modulation; and 3) characterizing factors involved in regulating the coupling between phorbol ester receptor occupancy and activation of protein kinase C.

Methods Employed:

Two cell lines are being used in these studies; a clonal strain of rat anterior pituitary mammotrophs (GH_4C_1) and a clonal strain of mouse anterior pituitary corticotrophs (AtT_{20}/D16v). Both types are maintained by routine passage of cells. Phorbol ester binding to intact cells, isolated cell membranes, and isolated cell cytosol is measured using [^{3}H]phorbol 12,13-dibutyrate(PDBu). Assay techniques for each type of sample have been described. Cell membrane and cytosol fractions are prepared by centrifugation of cell lysates at 100,000 x g for 60 minutes. Protein kinase C activity is measured by assaying the amount of ^{32}P transferred from ^{32}P-ATP to histone using a standard assay procedure.

Major Findings

1. Role of the cytosolic receptor in binding to intact cells.
The regulation of PDBu receptors in GH_4C_1 cells has been characterized in detail. Initially, affinities of several compounds for binding to intact cells were compared with corresponding potencies in eliciting cellular responses. For each of four ligands, 50% occupancy of the phorbol ester receptor resulted in 50% of the maximal obtainable biological response (in this case, a decrease in binding of epidermal growth factor). Therefore, occupancy of this receptor is closely associated with subsequent biological responses.

In order to define the role of the recently described cytosolic receptor in binding to intact cells, broken cell preparations are prepared and the binding parameters compared with those of intact cells. Cell lysates prepared in the presence of chelating agents have approximately 75% of the recovered receptors in the cytosol. In contrast, cell lysates prepared in the presence of excess Ca^{++} have approximately 75% of the recovered receptors in the membranes. In either case, the sum of the receptors in the cytosol plus membrane fractions is closely similar to the total receptors measured in parallel cultures of intact cells. These data indicate: 1) the receptor exists in equilibrium between the cytosol and membrane compartments and that Ca^{++} may shift the equilibrium to favor the membrane compartment; and 2) the cytosolic receptor contributes significantly to the total number of receptors measured in intact cells.

Characterization of the cytosolic receptor in vitro has shown that binding is dependent on phospholipids. This implies that in the living cell, the cytosolic receptor must form a membrane association to bind phorbol esters. To test this hypothesis, a comparison of the distribution of the receptor between the cytosol and membrane fractions of control and PDBu-treated cultures was made. The percentage of total recovered receptors in the membrane fraction was increased from approximately 20% in control cultures to approximately 45% in PDBu-treated cultures. These results indicate that PDBu-treatment can shift the equilibrium to favor the membrane compartment.

A high and low affinity form of the receptor has been identified both in intact cells and membrane fractions. In intact cells, the Kd for PDBu is approximately 10 nM when assayed at 37°. However, the affinity is decreased to a Kd of approximately 40 nM when assayed at 4°. Evidence that this difference is due to the formation of a high affinity receptor-ligand complex at 37° came from studying dissociation kinetics. Dissociation of bound ligand from cultures equilibrated at 4° is two-to-three fold faster than from cultures equilibrated at 37°. These data indicate that the receptor-ligand complex formed at 37° is tighter than at 4°.

In contrast to the temperature dependence of affinity in intact cells, no effect of temperature on binding to membranes was observed. However, the membrane receptor is of substantially lower affinity than that of intact cells; the Kd for PDBu is approximately 40 nM. Phosphatidylserine, which has been shown to be quite effective in reconstituting the cytosolic receptor, increases the affinity of the membrane receptor to a Kd of approximately 3 nM. These data indicate that the lipid environment can strongly influence receptor affinity and, furthermore, suggest that the increased affinity in intact cells at 37° vs 4° may be due to association with a preferred lipid environment.

Several lines of evidence suggest that phorbol ester binding and protein kinase C activities may reside on the same molecule. The possibility that this relationship may exist in GH_4C_1 cells was tested by comparing the potencies of PDBu for kinase activation and receptor occupancy. The ED_{50} for kinase activation (4 nM) was similar to the Kd (2 nM). These data are consistent with phorbol ester receptor occupancy leading to kinase C activation.

The data cited have been incorporated into a working model of phorbol ester action in GH_4C_1 cells: The receptor exists in equilibrium between the membrane and cytosolic compartments; exposure to phorbol esters stabilizes the membrane association of the receptor. This occurs at 4° or 37°; at 37° there is a temperature-dependent conversion to a high affinity receptor. This conversion may involve association with a preferred lipid environment based on the data showing that phosphatidylserine can increase the affinity of the membrane receptor; occupancy of the high affinity receptor is associated with activation of kinase C, which may mediate the subsequent biological effects of phorbol esters.

2. Mechanism of receptor down modulation.
Treatment of GH_4C_1 cells with thyrotropin-releasing hormone (TRH) causes a decrease in the number of phorbol ester receptors measured in intact cells with no change in affinity. TRH does not simply compete for phorbol ester binding;

rather it induces receptor down modulation. A surprising result was that the amount of receptors measured in lysates of down modulated cultures was not significantly different from lysates of control cultures, and by analogy, not different from intact cells of control cultures. These data indicate that down modulation involves the generation of a cryptic receptor state that is revealed upon cell lysis.

Distribution of the receptor between cytosol and membrane fractions of control and down modulated cultures revealed no difference. However, when control cultures are exposed to PDBu, a decrease in cytosolic receptors and an increase in membrane receptors occurs. In contrast, treatment of down modulated cultures with PDBu did not cause a large shift in distribution. These results indicate that down modulation is associated with decreased ability of PDBu to stabilize the membrane association of the cytosolic receptor.

3. Regulation of coupling between phorbol ester receptor occupancy and activation of protein kinase C.

Factors associated with regulation of the coupling between phorbol ester receptor occupancy and kinase C activation have been studied in mouse pituitary corticotrophs (D16 cells). Previous work had established a relationship between phorbol ester receptor occupancy and stimulation of ACTH release. However, prolonged exposure ($t_{1/2}$= 0.85 hours) to phorbol esters leads to specific desensitization to phorbol esters with no change in PDBu binding parameters. Desensitization to PDBu was not associated with desensitization to other secretagogues, ACTH depletion, or PDBu degradation. These results indicate that receptor occupancy can be uncoupled from cell responses.

The distribution of receptors between cytosol and membrane fractions indicates that PDBu-treatment causes a decrease in cytosolic receptor and an increase in membrane-associated receptor. A corresponding decrease in cytosolic kinase C activity was also measured. This effect of PDBu is noted with brief exposure times insufficient to cause desensitization, or longer times which correlate with desensitization. Therefore, a failure of PDBu to stabilize the membrane association is not a likely mechanism for desensitization.

A second potential mechanism for uncoupling involves a cell product that inhibits kinase but not binding activity. Binding can be measured in crude cytosol preparations; however, kinase C activity is completely masked. This masking is due to an inhibitor(s) which can be partially separated from the kinase with DE52 chromatography. The inhibitor can then be quantitated by measuring the amount of inhibition of kinase activity per unit volume. Using this approach, no quantitative difference in inhibitor concentrations in cytosols of D16 cells from control or desensitized cultures was noted. A role for the inhibitor in regulation of the coupling between binding and kinase activation is yet to be identified.

A third potential mechanism for uncoupling is differential phospholipid requirements for kinase activation and binding. A systematic study of the phospholipid requirements of binding and kinase activation has shown that certain mixtures of phospholipids can influence one and not the other. For example, mixed liposomes

of phosphatidylserine and phosphatidylcholine fully support the binding activity but not the kinase activity. Under these conditions, PDBu causes a 2-3 fold stimulation of kinase activity, and therefore, receptor occupancy and kinase activation by phorbol esters are coupled. With other mixtures, including phosphatidylserine alone, both kinase and binding activities are fully supported. No further stimulation of kinase with PDBu is observed. Therefore, receptor occupancy and kinase activation are uncoupled. It is feasible to consider that the lipid environment of the receptor in desensitized cultures is altered such that coupling of receptor occupancy to kinase activation is interrupted.

Significance to Biomedical Research and the Program of the Institute:

This project is designed to use model cell culture systems for detailed biochemical analysis of the mechanism of action of phorbol esters. It is anticipated that the general principles can then be applied to more complex systems such as whole skin or cultured keratinocytes. That is, the cultured cells used in this project should provide a useful model system for designing critical experiments with less readily available tissues in which phorbol esters have been shown to have tumor promoting properties.

GH_4C_1 cells are a particularly useful model system because they have well-characterized receptors and responses to a variety of effectors and hormones. They can be used to study the influence of several hormones on responses to phorbol esters. GH_4C_1 cells have receptors for glucocorticoids and compounds that work through the adenylate cyclase system, both of which have been suggested to influence phorbol ester actions. In addition, the effects of other compounds that may also activate protein kinase C, such as thyrotropin-releasing hormone, can also be explored. In this way, GH_4C_1 cells can be used to study the concerted cellular response to a variety of conditions.

Desensitization to tumor promoters is of potential importance in limiting susceptibility to carcinogenesis. For example, hamsters become refractory to phorbol ester-mediated hyperplasia. Based on data indicating that sustained hyperplasia is necessary for tumor promotion in mice, it seems likely that desensitization to phorbol esters in hamster skin is related to the resistance of this tissue to phorbol ester-promoted carcinogenesis. Biochemical analysis of this process in the easily obtainable cultured pituitary cells will provide a model for designing critical experiments to characterize the mechanism of desensitization in the less readily available hamster keratinocytes. Understanding the pathway of desensitization may also lead to pharmacological approaches to limiting cellular sensitivity to tumor promoters.

The overall significance to this project is that it will increase the understanding of the mechanism of phorbol ester action using cell culture model systems. This should provide insight into the biochemical pathways important in carcinogenesis in human tissues as well.

Proposed Course:

Experiments will be designed to further define the sequence of events associated with phorbol ester receptor occupancy using the described working model of

phorbol ester action as a guide. The photoaffinity probe, [20-^{3}H]phorbol 12-p-azidobenzoate 13-benzoate, has been shown to preferentially label phospholipids associated with the receptor. This will be used to directly test the hypothesis that the difference in affinity in intact cells at 37° and 4° is due to differences in the lipid environment of the receptor. Recent results from this section have identified diacylglycerol as the endogenous ligand for the phorbol ester receptor. The working model predicts that compounds that activate phospholipase C and cause diacylglycerol formation should also affect the subcellular distribution of binding and kinase C activities. This hypothesis will be tested in GH_4C_1 cells exposed to thyrotropin-releasing hormone, a known activator of phospholipase C.

The mechanism of phorbol ester receptor down modulation will be characterized further. The current results suggest that down modulation is associated with decreased ability to stabilize the membrane association of the cytosolic receptor. This difficulty could be due to a change in the lipid composition of the membranes and/or a covalent modification of the receptor itself. Plasma membrane lipids will be extracted from control and down modulated cultures and their efficacy in reconstituting binding and kinase will be measured. Efforts of others in this section are directed towards purification of the receptor and production of specific antibodies. It is anticipated that these antibodies could be used to assess whether there are biochemical differences between the receptors from control and down modulated cultures.

The mechanism of desensitization will be pursued along two courses. The first will involve further characterization of the kinase inhibitor. Experiments will be directed towards establishing the biochemical nature and also the specificity of the inhibitor. It is not yet known whether this inhibitor is an artifact of cell lysis or an endogenous regulatory component. The second course will be to further characterize the role of phospholipids in coupling of receptor occupancy to kinase activation.

Publications

Jaken, S., Shupnik, M. A., Blumberg, P. M. and Tashjian, A. H., Jr.: Relationship between mezerein-mediated biological responses and phorbol ester receptor occupancy. Cancer Res. 43: 11-14, 1983.

Phillips, M. A. and Jaken, S: Specific desensitization to tumor promoting phorbol esters in mouse pituitary cells: Evidence that desensitization is a two-step process. J. Biol. Chem. 258: 2875-2881, 1983.

ANNUAL REPORT OF

THE LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1982 to September 30, 1983

The major goals of the Laboratory of Cellular and Molecular Biology (LCMB) are to determine the etiology of naturally occurring cancers, to elucidate mechanisms of transformation by carcinogenic agents and to develop experimental strategies capable of preventing spontaneous and virus-induced tumors. The ultimate aim of these investigations is to apply approaches successful in animal model systems to the identification of causative agents of human malignancies and to the prevention of cancer in man. The primary emphasis of many ongoing investigations within the laboratory concerns RNA tumor viruses. These agents are unique among animal viruses in their mode of transmission and the intimate association that has evolved between them and cells of a large number and variety of vertebrate species. The etiologic role of this virus group has been established for naturally occurring cancers of many species, including some subhuman primates. Certain members of this virus group, so-called "replication-defective" transforming viruses, have arisen by a mechanism involving recombination with cellular genes. These genes, when incorporated within the retrovirus genome, exhibit transforming activity. Thus, these viruses offer an unparalleled opportunity to elucidate the processes by which cellular genes cause malignancies.

Research within the LCMB encompasses efforts to understand the processes involved in malignancy utilizing RNA tumor viruses as models. At the same time, studies are in progress to develop and apply the most sensitive and specific methods of tumor virus detection to the search for and investigation of genes related to oncogenic viruses in man.

During the past year, significant progress has been made in the investigation of replication-defective mammalian transforming retroviruses. These viruses have been isolated from a number of mammalian species, including subhuman primates. Our studies have led to the biological characterization of most of the known isolates of this virus group. In each case, these viruses have been shown to possess the capacity to transform cells, but to be replication-defective, requiring a type C RNA virus as a helper. During the past year, we have continued to apply recombinant DNA and nucleotide sequencing techniques to the elucidation of the structure and functions of these viruses and their cell-derived transforming genes.

Simian sarcoma virus (SSV) deletion mutants were constructed from a molecular clone containing the entire infectious provirus. Transfection analysis of these mutants localized the SSV-transforming gene to a small region of the viral genome encompassing its cell-derived sequence (v-sis). Antiserum to a peptide synthesized on the basis of the predicted amino acid sequence of the SSV-transforming gene detected a 28,000-dalton protein that was specifically expressed in SSV-transformed cells and that corresponded in size to that predicted from the v-sis-coding sequence. The v-sis gene product, designated p28sis, was not a phosphoprotein, nor did it possess detectable protein kinase activity. These findings distinguish p28sis from a number of other retroviral onc proteins.

The complete nucleotide sequence of the proviral genome of SSV has been determined. Like other transforming viruses, SSV contains sequences derived from its helper virus, simian sarcoma-associated virus (SSAV), and a cell-derived v-sis insertion sequence. By comparison with the sequence of Moloney murine leukemia virus, it was possible to precisely localize and define sequences contributed by SSAV during the generation of SSV. Comparative sequence analysis of SSV and SSAV showed that SSAV provides regulatory sequences for initiation and termination of transcription of the SSV-transforming gene. Moreover, coding sequences for the putative protein product of this gene appear to initiate from the amino terminus of the SSAV env gene. Antibodies to synthetic peptides derived from the carboxy and amino termini of the putative protein predicted by the open reading frame identified within v-sis specifically detect a 28,000 dalton protein, p28sis, in SSV-transformed cells. These and other findings confirm the predicted amino acid sequence of this protein and localize it to the coding region of the SSV-transforming gene.

The integrated Abelson murine leukemia virus (A-MuLV) genome cloned in bacteriophage λgtWES·λB was used to localize viral genetic sequences required for transformation. Comparison of the biological activity of cloned A-MuLV genomic and subgenomic fragments showed that subgenomic clones that lacked the 5' long terminal repeat (LTR) and adjoining sequences (300-base pairs downstream of the repeat) were not biologically active. In contrast, subgenomic clones that lacked the 3' LTR and as much as 1.3-kilobase pairs of the A-MuLV cell-derived abl gene were as efficient as wild-type viral DNA in transformation. The A-MuLV-encoded polyprotein P120 and its associated protein kinase activity were detected in transformants obtained by transfection with Cla I, Bam HI and Hind III subgenomic clones. In contrast, individual transformants obtained with subgenomic Sal I clones expressed A-MuLV proteins ranging in size from 82,000 to 95,000 daltons. Each demonstrated an associated protein kinase activity. These results provide direct genetic evidence that only the proximal 40% of abl with its associated 5' helper viral sequences is required for fibroblast transformation.

BALB and Harvey murine sarcoma viruses (MSV) comprise a family of retroviruses whose mouse- and rat-derived onc genes are closely related. These viruses induce sarcomas and erythroleukemias in susceptible animals. An in vitro colony assay that detects transformation of lymphoid cells by A-MuLV was used to demonstrate that BALB- and Harvey-MSV transform a novel hematopoietic cell both in culture and in vivo. Bone marrow colony formation was sarcoma virus-dependent, followed single-hit kinetics, and required the presence of mercaptoethanol in the agar medium. BALB- and Harvey-MSV-induced colonies could be established in culture as continuous cell lines that demonstrated unrestricted self-renewal capacity and leukemogenicity in vivo. The cells had a blast cell morphology and lacked detectable markers of mature cells within the myeloid or erythroid series. They also lacked detectable immunoglobulin μ chain or Thy-1 antigen, markers normally associated with committed cells of the B- and T-lymphoid lineages, respectively. However, the transformants contained very high levels of terminal deoxynucleotidyl transferase (TdT), an enzyme believed to be specific to early stages within the lymphoid differentiation pathway. This phenotype distinguishes these

BALB- and Harvey-MSV transformants from any previously reported hematopoietic targets of transforming retroviruses, including the pre-B lymphoid cell transformed by A-MuLV under identical assay conditions. These newly identified lymphoid progenitor cell transformants may provide an important means of studying early stages of lymphoid ontogeny and the possible role of TdT in lymphoid development.

Clonal BALB/c mouse epidermal keratinocyte (BALB/MK) cell lines were established in tissue culture. Despite their aneuploid nature, the lines were nontumorigenic, and retained in vitro properties similar to those of primary diploid keratinocytes. These included the constitutive expression of keratin and terminal differentiation in response to a calcium concentration greater than 1.0 mM in the medium. The cells also demonstrated an absolute requirement for nanomolar concentrations of epidermal growth factor (EGF) for their proliferation. BALB or Kirsten murine sarcoma viruses are acute transforming retroviruses, which have been shown to transform fibroblastic and hematopoietic cells. Infection of BALB/MK or its clonal sublines with either virus leads to the rapid acquisition of EGF-independent growth. The cells concomitantly lose their sensitivity to calcium-induced terminal differentiation. Thus, these retroviruses can rapidly confer upon epithelial keratinocytes in culture growth properties that resemble those of malignant epidermoid carcinoma cells.

Snyder-Theilen feline sarcoma virus (ST-FeSV) codes for a protein kinase with specificity for tyrosine residues, properties analogous to those of the transforming gene product of Abelson murine leukemia virus. ST-FeSV was demonstrated to transform murine hematopoietic cells under in vitro assay conditions which detect lymphoid cell transformation by Abelson murine leukemia virus. Bone marrow colony formation was shown to require ST-FeSV, follow single-hit kinetics, and require the presence of mercaptoethanol in the agar medium. ST-FeSV-induced colonies could be established in culture as continuous cell lines that demonstrated unrestricted self-renewal capacity and leukemogenicity in vivo. The hematopoietic blast cells transformed by ST-FeSV in culture appeared to be at an early stage of B cell differentiation. They possessed Lyb 2 surface antigens, were dependent on mercaptoethanol for growth, and contained only low levels of TdT. Moreover, a large fraction of the lines synthesized immunoglobulin μ chain in the absence of light chains. Thus, the phenotype of ST-FeSV hematopoietic transformants was indistinguishable from that of the pre-B lymphoblast transformants induced by Abelson murine leukemia virus. These findings indicate that the in vitro functional similarities in the onc gene products of ST-FeSV and Abelson murine leukemia virus may reflect a common pathway by which they exert their oncogenic potential.

Gardner-Rasheed feline sarcoma virus (GR-FeSV) is an acute transforming retrovirus which encodes a gag-onc polyprotein possessing an associated tyrosine kinase activity. The integrated form of this virus, isolated in the Charon 21A strain of bacteriophage λ, demonstrated an ability to transform NIH/3T3 cells at high efficiency upon transfection. Foci induced by GR-FeSV DNA contained rescuable sarcoma virus and expressed GR-P70, the major GR-FeSV translational product. Localization of LTRs within the DNA clone made it possible to establish the length of the

GR-FeSV provirus as 4.6 kbp. Analysis of heteroduplexes formed between λ-FeLV and λ-GR-FeSV DNAs revealed the presence of a 1,700-bp FeLV-unrelated segment, designated v-fgr, within the GR-FeSV genome. This region was sufficient in size to encode a protein of approximately 68,000 daltons and was localized immediately downstream of the FeLV gag gene-coding sequences present in GR-FeSV. Thus, it is likely that this 1.7-kbp stretch encodes the onc moiety of GR-P70. Utilizing probes representing v-fgr, homologous sequences were detected in the DNAs of diverse vertebrate species, implying that v-fgr originated from a well-conserved cellular gene. The number of cellular DNA fragments hybridized by v-fgr-derived probes indicated either that proto-fgr is distributed over a very large region of cellular DNA or represents a family of related genes. By molecular hybridization, v-fgr was not directly related to the onc genes of other known retroviruses having associated tyrosine kinase activity.

Oncoviruses have been shown to be etiologically involved in naturally occurring tumors of a wide variety of vertebrate species. They appear to exert their oncogenic potential under conditions in which there is poor host cell control of virus replication. This is the case for horizontally transmitted oncoviruses of birds, rodents, arylodictyles, carnivores and primates. It has become evident that oncoviruses can interact with their hosts in a manner which appears to be unique among viruses of vertebrates. In many species, including primates, oncoviruses are transmitted from one generation to the next in an unexpressed form within the host cell genome. Under such conditions, these endogenous viruses appear to be subject to regulatory processes analogous to those affecting cellular genes. Over the past several years, our laboratory has isolated a number of new endogenous oncoviruses. Moreover, we have investigated the relationships of such viruses to known oncoviruses, as well as the distribution of related viral sequences within vertebrate cellular DNAs by molecular hybridization techniques.

The distribution of endogenous type C RNA viruses was studied in inbred strains of mice and some subspecies of Mus musculus of different geographic origins. The following groups of inducible viruses were characterized by their host range and immunological properties: (1) viruses indistinguishable from one of the three prototype viruses endogenous to BALB/c mice; (2) viruses coding for proteins immunologically related to different prototype endogenous viruses; and (3) viruses whose p12 structural proteins were immunologically indistinguishable from that of BALB:virus-2, but whose p30 major structural proteins and envelope glycoproteins differed immunologically from those of previously characterized endogenous viruses. These findings suggest that endogenous viruses have undergone numerous genetic interactions during the process of evolution leading to inducible viruses of present day mouse strains. A class of xenotropic virus spontaneously released by NZB mice is endogenous to, but not inducible from, embryo cells of other previously studied mouse strains. Viruses which could not be distinguished from the NZB xenotropic virus by host-range analysis or radioimmunological techniques were chemically inducible from embryo cells of several mouse strains originating in Asia and Europe. These results indicate that the biological regulatory mechanisms that affect expression of this virus have evolved differently in such strains from control mechanisms that developed in standard inbred strains.

The closed circular form of the endogenous squirrel monkey type D retrovirus (SMRV) was molecularly cloned in a bacteriophage vector. The restriction map of the biologically active clone was determined and was found to be identical to the parental SMRV linear DNA, except for the deletion of one LTR. Restriction enzyme analysis and Southern blotting indicated that the SMRV LTR is approximately 300-bp long. The SMRV restriction map was oriented to the viral RNA by using gene-specific probes from murine mammary tumor virus. Restriction enzyme digests of a variety of vertebrate DNAs were analyzed for sequence homology to SMRV by using the cloned SMRV genome as a probe. Consistent with earlier studies, multiple copies of SMRV were detected in squirrel monkey DNA. Homologous restriction fragments were also detected in the DNAs of other primate species.

Sequences related to the mouse mammary tumor virus (MMTV) genome have been detected in fragments of restricted human cellular DNA. These results were obtained by using recombinant DNA containing the MMTV proviral genome and lowering the stringency of blot-hybridization conditions. The MMTV genome also reacts with unique families of fragments in restricted cellular DNA from other mammalian species but not with salmon sperm DNA. A clone that reacted with labeled MMTV proviral DNA was selected from a human DNA library in Charon 4A. Under stringent conditions, a 3.7-kilobase MMTV-related Eco RI fragment of this clone hybridized with many of the same Eco RI restriction fragments of human cellular DNA detectable with MMTV proviral DNA under low-stringency conditions. Specific fragments of the human clone were shown to contain sequences related to the molecularly cloned gag, pol, and env regions of the MMTV genome.

Research within the LCMB is also aimed at elucidating how leukemia viruses cause malignancies. The integrated proviral genome of Rauscher murine leukemia virus was molecularly cloned in a bacteriophage Charon 4A vector after the proviral sequences were enriched by sequential RPC-5 column chromatography and sucrose gradient centrifugation. A recombinant DNA clone, λ-RV-1, possessing a 12-kbp Eco RI insert, was shown to contain the entire 8.8 kbp-pair leukemia virus genome flanked by rat cellular sequences at the 5' and 3' ends. This DNA fragment was biologically active, inducing the release of virion-associated reverse transcriptase activity with as little as 10 ng of DNA insert. The virus induced XC plaque formation at high titers on NIH/3T3 and BALB/3T3 cells and demonstrated identity with the parental virus in radioimmunoassays for the highly type-specific gag gene coded p12 protein. The molecularly cloned Rauscher murine leukemia virus should be useful in studying the molecular mechanisms involved in the transformation of specific lymphoid target cells by chronic mouse leukemia viruses.

LCMB scientists have also been successful in new research thrusts utilizing knowledge gained from studies in model systems to investigate mechanisms involved in naturally occurring human malignancies. One major effort emanated from research on acute transforming retroviruses. We have utilized the cell-derived onc genes of such viruses as probes for the detection and eventual isolation of the normal homologs of such genes in human DNA. We have also utilized onc genes as probes to identify the chromosomal locations of related human cellular genes.

The primate cell-derived transforming gene (v-sis) of SSV is represented as a single copy marker within cellular DNAs of mammalian species, including human. The human analogue of v-sis can be distinguished from its rodent counterparts by Southern blotting analysis of Eco RI-restricted DNAs. By testing for the presence of the human v-sis-related fragment, c-sis (human) in somatic cell hybrids possessing varying numbers of human chromosomes, as well as in segregants of such hybrids, it was possible to assign c-sis to human chromosome 22.

Human DNA was analyzed for the presence of sequences homologous to the transforming gene (v-mos) of Moloney-MSV. A single 2.5-kbp Eco RI-generated fragment of human DNA was identified by using cloned v-mos as probe. This DNA was molecularly cloned in a bacteriophage vector. By heteroduplex and restriction enzyme analyses, this human DNA fragment, designated c-mos (human), contained a 0.65-kbp region of continuous homology with v-mos and was present as a single copy in human DNA. By testing for the presence of c-mos (human) in somatic cell hybrids possessing various numbers of human chromosomes, as well as in subclones of such hybrids, it was possible to assign c-mos (human) to human chromosome 8.

The development of DNA-mediated gene transfer techniques has made it possible to identify transforming genes present in certain human tumor cells. Such genes have been shown to induce morphological transformation when used to transfect suitable assay cells. Recently, a transforming gene has been isolated by molecular cloning techniques from the T24 and EJ human bladder carcinoma cell lines. This bladder carcinoma oncogene has been shown to be of human origin, less than 6 kbp in size and closely related to the onc genes (v-bas and v-ras) of BALB- and Harvey-MSVs. These transforming retroviruses arose in nature by transduction of cellular genes from mouse and rat cells, respectively. To understand better the relationship of the T24 oncogene with other human cellular genes, we have determined the chromosomal location of its normal allele within the human genome, and showed that it is carried on chromosome 11 in normal cells.

The consistent appearance of specific chromosomal translocations in human Burkitt's lymphomas and murine plasmacytomas has suggested that these translocations might play a role in malignant transformation. We have demonstrated that transformation of these cells is frequently accompanied by the somatic rearrangement of a cellular analogue of an avian retrovirus transforming gene, c-myc. Moreover, c-myc was mapped to chromosome 8 band q24. This chromosomal segment is involved in the reciprocal Burkitt's translocations [t(8;14), t(8;22) and t(2;8)]. In two t(8;14) human Burkitt's cell lines, c-myc appears to have been translocated directly into a DNA restriction fragment that also encodes the immunoglobulin μ chain gene. In the case of a specific cloned fragment of DNA derived from a mouse plasmacytoma, we demonstrated directly that c-myc has been translocated into the immunoglobulin α switch region. Our data provide a molecular basis for considering the role that specific translocations might play in malignant transformation.

The development of DNA-mediated gene transfer techniques has made it possible to identify transforming genes in a variety of tumors, including some of human origin. During the past two years, LCMB scientists have succeeded in the identification and isolation of human tumor oncogenes. Transforming DNAs have been detected in human tumor cell lines of diverse origin, including carcinomas of the bladder, lung, and colon, as well as certain sarcomas. Transformants induced by such DNAs exhibited anchorage-independent growth and were tumorigenic and athymic in xeno-competent mice. Moreover, they contained DNA sequences and were able to transmit their malignant phenotype in additional cycles of transfection.

A transforming gene isolated from T24 human bladder carcinoma cells is closely related to the BALB-MSV onc gene (v-bas). This transforming gene is localized to a 4.6-kbp region and expressed as a 1.2-kbp polyadenylated transcript, which contains v-bas-related sequences. Moreover, antisera known to detect immunologically-related onc gene products of BALB- and Harvey-MSVs recognized elevated levels of a related protein in T24 cells. The normal human homologue of v-bas was found to be indistinguishable from the T24 oncogene by heteroduplex and restriction enzyme analysis. These results imply that rather subtle genetic alterations have led to the activation of the normal human homologue of v-bas as a human transforming gene.

The identification and isolation of oncogenes capable of inducing malignant transformation on transfection into rodent cells from human tumor cells has opened the possibility of deciphering the processes involved in human carcinogenesis. With the exception of three human lymphomas, the human transforming genes so far identified have been detected in established tumor cell lines, raising the possibility that they might have been activated during in vitro manipulation of the cells. However, unmanipulated human solid tumors, including carcinomas of the colon (two), lung, and pancreas, and an embryonal rhabdomyosarcoma possessed a common oncogene which, like that isolated from human LX-1 lung carcinoma cells, shares sequences with the onc gene of the Ki-MSV. We also demonstrated that several other human tumor cell lines, including those established from carcinomas of the colon (A2233), lung (A427 and A2182), gall bladder (A1604), and urinary bladder (A1698), possessed the same oncogene. Thus, a variety of human tumors, regardless of their clinical manifestations, contain a common transforming gene related to the onc gene of a transforming retrovirus.

Twenty-two human hematopoietic tumors and tumor cell lines were surveyed for sequences capable of transforming NIH/3T3 cells by DNA transfection. A primary human acute myelogenous leukemia, a chronic myelogenous leukemia cell line and cell lines derived from three independent acute lymphocytic leukemias demonstrated oncogenes capable of conferring the transformed phenotype to NIH/3T3 cells through serial cycles of transfection. One of three transforming genes associated with acute lymphocytic leukemia cells (classified as thymocyte developmental stage II) was identified as the activated cellular homologue of the Ki-MSV virus onc gene, kis, a member of the ras family of onc genes. A transforming gene, which was demonstrated to be common to several human myeloid and lymphoid tumor cells, was shown to be a distantly related member of the ras gene family. Thus, the NIH/3T3 transfection assay commonly detects related oncogenes in human hematopoietic tumor cells. Moreover, the activation of these oncogenes appears to be independent of the specific stage of cell differentiation or tumor phenotype.

The genetic change that leads to the activation of the oncogene in T24 human bladder carcinoma cells was shown to be a single point mutation of guanosine into thymidine. This substitution results in the incorporation of valine instead of glycine as the 12th amino acid residue of the T24 oncogene-encoded p21 protein. Thus, a single amino acid substitution appears to be sufficient to confer transforming properties on the gene product of the T24 human bladder carcinoma oncogene.

A transforming gene related to c-bas/has (human) was cloned in biologically active form from a human lung carcinoma-derived cell line Hs242. Recombinants were constructed between the Hs242 oncogene and its normal allele in order to localize the genetic lesion that led to the acquisiton of transforming properties. In vitro transformation assays, coupled with primary nucleotide sequence analysis of these recombinants, revealed that the genetic change that led to the activation of the Hs242 oncogene is a point mutation in the second exon. This point mutation results in the incorporation of leucine instead of glutamine in the 61st amino acid of the predicted protein. No changes were observed in the first exon, the region of c-bas/has in which a point mutation is responsible for activation of the T24 and EJ bladder carcinoma oncogenes. Thus, single amino acid substitutions within different structural domains of the same protooncogene can independently be responsible for its malignant conversion under natural conditions.

DNAs of methylcholanthrene-induced mouse fibrosarcomas were analyzed for transforming activity in the NIH/3T3 transfection assay. Two of four tumors were demonstrated to contain dominant transforming genes, indistinguishable in their pattern of restriction endonuclease resistance to inactivation of biologic activity. By blotting analysis and molecular hybridization with retroviral onc genes, these transforming genes were each shown to be related to the onc gene, kis, of Ki-MSV. The availability of a model system in which a defined carcinogen reproducibly leads to activation of kis as a transforming gene should be of value in elucidating the role of oncogenes in the neoplastic process.

LCMB efforts to elucidate mechanisms of carcinogenesis are complemented by the program of the In Vitro Carcinogenesis Section which is directed toward elucidating through cell culture studies the mechanism(s) of neoplastic transformation in human cells. Major new findings of the past year are in two research areas: mechanisms of DNA damage and repair, and environmental influences on proliferation and expression of differentiated function in cultured cells, particularly epithelial cells, for analysis of neoplastic transformation in vitro.

Current emphasis is on the characterization in cultured human cells of chromatid DNA repair lesions found to be associated with malignant transformation and/or susceptibility to cancer. Since a chromatid apparently contains a single continuous DNA double strand, chromatid breaks and gaps represent unrepaired DNA strand breaks. Last year we reported that human skin fibroblasts transformed to malignant cells in culture had significantly more chromatid breaks and gaps than their normal counterparts when x-irradiated specifically during G_2 phase or within 1.5 hours of metaphase. These observations, together with results from use of DNA repair inhibitors, implicated deficiencies in DNA repair in the malignant cells as we had found

previously in mouse fibroblasts transformed in culture. These observations have been extended to cell lines from diverse human tumors, including sarcomas, carcinomas, glioblastomas, etc., as compared with numerous lines of normal fibroblasts. Further, skin fibroblasts from cancer-prone individuals, including those with ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia, Gardner's syndrome, familial polyposis or xeroderma pigmentosum, show similar defects in chromatid DNA repair. It thus appears that acquisition of defects in chromatid DNA repair operative during G_2-prophase is a prerequisite, possibly the initiating step, for carcinogenesis in human cells. In addition, the formation, consequences, and mechanism of repair of DNA-protein crosslinks are being studied in mouse and human fibroblasts. DNA-protein crosslinks are induced by numerous agents including x-rays, ultraviolet light, visible fluorescent light, and a wide variety of chemical carcinogens. The only known way to repair bulky DNA adducts is by the nucleotide excision pathway. It has been reported, as expected, that excision-deficient XP12BE, a xeroderma pigmentosum (XP), group A, cell line is deficient in repair of DNA-protein crosslinks. However, we have found that eventually XP12BE, as well as other XP groups, including C, D, E, and the XP variant, are able to repair completely the DNA-protein crosslinks induced by 20 μM trans-platinum. Since the excision capacity of the group A cells has been estimated at less than 2% of normal cells, their eventual repair of DNA protein crosslinks suggests that another mechanism is involved. From use of the metabolic inhibitors cycloheximide and aphidicolin, and from kinetic studies of crosslink repair and DNA replication in mouse L1210 cells and normal human fibroblasts, it appears that this second pathway requires protein synthesis. Further, though activity of this pathway for crosslink repair is enhanced in cells cycling rapidly, DNA replication per se is not essential. A mechanism for operation of this pathway is proposed that is based on extensive experimental findings to date. In another unrelated study, exposure of XP-A cells to low-intensity fluorescent light (wavelength >300 nm) was found to cause a greater degree of cell killing and/or cytostasis than observed in normal human fibroblasts. Further analysis of this photosensitivity is in progress.

Because of the relevance of epithelial cells to human carcinogenesis, epithelial cell proliferation and expression of differentiated function are of particular interest. Defining the environmental conditions for sustained expression of differentiated function in culture is necessary for a transformation system with epithelial cells because of the possible relationship between transformation and loss of differentiated function. One objective was to define conditions for rapid proliferation of basal cells and identify conditions that trigger maturation or terminal differentiation in epidermal keratinocytes. In studies to date, normal human and C3H mouse epidermal keratinocytes in mass culture proliferate more rapidly to higher cell density if the calcium ion concentration is increased well above levels reported for optimal clonal growth. Further, limiting protein synthesis by treatment with cycloheximide appears to trigger terminal differentiation in mouse keratinocytes; even at low calcium ion concentration normal and neoplastic mouse keratinocytes maintain capacity for terminal differentiation. In studies with monkey kidney epithelium, dissolved oxygen concentration modulates hemicyst formation, a manifestation of transepithelial ion and fluid movement, i.e., a function of secreting epithelium. Also, oxygen consumption by epithelial cells is

significantly greater than that of comparable populations of fibroblasts, a property that lowers the risk of oxidative injury to epithelium in culture. Increased cycling is currently attained in normal cultures of human epidermal keratinocytes transfected with an SV-40 plasmid. In a collaborative study, a transformation system has also been developed with human epidermal keratinocytes infected with a hybrid SV-40 virus.

In addition to their intramural research efforts, investigators within the LCMB serve on the editorial boards of major journals in their fields, serve as members of various review bodies, and participate in a large number of collaborative efforts with scientists in laboratories throughout the country. The ultimate goal of these multidisciplinary studies of virus-induced and spontaneously occurring cancers is to apply the basic information derived to its most important application, the prevention of cancer in man.

CONTRACT NARRATIVE
LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1983

HAZLETON LABORATORIES AMERICA, INC. (NO1-CP-01017)

Title: Support Services for the Laboratory of Cellular and Molecular Biology

Current Annual Level: $705,000

Man Years: 14

Objectives: The purpose of the contract is to provide support services for research conducted by the LCMB.

Major Contributions: Since the purpose of this contract is to provide support services for research conducted by LCMB, a discussion of major contributions is inappropriate.

Proposed Course: To continue to provide timely, high quality support services for the LCMB.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP04805-13 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cocarcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
A. Hellman Assistant Chief, LCMB, NCI

COOPERATING UNITS *(if any)*
Food and Drug Administration
National Center for Medical Diseases and Public Health

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recent epidemiological reports have suggested an excess morbidity or mortality from leukemia among male workers in electrical occupations. There is little biological evidence to suggest why electrical workers might have an extra risk of leukemia. There is, however, good evidence that certain pulsed electromagnetic fields (EMF) accelerate bone or wound healing. The biological basis of this phenomenon is also not understood. For the latter case, it has been suggested that alteration of cell membrane structure and/or receptor sites for hormones and other growth factors may be involved. Since cell membrane integrity is important in the functional response of cells of the immune system, we are looking for some possible common parameters that may account for both biological phenomena, that is, enhanced leukemia and accelerated bone and wound healing.

Utilizing two parameters of the immune system, we are investigating the influence of EMF on macrophage chemotaxis and on T cell blastogenesis. In both instances, we note some inhibitory effects brought about by 5.2 msec bursts of bipolar repeat 15 HZ bursts of energy.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

A. K. Fowler — Chief, Developmental Biology and Biochemistry Section — LCC, NCI

Objectives:

Bone and wound healing are reported to be accelerated when the affected areas are exposed to certain pulsed electromagnetic fields (EMF). Similarly, recent epidemiological reports suggest an excess of morbidity and mortality from leukemia among male workers in electrical occupations. We are attempting to gain a better biological understanding underlining both of these observations.

Methods Employed:

Certain cells of the immune system are subjected to EMF and their biological responses measured.

Major Findings:

Cultures were exposed between 2 10 x 10 cm coils placed 8 cm apart driven in parallel by the generator. For macrophage chemotaxis studies, thioglycollate-stimulated peritoneal exudate cells were collected from adult female ICR mice. Ninety-five percent of the cells were macrophages, as identified by esterase activity. Chemotaxis was measured by macrophage migration toward a complement-derived chemotactic factor using a modified Boyden chamber. There was an EMF exposure-dependent decrease in macrophage chemotaxis, with levels approximately 70% and 50% of exposed control values after 2 and 3 hours exposure, respectively. Maximal inhibition occurred within 3 hours, since a further decrease in chemotaxis was not observed after 4 hours of EMF exposure. Blastogenic transformation was measured by PHA-stimulated ^{3}H-thymidine incorporation in splenic lymphocytes obtained from adult female BALB/c mice. After addition of PHA, lymphocytes were exposed to the EMF. A 5-fold decrease in lymphocyte blast transformation was observed after 48 hours of EMF exposure, a time of optimal blastogenesis in unexposed controls. Neither macrophage nor lymphocyte viability were affected by EMF exposure, as determined by trypan blue dye exclusion.

Significance to Biomedical Research and the Program of the Institute:

These data indicate that macrophage and lymphocyte biofunction in vitro provide model systems for examining (1) the interaction of EMF with cells in vitro, thus aiding elucidation of mechanisms involved in such interaction, and (2) the phenotypic cellular relationships involved in immune surveillance.

Proposed Course:

Currently, there is no biological dosimeter to measure EMF. Since we have just initiated this study, we will attempt to delineate parameters of dose effects. We will then attempt to define the biological significance of EMF in order to gain some understanding of this observed non-ionizing radiation contribution to bone and wound healing, as well as its possible association with a reported increase in acute myeloid and chronic lymphoid leukemia.

Publications:

Saviolakis, J. A., Strickland, J. E., Paisely, T., Hellman, A. and Fowler, A. K.: Expression of oncornavirus RNA in mouse uterus during pregnancy. Biol. Reprod. 26: 806-812, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04930-12 LCMB

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Biology of Natural and Induced Neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation):
P. Arnstein Veterinary Director, LCMB, NCI

COOPERATING UNITS *(if any)*:
R. W. Emmons, Calif. Dept. Health Services; A. Hackett, Peralta Cancer Inst.; R. Gilden and D. Fish, FCRF; M. Gardner, J. Levy and H. Rubin, U. Calif; R. Owens, Naval Bio. Res. Lab.

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In vivo studies using cloned genetically defined mouse leukemia retroviruses administered to newborn mice of known backgrounds yielded data on pathogenicity of replication-competent Rauscher murine leukemia virus (R-MuLV), Moloney murine leukemia virus (M-MuLV), and their recombinants. R-MuLV is extremely pleiotropic, capable of inducing lymphoid, erythroid and granulocytic malignant neoplasms at approximately equal rates. M-MuLV is almost always lymphomagenic. M-MuLV/R-MuLV recombinants may resemble either parent or produce intermediate frequencies, depending on the segments of the genome represented in the hybrid.

In vitro transformation experiments, using oncoviruses (with and without promoters) to infect normal primate cultures, continue to produce greatly altered morphologic and growth patterns; no fully malignant transformants have been proven to date.

Human and animal xenografts on athymic nude mice have been used to study tumor progression, malignant transformation, and to characterize spontaneous cancer of particular theoretical or practical significance.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
J. S. Rhim	Research Microbiologist	LCMB, NCI
T. Storch	Research Associate	LCMB, NCI

Objectives:

1. Conduct experiments to determine the in vivo biology of selected genetically mapped murine tumor viruses. The goal is to correlate specific viral genomic sequences with a particular effect on the host (e.g., in vivo replication, viremia, target cell preference, tumor induction, and tumor type induced. The viruses for these studies are selected or especially engineered by molecular biology techniques in vitro.

2. Characterize morphologic transformation in primate cells and selected nonprimate cultures. Correlate morphologic transformation with transplantability and tumor production in athymic nude mice.

3. Document tumor induction (neoplastic graft) in athymic nude mice using spontaneous human cancers as well as selected animal specimens; correlate positive neoplastic graft results with malignancy-related gene sequences (oncogenes) and viruses detected.

Methods Employed:

1. In vitro portions of the viral gene experiments are collaborative studies in Dr. Aaronson's and Dr. Storch's projects (Nos. Z01CP04940-16 LCMB and Z01CP05112-04 LCMB). Candidate inocula are furnished in coded vials as deep-frozen aliquots. Newborn mice are inoculated at approximately 24 hours of age with the cloned retroviruses by the intraperitoneal-subcutaneous routes. All inoculated mice are subsequently monitored by periodic physical examination and appropriate periodic blood sampling until onset of disease or, in case of nonpathogenic clones, for the designated holding periods up to the normal life span of the breed of mouse. Candidate leukemic mice are exsanguinated, all tissues examined for neoplasia, and appropriate specimens processed for virus isolation, cell culture and microscopic characterization.

2. Transformation of normal primate cells to pre-neoplastic and neoplasm-like morphology is followed in tissue culture and tested in athymic nude mice. Some experiments are collaborative with Dr. Rhim's project (No. Z01CP05060-05 LCMB). Normal, karyologically defined cultures are exposed to oncoviruses and/or chemical carcinogens, passaged in parallel with controls and monitored for altered morphology and cultural characteristics (acquisition of anchorage independence, loss of contact inhibition) and periodically inoculated into athymic nude mice.

3. Candidate tissues and cultures derived from spontaneous neoplasms are either grafted directly as a whole tissue implant or established in cell culture and then inoculated after adequate replication in vitro. Some of the materials to be tested are furnished by collaborating NCI investigators and by University of California colleagues. A meaningful xenograft malignancy test usually employs 4 athymic nude mice given 10^6 to 10^7 viable test cells. If progressive tumors result, they are characterized histopathologically as well as by other criteria as indicated (karyology, antigenicity, virus content).

Major Findings:

R-MuLV oncogenesis: Recently summarized data clearly show that cloned replication-competent R-MuLV is capable of producing 3 major leukemia types with almost equal frequency. Of a total of 118 reticuloendothelial neoplasms induced, there were 35 lymphomas (30%), 38 granulocytic leukemias (32%), and 22 erythroleukemias (19%). The remaining 23 (19%) were poorly differentiated or mixed cell leukemias (the majority probably of the erythroid series). The cases are distributed among mice of different genetic backgrounds: NIH Swiss normal and athymic nude (T-cell deficient); 129J normal and athymic nude (T-cell deficient); N II nu normal and athymic nude (T- and B-cell deficient).

It should be borne in mind that the virus stock had been derived from plaque-purified seed recloned 3 times. The different cancers produced are, therefore, not the result of segregation of multiple viral strains, but rather real polymorphism in pathogenicity of a single clone of R-MuLV. In all individuals tested, viremia preceded the onset of disease.

Permanent Rauscher lymphoma tissue culture lines were established from some of the diseased lymph nodes for cytological analysis and other laboratory studies.

M-MuLV oncogenesis: In contrast to the R-MuLV data, M-MuLV is much more uniform in its pathogenicity. Using the same hosts as for R-MuLV, the cloned Moloney stock induced lymphomas almost exclusively. Among 125 reticuloendothelial tumor fatalities induced by M-MuLV, there were 115 lymphomas (92%), 7 erythroleukemias (5.6%), and 3 granulocytic leukemias (2.4%). In mouse strains with thymus, the disease could further be characterized as T-cell lymphoma. Athymic nude mice (profoundly deficient or devoid of T-cells) averaged longer incubation periods and developed predominantly pre-B or B-cell lymphomas. All inoculated animals tested were viremic by one month of age, long before the onset of leukemias. Permanent Moloney lymphoma tissue culture lines were established from representative cases for further viral and cytological analyses.

M-MuLV/R-MuLV recombinants: Viruses which were produced by transfection of virus-coding DNA of specific portions of both M- and R-MuLV were cloned and tested for pathogenicity in strains of mice in which the parent Rauscher and Moloney stocks had been tested previously. Preliminary data are available on two such recombinants: TFX 169-13, in which the Moloney genome had greater contribution than Rauscher, produced only slightly different distribution of cases than the M-MuLV parent: 30/37 (81%) lymphomas, 4/37 (11%) granulocytic leukemias, and 3/37 (8%) poorly differentiated or multiple-cell-type leukemias; and TFX 165-14, where Rauscher contributed a more extensive segment of the genome, gave rise to 24/43 (56%) lymphomas, 6/43 (14%) granulocytic leukemias, 9/43 (21%) erythroleukemias, and 4/43 (9%) poorly differentiated or multiple cell types. This clone has a disease pattern which may be expected of a virus where the genomic sequence coding for pathogenicity is about 1/2 Moloney and 1/2 Rauscher.

Transformation studies: Additional normal human cultures of fetal and adult origin have been exposed to the oncogenic hybrid virus adeno 12/SV40, followed by simple subculture or by additional exposure to chemical carcinogens and/or promoters. Morphologic changes occurred in each experiment in the infected flasks, whereas the controls remained unaltered and ultimately senesced. No fully malignant transformants could be isolated. These studies will be terminated.

In collaboration with Dr. Rhim (No. Z01CP05060-05 LCMB) it was confirmed that replication-competent bovine leukemia virus (BLV) alone can induce morphologic and xenograft-malignant transformation in lamb cells.

Spontaneous human tumor studies: A total of 13 human tumor-derived cell cultures have been propagated and tested for malignancy by xenograft in nude athymic mice. Information on the ability of such cultures to form neoplasms in an immunotolerant host contributes to studies in progress on the nature of oncogenes in spontaneous cancers. Six have been confirmed malignant, 5 are still on test, and the remaining 2 were nonmalignant in the nude mouse.

In collaboration with the University of California, San Francisco, cancer research program (Dr. Jay Levy), attempts are in progress to establish and characterize Kaposi sarcoma (KS) malignant cells. Tumors are obtained from surgery from patients with acquired immunodeficiency syndrome (AIDS). When possible, viable tumor fragments are grafted immediately into nude mice and also cultured in vitro. Alternately, the tumors are established as short-term cultures and then inoculated as low passage tissue cultured cells. Of 10 tumors processed so far, no proven sarcoma cells could be demonstrated. One permanent B-lymphocytic line has been established from a lymph node biopsy of an AIDS patient. The line was transformed by Epstein-Barr virus and was tumorigenic in suckling nude athymic mice, but not in adults.

Significance to Biomedical Research and the Program of the Institute:

In vivo testing of genetically defined murine leukemia retroviruses in their species of origin (Mus musculus) furnishes important information on the role of different portions of the RNA code transcribed into cellular DNA in the host. The data obtained may help find loci of susceptibility in human tissues. Human cellular oncogenes and candidate human cancer viruses will also be effectively studied with the aid of data collected in the mouse experiments.

The ability (or inability) of cultures to form malignant growth by xenografting to nude mice is still one of the best measures of malignancy in cell cultures. This test can be used to prove successful transformation by viruses or chemicals, or establishment of cancer cultures of naturally occurring neoplasms from any species.

Proposed Course:

Continue collaborative studies described.

Publications:

Levy, J. A., Arnstein, P., Dirksen, E. R., Siperstein, M. and Wiley, M.: Differentiated mouse epithelial cell line with hepatocyte characteristics. Differentiation 22: 12-18, 1982.

Rhim, J. S., Kraus, M. and Arnstein, P.: Neoplastic transformation of fetal lamb kidney cells by bovine leukemia virus. Int. J. Cancer. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04940-16 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Viruses and Transforming Genes in Experimental Oncogenesis and Human Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. A. Aaronson Chief, LCMB, NCI

COOPERATING UNITS *(if any)*

M. Gardner, U. California, Davis; S. Rasheed, USC, Los Angeles; R. Gallo, LTCB, NCI; H. Antoniades, Harvard U. Sch. Pub. Hlth., Boston; M. Hunkapiller, Calif. Inst. Tech., Pasadena; R. F. Doolittle, U. California, San Diego

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goals of this project are to elucidate the mechanisms of action of tumor viruses and to determine the cellular alterations responsible for naturally occurring human malignancies. Topics of present interest include: (1) transforming genes of retroviruses and cancer cells; (2) the biology of endogenous retroviruses; (3) the molecular biology of retrovirus replication and transformation; and (4) the application of knowledge gained from these studies to the search for the causes and mechanisms involved in human neoplastic transformation.

Some of the most important insights have been developed in the past year. LCMB's accomplishments include: characterization of oncogenes in a number of human cancers and their homologies with defined oncogenes of transforming retroviruses; identification of genetic lesions responsible for activating protooncogenes of onc genes into transforming genes as single base alterations which change single amino acids within the proteins coded by the gene; identification of onc genes activated by carcinogen-induced tumors in model systems; mapping the chromosomal locations of a number of onc genes in the human genome and demonstrating the specific rearrangement of one of these genes in translocations associated with Burkitt's lymphoma; detecting the potential product of previously unidentified onc genes and characterization of their functions.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. R. Tronick	Microbiologist	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI
S. G. Devare	Visiting Associate	LCMB, NCI
M. Barbacid	Visiting Scientist	LCMB, NCI
K. C. Robbins	Expert	LCMB, NCI
J. Pierce	Sr. Staff Fellow	LCMB, NCI
T. Storch	Research Associate	LCMB, NCI
B. Weissman	Staff Fellow	LCMB, NCI
A. Srinivasan	Visiting Associate	LCMB, NCI
Y. Yuasa	Visiting Fellow	LCMB, NCI
D. C. Swan	Expert	LCMB, NCI
A. Eva	Visiting Associate	LCMB, NCI

Objectives:

1. To study the mechanisms of action of RNA tumor viruses and transforming genes; and

2. To apply knowledge gained from experimental systems to the search for etiologic agents and mechanisms involved in neoplastic transformation of human cells.

Methods Employed:

Standard and developmental techniques in virology, cell biology, immunology, and molecular biology.

Major Findings:

The integrated Abelson murine leukemia virus (A-MuLV) genome cloned in bacteriophage λgtWES·λB was used to localize viral genetic sequences required for transformation. Comparison of the biological activity of cloned A-MuLV genomic and subgenomic fragments showed that subgenomic clones that lacked the 5' long terminal repeat (LTR) and adjoining sequences (300-base pairs downstream of the repeat) were not biologically active. In contrast, subgenomic clones that lacked the 3' LTR and as much as 1.3-kilobase pairs of the A-MuLV cell-derived abl gene were as efficient as wild-type viral DNA in transformation. The A-MuLV-encoded polyprotein P120 and its associated protein kinase activity were detected in transformants obtained by transfection with Cla I, Bam HI and Hind III subgenomic clones. In contrast, individual transformants obtained with subgenomic Sal I clones expressed A-MuLV proteins ranging in size from 82,000 to 95,000 daltons. Each demonstrated an associated protein kinase activity. These results provide direct genetic evidence that only the proximal 40% of abl with its associated 5' helper viral sequences is required for fibroblast transformation.

Gardner-Rasheed feline sarcoma virus (GR-FeSV) is an acute transforming retrovirus which encodes a gag-onc polyprotein possessing an associated tyrosine kinase activity. The integrated form of this virus, isolated in the Charon 21A strain of bacteriophage λ, demonstrated an ability to transform NIH/3T3 cells at high efficiency upon transfection. Foci induced by GR-FeSV DNA contained rescuable sarcoma virus and expressed GR-P70, the major GR-FeSV translational product. Localization of LTRs within the DNA clone made it possible to establish the length of the GR-FeSV provirus as 4.6 kbp. Analysis of heteroduplexes formed between λ-FeLV and λ-GR-FeSV DNAs revealed the presence of a 1,700-bp FeLV-unrelated segment, designated v-fgr, within the GR-FeSV genome. This region was sufficient in size to encode a protein of approximately 68,000 daltons and was localized immediately downstream of the FeLV gag gene-coding sequences present in GR-FeSV. Thus, it is likely that this 1.7-kbp stretch encodes the onc moiety of GR-P70. Utilizing probes representing v-fgr, homologous sequences were detected in the DNAs of diverse vertebrate species, implying that v-fgr originated from a well-conserved cellular gene. The number of cellular DNA fragments hybridized by v-fgr-derived probes indicated either that proto-fgr is distributed over a very large region of cellular DNA or represents a family of related genes. By molecular hybridization, v-fgr was not directly related to the onc genes of other known retroviruses having associated tyrosine kinase activity.

Simian sarcoma virus (SSV) deletion mutants were constructed from a molecular clone containing the entire infectious provirus. Transfection analysis of these mutants localized the SSV-transforming gene to a small region of the viral genome encompassing its cell-derived sequence (v-sis). Antiserum to a peptide synthesized on the basis of the predicted amino acid sequence of the SSV-transforming gene detected a 28,000-dalton protein that was specifically expressed in SSV-transformed cells and that corresponded in size to that predicted from the v-sis-coding sequence. The v-sis gene product, designated p28^{sis}, was not a phosphoprotein, nor did it possess detectable protein kinase activity. These findings distinguish p28^{sis} from a number of other retroviral onc proteins.

The complete nucleotide sequence of the proviral genome of the SSV has been determined. Like other transforming viruses, SSV contains sequences derived from its helper virus, simian sarcoma-associated virus (SSAV), and a cell-derived (v-sis) insertion sequence. By comparison with the sequence of Moloney murine leukemia virus, it was possible to precisely localize and define sequences contributed by SSAV during the generation of SSV. Comparative sequence analysis of SSV and SSAV showed that SSAV provides regulatory sequences for initiation and termination of transcription of the SSV-transforming gene. Moreover, coding sequences for the putative protein product of this gene appear to initiate from the amino terminus of the SSAV env gene. Antibodies to synthetic peptides derived from the carboxy and amino termini of the putative protein predicted by the open reading frame identified within v-sis specifically detect a 28,000 dalton protein, p28^{sis}, in SSV-transformed cells. These and other findings confirm the predicted amino acid sequence of this protein and localize it to the coding region of the SSV-transforming gene.

Clonal BALB/c mouse epidermal keratinocyte (BALB/MK) cell lines were established in tissue culture. Despite their aneuploid nature, the lines were nontumorigenic, and retained in vitro properties similar to those of primary diploid keratinocytes. These included the constitutive expression of keratin and terminal differentiation in response to a calcium concentration greater than 1.0 mM in the medium. The cells also demonstrated an absolute requirement for nanomolar concentrations of epidermal growth factor (EGF) for their proliferation. BALB or Kirsten murine sarcoma viruses are acute transforming retroviruses, which have been shown to transform fibroblastic and hematopoietic cells. Infection of BALB/MK or its clonal sublines with either virus leads to the rapid acquisition of EGF-independent growth. The cells concomitantly lose their sensitivity to calcium-induced terminal differentiation. Thus, these retroviruses can rapidly confer upon epithelial keratinocytes in culture growth properties that resemble those of malignant epidermoid carcinoma cells.

BALB and Harvey murine sarcoma viruses (MSV) comprise a family of retroviruses whose mouse- and rat-derived onc genes are closely related. These viruses induce sarcomas and erythroleukemias in susceptible animals. An in vitro colony assay that detects transformation of lymphoid cells by A-MuLV was used to demonstrate that BALB- and Harvey-MSV transform a novel hematopoietic cell both in culture and in vivo. Bone marrow colony formation was sarcoma virus-dependent, followed single-hit kinetics, and required the presence of mercaptoethanol in the agar medium. BALB- and Harvey-MSV-induced colonies could be established in culture as continuous cell lines that demonstrated unrestricted self-renewal capacity and leukemogenicity in vivo. The cells had a blast cell morphology and lacked detectable markers of mature cells within the myeloid or erythroid series. They also lacked detectable immunoglobulin μ chain or Thy-1 antigen, markers normally associated with committed cells of the B and T lymphoid lineages, respectively. However, the transformants contained very high levels of terminal deoxynucleotidyl transferase (TdT), an enzyme believed to be specific to early stages within the lymphoid differentiation pathway. This phenotype distinguishes these BALB- and Harvey-MSV transformants from any previously reported hematopoietic targets of transforming retroviruses, including the pre-B lymphoid cell transformed by A-MuLV under identical assay conditions. These newly identified lymphoid progenitor cell transformants may provide an important means of studying early stages of lymphoid ontogeny and the possible role of TdT in lymphoid development.

Snyder-Theilen feline sarcoma virus (ST-FeSV) codes for a protein kinase with specificity for tyrosine residues, properties analogous to those of the transforming gene product of Abelson murine leukemia virus. ST-FeSV was demonstrated to transform murine hematopoietic cells under in vitro assay conditions which detect lymphoid cell transformation by Abelson murine leukemia virus. Bone marrow colony formation was shown to require ST-FeSV, follow single-hit kinetics, and require the presence of mercaptoethanol in the agar medium. ST-FeSV-induced colonies could be established in culture as continuous cell lines that demonstrated unrestricted self-renewal capacity and leukemogenicity in vivo. The hematopoietic blast

cells transformed by ST-FeSV in culture appeared to be at an early stage of B-cell differentiation. They possessed Lyb 2 surface antigens, were dependent on mercaptoethanol for growth, and contained only low levels of TdT. Moreover, a large fraction of the lines synthesized immunoglobulin μ chain in the absence of light chains. Thus, the phenotype of ST-FeSV hematopoietic transformants was indistinguishable from that of the pre-B lymphoblast transformants induced by Abelson murine leukemia virus. These findings indicate that the in vitro functional similarities in the onc gene products of ST-FeSV and Abelson murine leukemia virus may reflect a common pathway by which they exert their oncogenic potential.

The primate cell-derived transforming gene (v-sis) of SSV is represented as a single copy marker within cellular DNAs of mammalian species, including human. The human analogue of v-sis can be distinguished from its rodent counterparts by Southern blotting analysis of Eco RI-restricted DNAs. By testing for the presence of the human v-sis-related fragment, c-sis (human) in somatic cell hybrids possessing varying numbers of human chromosomes, as well as in segregants of such hybrids, it was possible to assign c-sis to human chromosome 22.

Human DNA was analyzed for the presence of sequences homologous to the transforming gene (v-mos) of Moloney-MSV. A single 2.5-kbp Eco RI-generated fragment of human DNA was identified by using cloned v-mos as probe. This DNA was molecularly cloned in a bacteriophage vector. By heteroduplex and restriction enzyme analyses, this human DNA fragment, designated c-mos (human), contained a 0.65-kbp region of continuous homology with v-mos and was present as a single copy in human DNA. By testing for the presence of c-mos (human) in somatic cell hybrids possessing various numbers of human chromosomes, as well as in subclones of such hybrids, it was possible to assign c-mos (human) to human chromosome 8.

The development of DNA-mediated gene transfer techniques has made it possible to identify transforming genes present in certain human tumor cells. Such genes have been shown to induce morphological transformation when used to transfect suitable assay cells. Recently, a transforming gene has been isolated by molecular cloning techniques from the T24 and EJ human bladder carcinoma cell lines. This bladder carcinoma oncogene has been shown to be of human origin, less than 6-kbp in size and closely related to the onc genes (v-bas and v-ras) of BALB- and Harvey-MSVs. These transforming retroviruses arose in nature by transduction of cellular genes from mouse and rat cells, respectively. To understand better the relationship of the T24 oncogene with other human cellular genes, we have determined the chromosomal location of its normal allele within the human genome, and showed that it is carried on chromosome 11 in normal cells.

The consistent appearance of specific chromosomal translocations in human Burkitt's lymphomas and murine plasmacytomas has suggested that these translocations might play a role in malignant transformation. We have demonstrated that transformation of these cells is frequently accompanied by the somatic rearrangement of a cellular analogue of an avian retrovirus transforming gene, c-myc. Moreover, c-myc was mapped to chromosome 8 band q24. This chromosomal segment is involved in the reciprocal Burkitt's translocations [t(8;14), t(8;22) and t(2;8)]. In two t(8;14) human Burkitt's cell lines, c-myc appears to have been translocated directly into a DNA restriction fragment that also encodes the immunoglobulin μ chain gene. In the case of a specific cloned fragment of DNA derived from a mouse plasmacytoma, we demonstrated directly that c-myc has been translocated into the immunoglobulin α switch region. Our data provide a molecular basis for considering the role that specific translocations might play in malignant transformation.

A transforming gene isolated from T24 human bladder carcinoma cells is closely related to the BALB-MSV onc gene (v-bas). This transforming gene is localized to a 4.6-kbp region and expressed as a 1.2-kbp polyadenylated transcript, which contains v-bas-related sequences. Moreover, antisera known to detect immunologically related onc gene products of BALB- and Harvey-MSVs recognized elevated levels of a related protein in T24 cells. The normal human homologue of v-bas was found to be indistinguishable from the T24 oncogene by heteroduplex and restriction enzyme analysis. These results imply that rather subtle genetic alterations have led to the activation of the normal human homologue of v-bas as a human transforming gene.

The identification and isolation of oncogenes capable of inducing malignant transformation on transfection into rodent cells from human tumor cells has opened the possibility of deciphering the processes involved in human carcinogenesis. With the exception of three human lymphomas, the human transforming genes so far identified have been detected in established tumor cell lines, raising the possibility that they might have been activated during in vitro manipulation of the cells. However, unmanipulated human solid tumors, including carcinomas of the colon (two), lung, and pancreas, and an embryonal rhabdomyosarcoma possessed a common oncogene which, like that isolated from human LX-1 lung carcinoma cells, shares sequences with the onc gene of the Ki-MSV. We also demonstrated that several other human tumor cell lines, including those established from carcinomas of the colon (A2233), lung (A427 and A2182), gall bladder (A1604), and urinary bladder (A1698), possessed the same oncogene. Thus, a variety of human tumors, regardless of their clinical manifestations, contain a common transforming gene related to the onc gene of a transforming retrovirus.

Twenty-two human hematopoietic tumors and tumor cell lines were surveyed for sequences capable of transforming NIH/3T3 cells by DNA transfection. A primary human acute myelogenous leukemia, a chronic myelogenous leukemia cell line and cell lines derived from three independent acute lymphocytic leukemias demonstrated oncogenes capable of conferring the transformed phenotype to NIH/3T3 cells through serial cycles of transfection. One of three transforming genes associated with acute lymphocytic leukemia cells (classified as thymocyte developmental stage II) was identified as the activated cellular homologue of the Ki-MSV virus onc gene, kis, a member of the ras family of onc genes. A transforming gene, which was demonstrated to be common to several human myeloid and lymphoid tumor cells, was shown to be a distantly related member of the ras gene family. Thus, the NIH/3T3 transfection assay commonly detects related oncogenes in human hematopoietic tumor cells. Moreover, the activation of these oncogenes appears to be independent of the specific stage of cell differentiation or tumor phenotype.

A transforming gene related to c-bas/has (human) was cloned in biologically active form from a human lung carcinoma-derived cell line Hs242. Recombinants were constructed between the Hs242 oncogene and its normal allele in order to localize the genetic lesion that led to the acquisition of transforming properties. In vitro transformation assays, coupled with primary nucleotide sequence analysis of these recombinants, revealed that the genetic change that led to the activation of the Hs242 oncogene is a point mutation in the second exon. This point mutation results in the incorporation of leucine instead of glutamine in the 61st amino acid of the predicted protein. No changes were observed in the first exon, the region of c-bas/has in which a point mutation is responsible for activation of the T24 and EJ bladder carcinoma oncogenes. Thus, single amino acid substitutions within different structural domains of the same protooncogene can independently be responsible for its malignant conversion under natural conditions.

DNAs of methylcholanthrene-induced mouse fibrosarcomas were analyzed for transforming activity in the NIH/3T3 transfection assay. Two of four tumors were demonstrated to contain dominant transforming genes, indistinguishable in their pattern of restriction endonuclease resistance to inactivation of biologic activity. By blotting analysis and molecular hybridization with retroviral onc genes, these transforming genes were each shown to be related to the onc gene, kis, of Ki-MSV. The availability of a model system in which a defined carcinogen reproducibly leads to activation of kis as a transforming gene should be of value in elucidating the role of oncogenes in the neoplastic process.

The integrated proviral genome of Rauscher murine leukemia virus was molecularly cloned in a bacteriophage Charon 4A vector after the proviral sequences were enriched by sequential RPC-5 column chromatography and sucrose gradient centrifugation. A recombinant DNA clone, λ-RV-1, possessing a 12-kbp Eco RI insert, was shown to contain the entire 8.8 kbp-pair leukemia virus genome flanked by rat cellular sequences at the 5' and 3' ends. This DNA fragment was biologically active, inducing the release of virion-associated reverse transcriptase activity with as little as 10 ng of DNA insert. The virus induced XC plaque formation at high titers on NIH/3T3 and BALB/3T3 cells and demonstrated identity with the parental virus in radioimmunoassays for the highly type-specific gag gene-coded p12 protein. The molecularly cloned Rauscher murine leukemia virus should be useful in studying the molecular mechanisms involved in the transformation of specific lymphoid target cells by chronic mouse leukemia viruses.

The distribution of endogenous type C RNA viruses was studied in inbred strains of mice and some subspecies of Mus musculus of different geographic origins. The following groups of inducible viruses were characterized by their host range and immunological properties: (1) viruses indistinguishable from one of the three prototype viruses endogenous to BALB/c mice; (2) viruses coding for proteins immunologically related to different prototype endogenous viruses; and (3) viruses whose p12 structural proteins were immunologically indistinguishable from that of BALB:virus-2, but whose p30 major structural proteins and envelope glycoproteins differed immunologically from those of previously characterized endogenous viruses. These findings suggest that endogenous viruses have undergone numerous genetic interactions during the process of evolution leading to inducible viruses of present day mouse strains. A class of xenotropic virus spontaneously released by NZB mice is endogenous to, but not inducible from, embryo cells of other previously studied mouse strains. Viruses which could not be distinguished from the NZB xenotropic virus by host-range analysis or radioimmunological techniques were chemically inducible from embryo cells of several mouse strains originating in Asia and Europe. These results indicate that the biological regulatory mechanisms that affect expression of this virus have evolved differently in such strains from control mechanisms that developed in standard inbred strains.

The closed circular form of the endogenous squirrel monkey type D retrovirus (SMRV) was molecularly cloned in a bacteriophage vector. The restriction map of the biologically active clone was determined and was found to be identical to the parental SMRV linear DNA, except for the deletion of one LTR. Restriction enzyme analysis and Southern blotting indicated that the SMRV LTR is approximately 300 bp-long. The SMRV restriction map was oriented to the viral RNA by using gene-specific probes from murine mammary tumor virus. Restriction enzyme digests of a variety of vertebrate DNAs were analyzed for sequence homology to SMRV by using the cloned SMRV genome as a probe. Consistent with earlier studies, multiple copies of SMRV were detected in squirrel monkey DNA. Homologous restriction fragments were also detected in the DNAs of other primate species.

Significance to Biomedical Research and the Program of the Institute:

The systems that are being intensively investigated have provided a much better understanding of the biology and biochemistry of malignant transformation. It is felt that a clear understanding of these phenomena will significantly speed progress in the search for causes of human cancer as well as mechanisms involved in neoplastic transformation of human cells.

Proposed Course:

To continue research already in progress in the following major areas: (1) mechanisms of action of mammalian sarcoma and leukemia viruses; (2) regulation and functions of endogenous retroviral sequences in mammalian cells; (3) determination of the role of human homologues of retroviral transforming genes in human neoplasia; and (4) application of basic research advances to the investigation of mechanisms involved in malignant transformation of human cells.

Publications:

Aaronson, S. A.: Unique aspects of the interactions of retroviruses with vertebrate cells: C. P. Rhodes Memorial Lecture. Cancer Res. 43: 1-5, 1983.

Aaronson, S. A., Dunn, C. Y., Ellmore, N. W. and Eva, A.: Retroviral onc genes in human neoplasia. In O'Connor, T. E. and Rauscher, F. J. (Eds.): Oncogenes and Retroviruses: Evaluation of Basic Findings and Clinical Potential. New York, Alan R. Liss, Inc., 1983, pp. 207-222.

Aaronson, S. A., Reddy, E. P., Robbins, K. C., Devare, S. G., Swan, D. C., Pierce, J. H. and Tronick, S. R.: Retroviruses, onc genes and human cancer. In Harris, C. C. and Autrup, H. N. (Eds.): Human Carcinogenesis. New York, Academic Press. (In Press)

Aaronson, S. A., Storch, T. G., Balachandran, R. and Reddy, E. P.: Different hematopoietic target cells for transformation by replication-competent murine leukemia viruses. In Marchesi, V. T. and Gallo, R. C. (Eds.): Progress in Clinical and Biological Research: Differentiation and Function of Hematopoietic Cell Surfaces. New York, Alan R. Liss, Inc., 1982, pp. 251-261.

Chiu, I. M., Andersen, P. R., Callahan, R., Aaronson, S. A. and Tronick, S.R.: Molecular cloning of the unintegrated squirrel monkey retrovirus genome: Organization and distribution of related sequences in primate DNAs. J. Virol. (In Press)

Devare, S. G., Reddy, E. P., Law, D. J., Robbins, K. C. and Aaronson, S. A.: Nucleotide sequence of the simian sarcoma virus genome: Demonstration that its acquired cellular sequences encodes the putative transforming gene product, p28sis. Proc. Natl. Acad. Sci. USA 80: 731-735, 1983.

Eva, A. and Aaronson, S. A.: Frequent activation of c-kis as a transforming gene in fibrosarcomas induced by methylcholanthrene. Science 220: 955-956, 1983.

Eva, A., Tronick, S. R., Gol, R. A., Pierce, J. H., and Aaronson, S. A.: Transforming genes of human hematopoietic tumors: Frequent detection of ras-related oncogenes whose activation appears to be independent of tumor phenotype. Proc. Natl. Acad. Sci. USA. (In Press)

Habara, A., Reddy, E. P. and Aaronson, S. A.: Rauscher-murine leukemia virus: Molecular cloning of infectious integrated proviral DNA. J. Virol. 44: 731-735, 1982.

McBride, O. W., Swan, D. S., Robbins, K. C., Prakash, K. and Aaronson, S. A.: Chromosomal mapping of tumor virus tranforming gene analogues in human cells. In Pearson, M. L. and Sternberg, N. L. (Eds.): Gene Transfer and Cancer 1982. New York, Raven Press. (In Press)

McBride, O. W., Swan, D. C., Santos, E., Barbacid, M., Tronick, S. R. and Aaronson, S. A.: Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome 11. Nature 300: 773-774, 1982.

Merregaert, J. and Aaronson, S. A.: Characterization of inducible type C RNA viruses of mouse strains from different geographic areas. Virology 123: 165-174, 1982.

Naharro, G., Tronick, S. R., Rasheed, S., Gardner, M. B., Aaronson, S. A. and Robbins, K. C.: Molecular cloning of integrated Gardner-Rasheed feline sarcoma virus: Genetic structure of its cell-derived sequence differs from that of other tyrosine kinase coding onc genes. J. Virol. (In Press)

Pierce, J. and Aaronson, S. A.: BALB- and Harvey-MSV transformation of a novel lymphoid progenitor cell. J. Exp. Med. 156: 873-887, 1982.

Pierce, J. H. and Aaronson, S. A.: In vitro transformation of murine pre-B lymphoid cells by Snyder-Theilen feline sarcoma virus. J. Virol. 46: 993-1002, 1983.

Prakash, K., McBride, O. W., Swan, D. C., Devare, S. G., Tronick, S. R. and Aaronson, S. A.: Molecular cloning and chromosomal mapping of a human locus related to the transforming gene of Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 79: 5210-5214, 1982.

Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Aaronson, S. A. and Barbacid, M.: Oncogenes in solid human tumors. Nature 300: 539-542, 1982.

Robbins, K. C., Devare, S. G., Reddy, E. P., and Aaronson, S. A.: In vivo identification of the transforming gene product of simian sarcoma virus. Science 218: 1131-1133, 1982.

Santos, E., Tronick, S., Aaronson, S. A., Pulciani, S. and Barbacid, M.: The T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature 298: 343-347, 1982.

Srinivasan, A., Dunn, C. Y., Yuasa, Y., Devare, S. G., Reddy, E. P. and Aaronson, S. A.: Abelson murine leukemia virus: Structural requirements for transforming gene function. Proc. Natl. Acad. Sci. USA 79: 5508-5512, 1982.

Swan, D. C., McBride, O. W., Robbins, K. C., Keithley, D. A., Reddy, E. P. and Aaronson, S. A.: Chromosomal mapping of the simian sarcoma virus onc gene analogue in human cells. Proc. Natl. Acad. Sci. USA 79: 4691-4695, 1982.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S. A. and Leder, P.: Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt's lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. USA 79: 7837-7841, 1982.

Weissman, B. E. and Aaronson, S. A.: BALB and Kirsten murine sarcoma viruses alter growth and differentiation of EGF-dependent BALB/c mouse epidermal keratinocyte lines. Cell 32: 599-606, 1983.

Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. S., Reddy, E. P. and Aaronson, S.A.: Acquisition of transforming properties by alternative point mutations within c-bas/has human protooncogene. Nature. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04941-11 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biochemical Characterization of Retroviruses and onc Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Steven R. Tronick Head, Recombinant DNA Unit, LCMB, NCI

COOPERATING UNITS *(if any)*
Dr. M. Baluda, University of California, Los Angeles, CA

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to biochemically characterize retroviruses and retroviral and cellular onc genes in order to understand the mechanisms by which these viruses and cellular genes induce cancers. The role of these sequences in the etiology of human cancers is also under study. Studies currently in progress are the following: (1) biochemical characterization of replication-defective mammalian transforming viruses; (2) biochemical characterization of new isolates of retroviruses; (3) search for the presence of retroviral genes and gene products in human tumors; and (4) characterization of human onc genes in normal and malignant human cells.

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

Stuart Aaronson	Chief	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI
Alessandra Eva	Visiting Associate	LCMB, NCI
Keith Robbins	Expert	LCMB, NCI
Ing-Ming Chiu	Visiting Fellow	LCMB, NCI
Robert Callahan	Microbiologist	LTIB, NCI
Rose Gol	Visiting Fellow	LCMB, NCI
Y. Yuasa	Visiting Fellow	LCMB, NCI
David Swan	Expert	LCMB, NCI
G. Franchini	Visiting Scientist	LTCB, NCI
W. McBride	Microbiologist	LMB, NCI

Objectives:

1. To biochemically characterize mammalian transforming retroviruses.
2. To biochemically characterize cellular analogues of viral onc genes.
3. To biochemically characterize new isolates of retroviruses.
4. To determine the mechanisms of oncogenesis by retroviruses in their natural hosts.
5. To determine whether or not retroviruses and cellular onc genes play a role in the etiology of cancers of humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; molecular hybridization techniques to detect and characterize viral genomes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of cloned DNAs by restriction endonuclease mapping, nucleotide sequencing, and electron microscopy.

Major Findings:

1. Human DNA sequences related to the ras gene family were isolated from bacteriophage libraries. Clones representing c-K-ras-1 and c-K-ras-2 sequences were obtained. Probes prepared from these clones were used to analyze NIH/3T3 cells transformed by human tumor cell DNAs. It was shown that the c-K-ras-2 but not c-K-ras-1 was activated as a transforming gene in both solid and hematopoietic tumors. A new member of the human ras gene family was also isolated. Because of similarities to a transforming gene isolated from a human neuroblastoma cell line by other workers, this gene was designated c-N-ras. This clone was characterized structurally and probes prepared from it were used to screen transfected human tumor DNAs. The N-ras probe detected related sequences in transfectants that previously failed to hybridize with any other onc gene probes. This study demonstrated the presence of N-ras genes in several different types of hematopoietic tumors,

carcinomas, and sarcomas. Furthermore, it was demonstrated that different ras genes can be activated in the same human tumors and that different human tumors can possess the same activated ras gene. In collaborative studies, these and other ras probes were used to localize c-H-ras-1, c-K-ras-1, c-K-ras-2, and c-N-ras to human chromosomes 11, 6, 12, and 1, respectively.

2. The human myb gene analogue was cloned and structurally characterized in collaboration with G. Franchini. Five myb-related regions, corresponding to 900 bp of v-myb sequences, were localized within an 8.0-kbp stretch of human DNA. The clones isolated represented 35 kbp of the human genome and probes prepared from various parts of this region were used to analyze myb gene expression in the MOLT-4 human cell line. A 4.0-kbp transcript was detected with a v-myb probe and also by probes spanning 35 kbp of human DNA, suggesting that the c-myb locus contains coding sequences in addition to those found in AMV.

3. Probes prepared from the human myc analogue were used to map this oncogene to human chromosome 8 (q24) and to demonstrate that in Burkitt's lymphoma myc gene locus is translocated to chromosome 14 in juxtaposition to immunoglobulin sequences.

4. The genome of SMRV was molecularly cloned and characterized. The sequences related to SMRV were demonstrated to be present in squirrel monkey DNA and in other vertebrates, including humans. By using molecular hybridization techniques, the genetic relationship of SMRV to the pol gene of mammalian types A and B and avian type C retroviruses was demonstrated, whereas env gene homologies between SMRV and mammalian type C viruses were detected. Homologies between SMRV and two classes of human endogenous retroviral genomes were also demonstrated. Nucleotide sequence analysis of SMRV is in progress.

Significance to Biomedical Research and the Program of the Institute:

Highly sensitive and specific biochemical probes for retroviral gene products and cellular onc genes generated in our studies have been extremely valuable in order to demonstrate the possible etiologic involvement of these viral and cellular genes in human cancers, and to study the role that retroviral gene products and cellular onc genes may play in normal cellular functions. The availability of molecularly cloned human gene fragments related to viral onc genes has greatly facilitated studies on the role these sequences play in the causation of human cancer.

Proposed Course:

A detailed biochemical and biological analysis of molecularly cloned human DNA analogues of retroviral onc genes will continue.

Recombinant DNA techniques are being applied and further developed to study other mammalian transforming viruses and cellular genes involved in transformation.

Publications:

Aaronson, S. A., Reddy, E. P., Robbins, K. C., Devare, S. G., Swan, D. C., Pierce, J. H. and Tronick, S. R.: Retroviruses, onc genes and human cancer. In Harris, C. C. and Autrup, H. N. (Eds.): Human Carcinogenesis. New York, Academic Press. (In Press)

Callahan, R., Drohan, W., Tronick, S. and Schlom, J.: Detection and cloning of human DNA sequences related to the mouse mammary tumor virus genome. Proc. Natl. Acad. Sci. USA 79: 5503-5517, 1982.

Chiu, I. M., Andersen, P. R., Aaronson, S. A. and Tronick, S. R.: Molecular cloning of unintegrated squirrel monkey retrovirus genome: Organization and distribution of related sequences in primate DNAs. J. Virol. (In Press)

Eva, A., Robbins, K. C., Andersen, P. R., Srinivasan, A., Tronick, S. R., Reddy, E. P., Ellmore, N. W., Galen, A. T., Lautenberger, J. A., Papas, T. S., Westin, E. W., Wong-Staal, F., Gallo, R. C. and Aaronson, S. A.: Transcription of retrovirus onc genes and analogues in human solid tumor cells. In Yohn, D. S. and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research 1981. New York/Amsterdam, Elsevier Biomedical, 1982, pp. 381-382.

Eva, A., Tronick, S. R., Gol, R. A., Pierce, J. H. and Aaronson, S. A.: Transforming genes of human hematopoietic tumors: Frequent detection of related oncogenes whose activation appears to be independent of tumor phenotype. Proc. Natl. Acad. Sci. USA. (In Press)

McBride, O. W., Swan, D. C., Santos, E., Barbacid, M., Tronick, S. R. and Aaronson, S. A.: Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome 11. Nature 300: 773-774, 1982.

Naharro, G., Tronick, S., Rasheed, S., Gardner, M., Aaronson, S. and Robbins, K.: Molecular cloning of integrated Gardner-Rasheed feline sarcoma virus: Genetic structure of its cell-derived sequence differs from that of other tyrosine kinase coding onc genes. J. Virol. (In Press)

Prakash, K., McBride, O. W., Swan, D. C., Devare, S. G., Tronick, S. R. and Aaronson, S. A.: Molecular cloning and chromosomal mapping of a human locus related to the transforming gene of Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 79: 5210-5214, 1982.

Santos, E., Tronick, S., Aaronson, S. A., Pulciani, S., and Barbacid, M.: The T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature 298: 343-347, 1982.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S. and Leder, P.: Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. USA 79: 7837-7841, 1982.

Westin, E., Wong-Staal, F., Gelmann, E., Baluda, M., Papas, T., Eva, A., Reddy, E. P., Tronick, S., Aaronson, S. A. and Gallo, R. C.: Expression of the abl, myc and amv genes in human hematopoietic cells. In Yohn, D. S. and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research 1981. New York/Amsterdam, Elsevier Biomedical, 1982, pp. 405-406.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04948-08 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Isolation and Characterization of Transforming Genes from Human Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
M. Barbacid Visiting Scientist, LCMB, NCI

COOPERATING UNITS *(if any)*
J. DiPaolo, LB, NCI; A. Puga, LOM, NIDR; E. Farber, U. Toronto, Toronto, Canada

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have successfully transmitted the malignant phenotype from tumor to morphologically normal cells via transfection with discrete fragments of tumor DNA. This experimental approach has allowed us to detect the presence of transforming genes in a variety of human tumors and human tumor cell lines. They include carcinomas of the bladder, colon, gall bladder, liver, lung and pancreas, as well as in a fibrosarcoma and an embryonal rhabdomyosarcoma. The transforming gene present in T24 cells in a human bladder carcinoma cell line has been isolated by molecular cloning techniques. We have demonstrated that it represents the human homologue of the oncogene present in the Harvey and BALB strains of murine sarcoma viruses. Finally, we have established that the T24 bladder oncogene is identical to its normal allele present in all human cells except for a single nucleotide, the basic chemical unit of the DNA molecule. Thus, a single mutational event is responsible for the malignant activation of this human transforming gene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

S. Pulciani	Visiting Fellow	LCMB, NCI
E. Santos	Visiting Fellow	LCMB, NCI
V. Notario	Visiting Associate	LCMB, NCI
S. Sukumar	Guest Worker	LCMB, NCI
J. C. Lacal	Guest Worker	LCMB, NCI
D. Martin-Zanca	Visiting Fellow	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI
S. A. Aaronson	Chief	LCMB, NCI

Objectives:

1. To determine the incidence of transforming gene activation in human tumors;

2. To isolate by molecular cloning techniques human transforming genes;

3. To establish the mechanism of activation of these human oncogenes;

4. To characterize their transcriptional and translational products;

5. To determine the role of p21, the gene product of a variety of human transforming genes, on the biochemical processes that lead to malignant transformation;

6. To search for alternative assay systems that will allow the detection of human oncogenes other than those of the *ras* family;

7. To develop in vitro and in vivo model systems in which transforming genes can be induced in a specific and reproducible manner; and

8. To investigate which of the multiple steps needed for the induction of malignancy involves the activation of oncogenes.

Methods Employed:

The following techniques have been utilized:

1. DNA-mediated gene transfer (transfection) utilizing NIH/3T3 as the recipient cells;

2. Tumorigenicity assays, such as growth in semisolid media and tumor formation in suitable animals;

3. Southern and northern analysis of DNA and RNA molecules, respectively;

4. Molecular cloning of defined DNA segments, utilizing both λ and λ10J9 vectors;

5. Selecting of defined cloned DNA fragments in pBR322;

6. DNA sequence analysis following the Maxam and Gilbert protocol; and

7. Immunoprecipitation and SDS-polyacrylamide analysis of the gene products of human transforming genes.

Major Findings:

1. A transforming gene isolated from T24 human bladder carcinoma cells is closely related to the BALB murine sarcoma virus (MSV) onc gene (v-bas). This transforming gene is localized in a 4.6-kilobase pair (kbp) region and is expressed as a 1.2 kbp-polyadenylated transcript, which contains v-bas-related sequences. Moreover, antisera known to detect the immunologically related onc gene products of BALB- and Harvey-MSVs recognized elevated levels of a related protein in T24 cells. The normal human homologue of v-bas was found to be indistinguishable from the T24 oncogene by heteroduplex and restriction enzyme analysis. These results imply that rather subtle genetic alterations have led to the activation of the normal human homologue of v-bas as a human transforming gene.

2. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. The genetic change that leads to the activation of the oncogene in T24 human bladder carcinoma cells is shown to be a single point mutation of deoxyguanosine into thymidine. This substitution results in the incorporation of valine instead of glycine as the twelfth amino acid residue of the T24 oncogene-encoded p21 protein. Thus, a single amino acid substitution appears to be sufficient to confer transforming properties on the gene product of the T24 human bladder carcinoma oncogene.

3. The identification and isolation of oncogenes capable of inducing malignant transformation on transfection into rodent cells from human tumor cells has opened the possibility of deciphering the processes involved in human carcinogenesis. With the exception of three human lymphomas, the human transforming genes so far identified have been detected in established tumor cell lines, raising the possibility that they might have been activated during in vitro manipulation of the cells. However, we have found that unmanipulated human solid tumors, including carcinomas of the colon (two), lung, and pancreas, and an embryonal rhabdomyosarcoma, also contain dominant transforming genes. The carcinomas of the lung and pancreas and the rhabdomyosarcoma possessed a common oncogene which, like that isolated from human LX-1 lung carcinoma cells, shares sequences with the onc gene of the Kirsten strain of MSV. We have also shown that several other human tumor cell lines, including those established from carcinomas of the colon (A2233), lung (A427 and A2182), gall bladder (A1604), and urinary bladder (A1698), possess the same oncogene. Thus, a variety of human tumors, regardless of their clinical manifestations, contain a common transforming gene.

4. We have localized the normal allele of the T24 bladder carcinoma oncogene to human chromosome 11. The development of DNA-mediated gene transfer techniques has made it possible to identify transforming genes present in certain human tumor cells. Such genes have been shown to induce morphological transformation when used to transfect suitable assay cells. Recently, a transforming gene has been isolated by molecular cloning techniques from the T24 and EJ human bladder carcinoma cell lines. This bladder carcinoma oncogene has been shown to be of human origin, less than 6 kilobase pairs (kbp) in size, and closely related to the onc genes (v-bas and v-ras) of BALB- and Harvey-MSVs. These transforming retroviruses arose in nature by transduction of cellular genes from mouse and rat cells, respectively. To understand better the relationship of the T24 oncogene with other human cellular genes, we have determined the chromosomal location of its normal allele within the human genome. We have established that it is carried on chromosome 11 in normal cells.

5. We have recently shown that malignant activation of the c-has/bas protooncogene in T24 human bladder carcinoma cells was mediated by a single point mutation. A deoxyguanosine located at position 35 of the first exon of this protooncogene was substituted by thymidine. These findings predicted that the resulting oncogene would code for a structurally altered p21 protein containing valine instead of glycine as its twelfth amino acid residue. We now report the spontaneous activation of the human c-has/bas protooncogene during transfection of NIH/3T3 cells. As in T24 cells, this in vitro-activated oncogene also acquired malignant properties by a single point mutation. In this case, we have detected a G → A transition, which occurred at the same position as the mutation responsible for the activation of the T24 oncogene. These results predict that the p21 protein coded for by the spontaneously activated c-has/bas gene will incorporate aspartic acid as its twelfth amino acid residue. Computer analysis of the secondary structure of c-has/bas-coded p21 proteins indicates that substitution of the glycine residue located at position 12, not only by aspartic acid or valine, but also by any other amino acid, would result in the same structural alteration. These findings indicate that a specific conformational change is sufficient to confer transforming properties to this p21 protein. Moreover, they predict that any mutation affecting the coding properties of the twelfth codon of the c-has/bas protooncogene will lead to its malignant activation.

Significance to Biomedical Research and the Program of the Institute:

The identification of transforming genes in human tumors represents a significant landmark in the understanding of carcinogenesis. Moreover, the isolation of these human oncogenes, as well as their normal counterparts, by molecular cloning techniques has made it possible to establish the molecular events that led to the acquisition of their malignant properties. Our findings that a single point mutation is responsible for the transforming properties of a human bladder carcinoma oncogene have been described by Nature as "one of the most startling discoveries so far in the big and frustrating reach for an understanding of cancer." These scientific

developments have been covered by the major newspapers and magazines of the United States as well as of other countries, including England, West Germany, Spain, France, Japan, etc.

Proposed Course:

1. To investigate whether these oncogenes play a dominant role in tumor development;

2. To determine what stage in tumor development involves the activation of oncogenes;

3. To search for human oncogenes that are not members of the ras gene family;

4. To establish in vivo and in vitro model systems (chemical carcinogenesis) in which oncogenes can be activated in a reproducible and specific manner, in order to establish their role in the development of neoplasia; and

5. To understand the normal and transforming roles of p21, the gene product of the ras family of human transforming genes.

Publications:

McBride, O. W., Swan, D. C., Santos, E., Barbacid, M., Tronick, S. R. and Aaronson, S. A.: Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome 11. Nature 300: 773-774, 1982.

Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Aaronson, S. A. and Barbacid, M.: Oncogenes in solid human tumors. Nature 300: 539-542, 1982.

Pulciani, S., Santos, E., Lauver, A., Long, L. K. and Barbacid, M.: Transforming genes in human tumors. J. Cell. Biochem. 20: 51-61, 1982.

Rapp, U. R. and Barbacid, M.: Activation of a type C virus particle in cells from the inbred mouse strain 129/J: Antigenic relationship with the horizontally transmitted type C viruses of primates. Arch. Virol. (In Press)

Reddy, E. P., Reynolds, R. K., Santos, E. and Barbacid, M.: A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature 300: 149-152, 1982.

Santos, E., Pulciani, S. and Barbacid, M.: Characterization of a human transforming gene isolated from T24 bladder carcinoma cells. Fed. Proc. (In Press)

Santos, E., Reddy, E. P., Pulciani, S., Feldmann, R. and Barbacid, M.: Spontaneous activation of a human protooncogene. Proc. Natl. Acad. Sci. USA. (In Press)

Santos, E., Tronick, S., Aaronson, S. A., Pulciani, S. and Barbacid, M.: The T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature 298: 343-347, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP04950-08 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of SSV-Encoded onc Gene and Its Cellular Homologue in Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
S. G. Devare Visiting Associate, LCMB, NCI

COOPERATING UNITS *(if any)*
Smith, Kline and French Labs., Philadelphia, PA; Mol. Genet. Div. Ctr. for Blood Res. and Dept. Nutr., Harvard U. Sch. Pub. Hlth., Boston, MA; Div. Biol., Calif. Inst. Tech., Pasadena, CA

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The primary nucleotide sequence of the biologically active proviral genome of simian sarcoma virus (SSV) has been determined in order to understand the molecular mechanisms of transformation by this only known primate retrovirus with oncogenic potential. These studies have revealed that the cellular oncogenic sequences acquired by SSV are activated in the virus-transformed cells by virtue of their regulatory sequences for initiation and termination of transcription and translation provided by the retrovirus. The availability of predicted amino acid sequences for the SSV transforming gene, as deduced from nucleotide sequence analysis, and its comparison to proteins with known amino acid sequences of proteins with known cellular function, made it possible to demonstrate that the SSV transforming gene shares primary structural homology with a potent mitogen, a platelet-derived growth factor (PDGF). These results, thus, constitute the first demonstration of an onc gene product encoded by a retrovirus which has been identified to correspond to a cellular gene with known function. Previous studies from our laboratory have detected SSV onc gene transcripts in specific human malignancies, such as fibrosarcomas, osteosarcomas, and glioblastomas. These findings corroborate the present finding that the SSV onc gene may encode for a protein with mitogenic activity similar to PDGF which exerts its growth-promoting activity on fibroblasts, tumor muscle cells, and glial cells. These results suggest that malignant transformation by the SSV onc gene or its cellular homologues, in certain instances, may involve constitutive expression of a protein which could act as a potent mitogen similar to PDGF.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

S. R. Tronick	Head, DNA Recombinant Unit	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI
K. C. Robbins	Expert	LCMB, NCI
K. Prakash	Visiting Fellow	LCMB, NCI
A. Srinivasan	Visiting Associate	LCMB, NCI
S. A. Aaronson	Chief	LCMB, NCI

Objectives:

To study genomic organization and regulatory signals of replication-defective mammalian transforming retroviruses in order to evaluate the role of their acquired cellular sequences in oncogenesis. To express specific oncogenes in procaryotic systems to obtain transforming gene products in large amounts to study functional aspects of molecular mechanisms of tumorigenesis.

Methods Employed:

Primary nucleotide sequence analysis of molecularly cloned retroviral genes to identify regulatory elements involved in their expression, and construction of plasmid vector systems for efficient expression of eukaryotic genes in procaryotes to obtain transforming gene products in large amounts.

Major Findings:

Nucleotide sequence of the SSV genome: SSV is the only primate virus representative of the class of replication-defective acute transforming retroviruses. This virus arose by recombination of the nondefective simian sarcoma-associated virus (SSAV) with cellular sequences (sis) present within the woolly monkey genome. By deletion mutant analysis, v-sis sequences have been shown to be essential for SSV transforming activity. In an effort to better understand its structural organization, as well as the molecular mechanisms involved in SSV transformation, the primary nucleotide sequence of the cloned integrated SSV genome was determined.

The 5779-bp SSV genome contained 504-bp long terminal repeats (LTRs). By comparison with the sequence of Moloney murine leukemia virus, it was possible to precisely localize and define other sequences contributed by SSAV during the generation of SSV. Sequence comparison revealed that: (a) the entire SSAV gag gene was present in the SSV genome; (b) the polymerase gene has undergone a single large deletion at its amino terminus, leaving a stretch of 882 nucleotides which corresponded to the carboxy terminus of this gene. This sequence also included the overlapping amino terminus of the envelope gene whose reading frame extended into v-sis; and (c) to the right of the 3' v-sis SSAV junction it was possible to identify sequences which showed significant homology with the carboxy terminal region of the MuLV env gene.

Examination of the region of the SSV genome to which its transforming gene has been localized indicated a long open reading frame. This 678 nucleotides-long reading frame was initiated within SSAV sequences to the left of the 5' SSAV v-sis junction and terminated with an ochre codon within v-sis. By sequence comparison with Moloney-MuLV, the open reading frame to the left of v-sis was identified as initiating from the amino terminal region of the SSAV env gene. Antibodies to synthetic peptides derived from the carboxy and amino termini of the putative protein predicted by the open reading frame identified within v-sis specifically detect a 28,000 MW protein, $p28^{sis}$, in SSV-transformed cells. These and other findings confirm the predicted amino acid sequence of this protein and localize it to the coding region of the SSV-transforming gene.

Expression of SSV-transforming gene product in bacteria: Identification of the transforming gene product $p28^{sis}$ provided an important approach in studies to elucidate molecular mechanism(s) of transformation by SSV. Though readily detected by immunoprecipitation, the amount of $p28^{sis}$ synthesized in SSV-transformed rat or marmoset cells was not high enough to be purified from these cells. In order to study the structural and functional properties of $p28^{sis}$ in the process of oncogenesis by SSV, it was essential to obtain this protein in sufficient quantities. The development of recombinant technology has opened new avenues to deal with this problem. An important approach in these studies has been the use of a multicopy vector system constructed to achieve efficient expression of cloned genes in bacteria. Thus, the sis gene sequences derived from the molecularly cloned SSV genome were ligated to a bacterial plasmid, developed by A. J. Shatzman and M. Rosenberg, which served as the multicopy vector system. This plasmid is constructed in such a way that it contains a very strong promoter and translation initiation site from bacteriophage lambda. The sis gene is thus under the regulation of a bacterial promoter and can be manipulated by the temperature-sensitive repressor carried by the host Escherischia coli AR58, a lambda cI578 lysogen. Using this system, it was possible to translate $p28^{sis}$ efficiently in bacteria. The amount of protein synthesized can be easily visualized by labelling $p28^{sis}$ with ^{35}S-methionine, since it constitutes the major protein synthesized during induction. Further, identity of the protein synthesized by bacteria, using the sis expression plasmid construct, was confirmed by immunoprecipitation with antibodies to amino and carboxy terminal synthetic peptides of $p28^{sis}$. The specificity of immunoprecipitation was demonstrated by inhibition of precipitation of $p28^{sis}$ when antisera were preincubated with either amino or carboxy terminal peptides. It should now be possible to obtain $p28^{sis}$ in large amounts to study its functional properties and role in transformation.

Significance to Biomedical Research and the Program of the Institute:

SSV is the only known retrovirus of primate origin with transforming potential. The nucleotide sequence analysis of the proviral genome is of importance for understanding the molecular mechanism of transformation by this retrovirus. The studies on the transforming gene of SSV have provided an invaluable approach toward understanding the role of oncogenes in the etiology of human cancer, since these onc gene sequences are conserved

among vertebrates. Moreover, the studies in our laboratory have demonstrated that the SSV-transforming gene is transcribed in certain human malignancies, like fibrosarcomas, osteosarcomas, and glioblastomas. Recently, in collaboration with R. Doolittle, Michael Hunkapillar and H. Antoniades, we have found that the predicted amino acid sequence of the transforming gene product encoded by SSV has a very high degree of relatedness with the platelet-derived growth factor (PDGF), a potent mitogen for fibroblasts. Though the complete amino acid sequence for PDGF has not been deduced to date, in one peptide, 87% correspondence in the amino acid sequence was observed. This demonstration that PDGF and the SSV-transforming protein are closely related suggests that malignant transformation by SSV may involve constitutive expression of a protein which could act as a potent mitogen similar to PDGF. It is of interest to note that the primary targets of the PDGF for its growth-promoting activity are fibroblasts, smooth muscle cells, and glial cells. It is in the malignant cells of humans where SSV-specific transcripts have been detected in our laboratory. Thus, this constitutes the first demonstration of an onc gene product corresponding to a cellular gene with known function. These studies are of special significance and will have far-reaching consequences in understanding the mechanisms of human malignancies.

Proposed Course:

The molecular mechanism of transformation by a viral onc gene and its cellular homologue represents a comprehensive approach toward understanding the process of human malignancies. The studies on SSV provide a unique system for such research efforts, in view of the present findings that the onc gene of SSV shows a close amino acid sequence homology with a potent mitogen, PDGF. The development of a bacterial expression system provides an important opportunity to study the SSV onc gene product for its correlation with PDGF. Studies will be directed toward evaluating the biological activity of the SSV onc gene product produced in bacteria. In particular, the role of the constitutive expression of the SSV onc gene product in human malignancies will be evaluated in depth.

Publications:

Aaronson, S. A., Reddy, E. P., Robbins, K. C., Devare, S. G., Swan, D. C., Pierce, J. H. and Tronick, S. R.: Retroviruses, onc genes and human cancer. In Harris, C. C. and Autrup, H. N. (Eds.): Human Oncogenesis. New York, Academic Press. (In Press)

Deobagkar, D. N., Muralidharan, K., Devare, S. G., Kalghatgi, K. K. and Chandra, H. S.: The mealybug chromosome system I: Unusual methylated bases and dinucleotides in DNA of a Planococcus species. J. Biosci. 4: 513-526, 1982.

Devare, S. G., Reddy, E. P., Law, J. D., Robbins, K. C. and Aaronson, S. A.: Nucleotide sequence of the simian sarcoma virus genome: Demonstration that its acquired cellular sequences encode the putative transforming gene product, p28sis. Proc. Natl. Acad. Sci. USA 80: 731-735, 1983.

Prakash, K., McBride, O. W., Swan, D. C., Devare, S. G., Tronick, S. R. and Aaronson, S. A.: Molecular cloning and chromosomal mapping of a human locus related to the Moloney murine sarcoma virus transforming gene. Proc. Natl. Acad. Sci. USA 79: 5210-5216, 1982.

Robbins, K. C., Devare, S. G., Reddy, E. P. and Aaronson, S. A.: In vivo identification of the transforming gene product of simian sarcoma virus. Science 218: 1131-1133, 1982.

Srinivasan, A., Dunn, C. Y., Yuasa, Y., Devare, S. G., Reddy, E. P. and Aaronson, S. A.: Abelson murine leukemia virus: Structural requirements for transforming gene function. Proc. Natl. Acad. Sci. USA 79: 5508-5512, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04951-07 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunological Characterization of Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. E. Dahlberg Microbiologist, LCMB, NCI

COOPERATING UNITS *(if any)*

U. California School of Veterinary Medicine, Davis, CA;
Animal Research Center, Hebrew University, Rehovot, Israel

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hybridoma technology has been utilized to produce monoclonal antibodies to the structural proteins of herpesvirus saimiri. These antibodies have been used to analyze virus structure and replication, and by utilizing both electrophoresis and immunofluorescence, it has been possible to categorize various virus polypeptides into membrane glycoproteins, nuclear antigens, and non-glycosylated structural proteins.

Lentiviruses, a group of retroviruses which cause various diseases, including cancer, in domestic animals, have also been analyzed. Radioimmunoassays to the major structural proteins of several of these viruses have been used to investigate the genetic relatedness between different strains of virus and to provide sensitive assays to assess the virological status of tissues from diseased and experimentally inoculated animals. We have shown that sheep pulmonary adenomatosis virus (SPAV) is closely related to the caprine arthritis-encephalitis virus (CAEV) and to progressive pneumonia virus (PPV) of sheep. Furthermore, equine infectious anemia virus (EIAV) was shown, for the first time, to be distantly related to the sheep and goat retroviruses, CAEV and PPV.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Dharam Ablashi	Microbiologist	LCMB, NCI
Abraham Yaniv	Visiting Scientist	LCMB, NCI
Arnona Gazit	Visiting Fellow	LCMB, NCI Vet. Med., Davis

Objectives:

1. To utilize hybridoma technology to develop monoclonal antibodies useful in characterizing viral structural and nonstructural antigens; to obtain monoclonal antibodies to cellular antigens and/or synthetic oligopeptides which represent proteins associated with malignant transformation.

2. To further characterize, primarily by immunological means, retroviruses of the lentivirus group, and their immunological relatedness to each other and to other retroviruses; to analyze, in collaboration with laboratories in California and Israel, how these viruses are naturally transmitted and are involved in the pathogenesis of disease in their natural hosts; to clone the genome of these viruses to analyze genomic structure, and to facilitate the development of effective vaccines.

Methods Employed:

The methodology required to develop monoclonal antibody-producing hybridoma cell lines includes maintaining mycoplasma-free cell lines, cell fusion techniques, preparation of thymocyte conditioned medium, single cell cloning procedures, and a variety of immunological assays to screen for antibody-producing cells. These include solid phase immunoassays, ELISA tests, and immunoprecipitation followed by analysis on polyacrylamide gels.

The lentiviruses are grown in 2-8 liter batches and purified by a combination of ultrafiltration and isopycnic gradient centrifugation. Purified virus is used both as a source of protein for purification of virion proteins, in order to develop intraspecies and interspecies radioimmunoassays, and as a source of genomic RNA, to allow preparation of cDNA probes to permit cloning of proviral DNA from infected cells.

Major Findings:

1. In collaboration with Dr. Ablashi, a battery of monoclonal antibodies has been obtained which immunoprecipitate a high percentage of the structural antigens of the oncogenic primate herpesvirus, herpesvirus saimiri (HVS). These antibodies have been shown to fall into 11 unique groups, with up to six independent isolates present within a group. Eight of these groups are represented by monoclonal antibodies which precipitate two or more antigens. Although none of these antibodies

are able to neutralize HVS, one has been shown to identify a set of three tunicamycin-sensitive glycoproteins. Fluorescent antibody analysis demonstrates that these antigens are localized in the nuclear membranes of infected but not control cells, suggesting that these glycoproteins are present in an internal virion membrane, and therefore would not be capable of eliciting neutralizing antibody.

An additional monoclonal antibody of interest recognizes antigens present in the nucleus of infected cells, as shown by immunofluorescence, but not in uninfected owl monkey kidney cells. This antibody precipiates two proteins, with molecular weights of about 150,000 and 14,000 daltons, from extracts of infected cells, but does not precipitate significant amounts of either of these two antigens from extracts of radioactive purified virus. We are currently obtaining HVS-transformed nonproducer cells to investigate whether this antibody might be reacting with either a nonstructural viral antigen such as a DNA polymerase, or with a T antigen. As yet, no T antigen has been described for HVS-transformed cells.

The panel of 11 monoclonal antibodies was used to screen a variety of herpesviruses for the presence of related antigens. A roster of the viruses tested included herpes simplex, herpes platyrhinnae, varicella zoster, human cytomegalovirus, herpesvirus ateles, and herpes aotus. None of the monoclonals reacted with any of the antigens of these viruses. Four of the 11 monoclonal antibodies reacted with extracts of cells infected with the OMI strain of HVS (characterized by low infectivity in culture and high tumor incidence in marmosets).

More recently, attempts are being made to produce monoclonal antibodies which recognize selected portions of onc gene proteins. Mice are immunized with chemically synthesized oligopeptides and resultant hybridomas are screened first for reactivity of the peptide and then are tested for their ability to immunoprecipitate the protein of interest. At this time, a number of hybridomas which recognize a peptide representing a portion of the N-terminal region of the BALB-MSV bas gene p21 are being tested. Monoclonal antibodies to such proteins will offer advantages over currently available sera from tumor-bearing rats and from rabbits immunized with the peptide both in terms of specificity and markedly higher titers. This in turn might allow assays such as immunofluorescence to be carried out which are not otherwise possible.

2. The lentiviruses are a subgroup of the retroviruses which appear to principally infect domestic ungulates. All of the known members cause a significant amount of disease and are of great economic importance. This laboratory has been studying the caprine arthritis-encephalitis virus (CAEV), which causes severe mortality and morbidity in young goats, and arthritis in older chronically infected animals. We have also been interested in the equine infectious anemia virus (EIAV) which can cause severe anemia in horses. Within the last year, K. Perk at the Hebrew University, Israel, has described the transmission of pulmonary adenomatosis into young sheep with sucrose gradient banded virus isolated from cell cultures infected with pulmonary tumor extracts. This new isolate, termed

sheep pulmonary adenomatosis virus (SPAV), was shown in this laboratory to be immunologically closely related to CAEV, and less closely related to the progressive pneumonia virus of sheep. This new virus is currently the only lentivirus thought to be capable of inducing cancer. The long latent period suggests that it is not an acutely transforming virus, and it will be of interest to study the mechanism of tumorigenesis in this system. In collaboration with K. Perk, SPAV has been grown and purified in this laboratory. The major virus protein, p28, is currently being purified to homogeneity. This will allow the development of a radioimmunoassay for SPAV that will permit the determination, with great sensitivity, of antigen and antibody levels in diseased animals and tissues, and to follow the consequences of experimental inoculation of virus more accurately than is otherwise possible.

Using a series of intra- and interspecies radioimmunoassays, we have also established that EIAV is distantly related to the other known lentiviruses. Previous to this observation, EIAV had not been shown to be related to any other retrovirus. The distant relationship between EIAV and the other known lentiviruses, which are themselves rather closely related, suggests that EIAV represents a virus that diverged from a common progenitor at a considerably earlier time than the other lentiviruses began to diverge from each other.

In a study with G. Thielen, U. California, Davis, tissue-grown virus was titered and then administered by different routes to newborn seronegative goats. Sera, lymphocytes, synovial fluid, and tissue samples, where appropriate, are being collected weekly and analyzed for CAEV antigen and antibodies by radioimmunoassay. To date, it is evident that the tissue culture adapted virus remains infectious in goats, but it is still too early to determine if this virus will induce disease. Virus freshly isolated from milk samples will be compared biochemically and biologically, and if significant differences in virulence are noted, efforts will be made to determine if the tissue culture adapted virus has value as a vaccine agent.

At the same time, in collaboration with A. Yaniv and A. Gazit, we are cloning the genomes of CAEV and EIAV. Availability of molecularly cloned viral genomes will facilitate a great many of these studies. In particular, it will allow a fresh approach to analyzing strain differences and evolutionary relatedness. We will sequence the glycoprotein genes to permit the synthesis of oligopeptides, which can then be tested for their ability to confer protection to test animals challenged with live virus. This approach has been successfully used to effectively produce protection against foot-and-mouth disease virus, and is preferable over methods involving the potential transfer of genetic material.

Significance to Biomedical Research and the Program of the Institute:

1. Hybridoma technology is rapidly fulfilling earlier promises that it would allow new approaches in immunology, biochemistry, cancer research and other areas that would otherwise be difficult or impossible. We have been using this technique to produce antibodies to a transforming herpesvirus that recognizes many of the structural antigens of the virion, and several nonstructural virus-specified proteins as well. These antibodies are being used to probe the architecture of the virus and the mechanism of how the virus proteins are synthesized and assembled. They will also be made available to other workers interested in HVS and related viruses. In addition, monoclonal antibodies to selected regions of virally derived onc genes are being isolated and tested for their ability to cross react with the transforming protein. These antibodies will be of enormous value in helping to understand how and where transforming genes function.

2. Lentiviruses are retroviruses which cause widespread disease in farm animals. A recent isolate which causes lung tumors (SPAV) is closely related to a goat virus (CAEV) which causes encephalitis and arthritis. It is important not only to understand how SPAV induces tumors, but also to understand why closely related viruses, in the same or different animals, do not. We are interested in developing better and more sensitive assays for these viruses to both facilitate the analysis of virus replication and mechanisms of pathogenesis, and to aid in diagnosis in the field. Cloning of the viral genomes will possibly allow the development of reagents useful as vaccines.

Proposed Course:

1. Analysis of monoclonal antibodies to HVS is nearly completed. One emphasis will be on analyzing how a monoclonal antibody recognizes two or more antigens. Most of the emphasis in this area will be on developing monoclonal antibodies to onc gene products. These will be used to analyze the location and level of the transforming proteins in normal and malignant cells.

2. The radioimmunoassays developed to detect the p28 proteins of the lentiviruses will be used to analyze the mechanisms of transmission and development of disease in experimentally inoculated animals. The viruses will be cloned to permit structural comparison, sequencing of critical portions of the genomes, and development of vaccine reagents.

Publications:

Faggioni, A., Ablashi, D. V., Armstrong, G., Dahlberg, J., Sundar, S. K., Rice, J. M. and Donovan, P. J.: Enhancing effect of N-methyl-N-nitrosoguanidine (MNNG) on Epstein-Barr virus (EBV) replication and comparison of short term and continuous TPA treatment of nonproducer and producer

cells for EBV antigen induction and/or stimulation. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Gazit, A., Yaniv, A., Dvir, M., Perk, K. and Dahlberg, J. E.: Caprine arthritis-encephalitis virus is a distinct virus within the lentivirus group. Virology 124: 192-195, 1983.

Pantazis, P., Pavlidis, N., Demetrakopoulos, G. E. and Dahlberg, J. E.: Morphological changes in cultured human leukemia cells (K 562) treated with the tumor promotor 12-O-tetradecanoylphorbol-13-acetate. Biol. Cell 46: 143-150, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04976-06 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chromatid DNA Repair and Neoplastic Potential of Human Cells in Culture

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

K. K. Sanford Chief, In Vitro Carcinogenesis Section, LCMB, NCI

COOPERATING UNITS *(if any)*

Pathology Dept., Howard U., College of Medicine, Washington, D.C.; Biometry Branch, DCCP, NCI; Computer Systems Laboratory, DCRT, NCI; Cytopathology Lab., Johns Hopkins U. Sch. Med.; FDA/NCDB/OB/DBB

LAB/BRANCH

Laboratory of Cellular and Molecular Biology, NCI

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	1.5	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project is to elucidate through cell culture studies mechanisms of neoplastic transformation in human cells. Current emphasis is on the characterization in cultured human cells of chromatid DNA repair lesions found to be associated with malignant transformation and/or susceptibility to cancer. Since a chromatid apparently contains a single continuous DNA double strand, chromatid breaks and gaps represent unrepaired DNA strand breaks. Last year we reported that human skin fibroblasts transformed to malignant cells in culture had significantly more chromatid breaks and gaps than their normal counterparts when x-irradiated specifically during G_2 phase or within 1.5 hours of metaphase. These observations, together with results from use of DNA repair inhibitors, implicated deficiencies in DNA repair in the malignant cells as we had found previously in mouse fibroblasts transformed in culture. These observations have been extended to cell lines from diverse human tumors, including sarcomas, carcinomas, glioblastomas, etc., as compared with numerous lines of normal fibroblasts. Further, skin fibroblasts from cancer-prone individuals, including those with ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia, Gardner's syndrome, familial polyposis or xeroderma pigmentosum show similar defects in chromatid DNA repair. It thus appears that acquisition of defects in chromatid DNA repair operative during G-2 prophase is a prerequisite, possibly the initiating step, for carcinogenesis in human cells. Another aspect of this project is the development of a transformation system with human epidermal keratinocytes for our further experimental studies. Additionally, computerized image analysis of living cells is being used to identify cytomorphologic markers of neoplastic transformation in culture.

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

R. Gantt	Research Chemist	LCMB, NCI
W. G. Taylor	Research Biologist	LCMB, NCI
F. M. Price	Biologist	LCMB, NCI
R. E. Tarone	Mathematical Statistician	BB, NCI
B. L. Trus	Research Chemist	CSL, DCRT
J. S. Rhim	Microbiologist	LCMB, NCI
D. L. Hogan	Biologist	LMB, NCI

Objectives:

The objective of this project is to elucidate through cell culture studies mechanisms of neoplastic transformation in human cells. Current emphasis is on the characterization in cultured human cells of chromatid DNA repair defects found to be associated with malignant transformation and/or susceptibility to cancer. Efforts are also directed toward developing a transformation system with human epidermal keratinocytes as an in vitro model of human cell carcinogenesis for characterizing changes in DNA repair capacity during neoplastic transformation. Additionally, computerized image analysis of living cells is being used to identify quantifiable cytomorphologic markers of neoplastic transformation as a nondestructive diagnostic tool for use in transfection and transformation studies and also to provide insights into mechanisms of carcinogenic change at the cellular level.

Methods Employed:

Chromatid breaks, gaps and interchanges following low-level x-irradiation (25, 50, 100 rad) or 2 to 5 hour exposure to low-intensity fluorescent light (effective wavelength 405 nm in visible range) are quantified in cells grown on coverslips in sealed Leighton tubes and processed in situ following experimental treatment. DNA repair inhibitors, caffeine and cytosine arabinoside, are used following irradiation to analyze mechanisms. Since repair of lesions is influenced by the stage of the cell cycle, and since the chromatid damage is scored in metaphase cells only, the stage of the cell cycle at the time of irradiation can be experimentally manipulated by varying the interval from treatment to fixation of cells.

In developing a transformation system with normal epidermal keratinocytes, it has been necessary to study in detail the calcium and numerous other requirements for proliferation and differentiation of human as well as mouse epidermal keratinocytes in culture. The plasmid PSV-3GPT has also been transfected to enhance cell cycling in human epidermal cells.

Computerized image analysis is being carried out on photomicrographs taken at high power of individual living normal and neoplastic cells growing in Dvorak chambers. Emphasis is on nuclear structure and cytoplasmic spread.

Major Findings:

The pursuit of this project has led to the following major new findings and accomplishments: Our results from irradiating cells during G_2 phase or within 1.5 hours of metaphase together with use of DNA repair inhibitors implicate deficiences in DNA repair during G_2 prophase in all human tumor cell lines examined (including sarcomas, carcinomas, melanomas, etc.) as compared with numerous lines of normal fibroblasts. Furthermore, skin fibroblasts from cancer-prone individuals, including those with ataxia telangiectasia, Fanconi's anemia, Bloom's syndrome, Gardner's syndrome, familial polyposis or xeroderma pigmentosum show simular defects in chromatid DNA repair during G_2 prophase. Kinetic studies of events during the first 6 hours following a 2-hour light exposure have indicated that one defect is in a stage of excision repair of base damage, possibly in polymerase or ligase activity. X-ray studies with xeroderma pigmentosum group A cells seem to indicate that endonuclease incision of DNA is required for chromatid gap formation. It appears from all of these studies that acquisition of defects in chromatid DNA repair operative during G_2 prophase is a prerequisite, possibly the initiating step, for carcinogenesis in human cells.

We have found that survival and proliferation of human epidermal keratinocytes in mass culture require a significantly higher calcium ion concentration than reported previously for colony growth. Quantitative procedures have been developed for growth of replicate mass cultures of human keratinocytes by use of nuclei isolation and enumeration. Dr. Rhim has successfully transformed cultures of these cells with a hybrid adeno-SV40 virus. Increased cycling of these cells is currently attained by transfection with an SV40 plasmid.

Significance to Biomedical Research and the Program of the Institute:

The discovery of a DNA repair defect(s) in human tumor cells and in skin fibroblasts from individuals genetically predisposed to a high risk of cancer suggests that the acquisition of defects in DNA repair operative during G_2 prophase is prerequisite, possibly the initiation step, for human carcinogenesis. Our cytogenetic assay may also provide a tool to identify individuals at high risk of cancer.

Since most human tumors are carcinomas of epithelial origin, the development of a transformation system with human epidermal keratinocytes provides a useful model for further studies of human cell carcinogenesis.

Proposed Course:

The following studies are planned or in progress:

1. To determine whether the heterozygote condition in those genetic disorders predisposing to a high risk of cancer can be detected by our cytogenetic assay.

2. To assess the genetic basis of DNA repair defects and their association with tumorigenicity of cell lines derived from fusions of malignant with normal human cells.

3. To determine whether human epidermal keratinocytes transfected with the SV40 plasmid are neoplastic and to assess their DNA repair capacities, as well as those of cells transformed by adeno 12-SV40.

4. To complete and analyze results from cytomorphologic studies.

Publications:

Parshad, R., Gantt, R., Sanford, K. K., Jones, G. M. and Tarone, R. E.: Repair of chromosome damage induced by x-irradiation during G_2 phase in a line of normal human fibroblasts and its malignant derivative. J. Natl. Cancer Inst. 69: 404-414, 1982.

Parshad, R., Sanford, K. K. and Jones, G. M.: Chromatid damage following G_{12} phase x-irradiation of cells from cancer-prone individuals implicates deficiency in DNA repair. Proc. Natl. Acad. Sci. USA. (In Press)

Parshad, R., Sanford, K. K., Jones, G. M. and Tarone, R. E.: Neoplastic transformation of human cells in culture associated with deficient repair of light-induced chromosomal DNA damage. Int. J. Cancer 30: 153-159, 1982.

Price, F. M., Taylor, W. G., Camalier, R. F. and Sanford, K. K.: Approaches to enhance proliferation of human epidermal keratinocytes in mass culture. J. Natl. Cancer Inst. 70: 853-861, 1983.

Sanford, K. K. and Evans, V. J.: A quest for the mechanism of "spontaneous" malignant transformation in culture with associated advances in culture technology. J. Natl. Cancer Inst. 68: 895-913, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04977-06 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Damage, Repair, and Neoplastic Conversion in Cultured Mouse and Human Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

R. R. Gantt Research Chemist, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The formation, consequences, and mechanism of repair of DNA-protein crosslinks are being studied in mouse cells and human fibroblasts, including cells from xeroderma pigmentosum (XP) patients and normal individuals. The mechanism of repair of crosslinks induced with 20 μM trans-platinum(II)-diaminedichloride is currently emphasized. It is known that DNA-protein crosslinks are induced by numerous agents, including x-rays, ultraviolet light, visible fluorescent light, and a wide variety of chemical carcinogens such as benzopyrene, methylmethane sulfonate, and AAF. These lesions induced by trans-platinum have been reported to result in transformation of 3T3 and 10T-1/2 mouse cells and they are repaired by the nucleotide excision pathway. Studies to assess the response of rapidly proliferating SV40-transformed XP20S cells and the slowly proliferating untransformed XP20S parental line to transplatinum-induced DNA-protein crosslinks indicate a pathway in addition to the nucleotide excision pathway exists. From use of the metabolic inhibitors cycloheximide and aphidicolin, and from kinetic studies of crosslink repair and DNA replication in mouse L1210 cells and normal human fibroblasts, it appears that this second pathway requires protein synthesis. Further, although activity of this pathway for crosslink repair is enhanced in cells cycling rapidly, DNA replication per se is not essential.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged in this Project:

K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB, NCI
W. G. Taylor	Research Biologist	LCMB, NCI

Objectives:

To identify primary changes in cellular and viral nucleic acids during photochemical, chemical, and viral carcinogenesis and to develop and apply techniques for assaying the repair responses of the cells. The use of human epithelial cells is emphasized where technically feasible and the study of the induction, consequences, and repair of DNA-protein crosslinks is currently stressed.

Methods Employed:

The standard laboratory techniques for measuring single and double strand DNA breaks, repair synthesis, base damage, base adducts, and crosslinks are used with the usual adaptations and innovations.

Major Findings:

Repair of DNA-protein crosslinks. The reagent trans-platinum (trans-platinum(II)diaminedichloride) induces DNA-protein crosslinks. We are investigating the mechanism(s) by which mammalian cells repair these crosslinks. The conditions chosen for these studies minimize cell toxicity to increase the relevance of the data to conditions of chronic exposure. The only known way to repair bulky DNA adducts is by the nucleotide excision pathway. It has been reported, as expected, that excision-deficient XP12BE, a xeroderma pigmentosum (XP), group A, cell line is deficient in repair of DNA-protein crosslinks. However, we have found that eventually XP12BE, as well as other XP groups including C, D, E, and the XP variant are able to repair completely the DNA-protein crosslinks induced by 20 μM trans-platinum. Since the excision capacity of the group A cells has been estimated at less than 2% of normal cells, the eventual repair of DNA-protein crosslinks suggests that another mechanism is involved. Many laboratories have reported enhanced repair of DNA damage in cells which are synthesizing DNA. This finding implicates a coupling of repair to DNA replication and/or the necessity for cell cycling, but doesn't differentiate between a simple enhancement of nucleotide excision repair or the presence of a second pathway. Previous investigations in our laboratory using mouse L1210 cells, normal human skin fibroblasts, and metabolic inhibitors including cycloheximide, α amanitin and aphidicolin showed that extent of DNA replication correlated with extent of DNA-protein crosslink repair, but that replication per se was not essential. Recent experiments with XP group A cells that eliminate the complications of simple nucleotide excision repair in normal cells indicate, again, that replication per se is not required but that a pathway other than nucleotide excision is available to repair the DNA-protein crosslinks at rates equivalent to those in normal cells. When DNA protein crosslinks are

induced with 20 μM trans-platinum in rapidly proliferating SV_{40}-transformed, group A, XP20S cells, the crosslinks are repaired at essentially the same rate as in normal human skin fibroblasts. DNA-protein crosslinks induced in the slowly proliferating untransformed parental line, XP20S, are repaired very slowly, similar to repair rates in XP12BE cells.

Since the SV_{40}-transformed XP20S cells remain highly sensitive to UV light (the hallmark of XP cells), the viral genome has not introduced new information directly affecting nucleotide excision repair capacity. Presumably then, a direct or indirect consequence of cell transformation such as cell cycling and/or a new expression of cell information present before transformation leads to the observed proficiency in DNA-protein crosslink repair. The second pathway cannot be post-replication repair because these experiments measure changes in the parental strand only, rather than daughter strand repair.

The simplest scheme to accommodate our findings at this time is the following: a second pathway exists which can repair trans-platinum-induced DNA-protein crosslinks. This pathway is activated, probably by induction of new protein, sometime prior to S phase in cycling cells in the absence of DNA damage. In the presence of DNA damage, apparently the damage itself, if sufficient, activates this pathway. Cell cycling (as measured by DNA replication) enhances this repair pathway, but DNA replication is not essential.

Significance to Biomedical Research and the Program of the Institute:

Reports of others show that DNA-protein crosslinks (trans-platinum induced) increase sister chromatid exchanges and transform 3T3 and 10T-1/2 mouse cells, observations which indicate important perturbations of DNA. These observations, taken together with our finding that repair of the crosslinks is by two pathways, one of which is cell-cycle dependent, have two important aspects. First, they suggest that DNA-protein crosslinks (which are induced by a wide variety of carcinogens including x-rays, light, and many chemicals such as benzopyrene, methylmethane sulfonate, AAF, etc.) may play a role in epigenetic events leading to malignant transformation. Second, DNA-protein crosslinks may accumulate with time in noncycling cells of animals if repair is dependent solely on cycling. In animals this accumulation would be expected to impair the function of organ systems containing significant numbers of nondividing cells, particularly at the level of mRNA production; a general decline of organ response would ensue.

Proposed Course:

1. Transfect human epithelial cells to obtain rapidly proliferating cells to compare DNA repair capacities with fibroblasts.

2. Develop substrate for a cell-free repair system to study DNA-protein crosslink repair.

3. Determine whether other types of DNA lesions are repaired by the second pathway detailed in this report.

4. Look for the accumulation of DNA-protein crosslinks in animals as a function of age or "deterioration."

Publications:

Parshad, R., Gantt, R., Sanford, K. K., Jones, G. M. and Tarone, R. E.: Repair of chromosome damage induced by x-irradiation during G_2 in normal human fibroblasts and their malignant derivatives. J. Natl. Cancer Inst. 69: 404-414, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04978-06 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Growth, Nutrition, and Neoplastic Transformation of Mammalian Cells In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

W. G. Taylor Biologist, LCMB, NCI

COOPERATING UNITS *(if any)*

B. L. Trus, CSL, DCRT

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The long-term objective of this program is to understand the mechanism(s) of neoplastic transformation through use of mammalial cells in culture. Because of the relevance of epithelial cells to human carcinogenesis, epithelial cell proliferation and functional expression are of interest. Defining environmental conditions for sustained expression of differentiated function in culture is pertinent because of possible relationship between transformation of epithelial cells and changes in differentiated function. Studies include: (a) Impact of environmental conditions on differentiated function in two model systems, maturation or terminal differentiation of mouse epidermal keratinocytes and formation of hemicysts, a manifestation of transepithelial ion and fluid movement, in secreting epithelium. Investigating conditions for rapid proliferation of basal cells and conditions that trigger terminal differentiation, we found that normal human and C3H epidermal keratinocytes in mass culture proliferate more rapidly to higher cell density if calcium ion concentration is increased above levels for optimal clonal growth. Further, limiting protein synthesis by cycloheximide treatment enhances terminal differentiation in mouse keratinocytes, and even at low calcium ion concentration normal and neoplastic mouse keratinocytes maintain a capacity for maturation. In monkey kidney epithelium dissolved oxygen concentration modulates hemicyst formation. Also, oxygen consumption by epithelial cells is significantly greater than that of fibroblasts, a property that lowers the risk of oxidative injury to epithelium in culture. (b) Association of focal adhesion sites to cell shape change characteristic of neoplastic transformation of mouse and human cells in culture. Photomicrographic data are being quantified by computer-assisted morphometric analysis. (c) Analysis of photosensitivity of xeroderma pigmentosum, Group A, cells (XP-A) to low intensity fluorescent light. Exposure of XP-A cells to low intensity fluorescent light (wave lengths >300 nm) caused a greater degree of cell killing and/or cytostasis than observed in normal human fibroblasts.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB, NCI
R. R. Gantt	Research Chemist	LCMB, NCI
F. M. Price	Biologist	LCMB, NCI

Objectives:

The objectives pursued during this year included: (a) assessment of the role of environmental factors on expression of differentiated function, either terminal differentiation by mouse epidermal keratinocytes or trans-epithelial ion transport (and fluid flux) by adult monkey kidney epithelium; (b) computer-assisted morphometric analyses of focal adhesion sites and cell shape changes characteristic of neoplastic transformation; and (c) analysis of photosensitivity of xeroderma pigmentosum Group A human fibroblasts and studies on the repair of transdiaminodichloroplatinum (II)-inducing DNA-protein crosslinks by these excision repair-deficient cells.

Methods Employed:

In this laboratory, methods for propagation of primary or low-passage human epidermal keratinocytes have been developed, including formulation of medium NCTC 168 for use without a feeder layer and methods for quantifying proliferation. Formation of cornified envelopes is assayed by solubilization of keratinocytes in detergent under reducing conditions (1% SDS and 20 mM DTT) followed by heating at 90°C to achieve maximum detergent-action, after which cornified envelopes are enumerated. Morphology of keratinocytes and appearance of hemicysts, a marker of secretory or transport activity by epithelium, is followed by light microscropy and staining procedures. The extent and distribution of focal adhesion sites are recorded by reflection-interference photomicrography and quantified by microdensimetric computerized image analysis. Utilization of dissolved oxygen is determined with a Clark electrode calibrated against standard humidified gas mixtures; for dynamic measurement T-60 pyrex flasks have been modified to allow continuous monitoring of dissolved oxygen by the adherent monolayer. DNA-protein crosslinks in normal and SV-40 transformed human fibroblasts are assayed by the alkaline elution procedure of Kohn, et al. Preliminary transfection studies of NIH/3T3 cells with calcium phosphate-precipitated T24 bladder carcinoma DNA have been initiated to establish a baseline for future studies.

Major Findings:

During the last year, several new findings have been made. We find that normal human and C3H mouse epidermal keratinocytes in mass culture proliferate more rapidly if the calcium ion concentration is increased to levels well above the optimal concentration for clonal growth. However, superior results are achieved in primary cultures of human epidermal keratinocytes if dispersed keratinocytes are initially grown in a clonal growth medium, MCDB 151, and after attachment and early proliferation, a more enriched culture medium is used. We conclude that the balance of nutrients and electrolytes must be adjusted to satisfy the proliferative requirements of a dynamically expanding keratinocyte population. Moreover, cornified envelope formation is most pronounced at high calcium ion concentration, but also occurs at low calcium ion levels, in cultures of neoplastic keratinocytes, and after addition of cycloheximide, suggesting limiting protein synthesis triggers cornified envelope formation. In studies with monkey kidney epithelium, dissolved oxygen is a critical requirement for transepithelial ion transport and fluid flux, manifest by hemicyst formation. Moreover, continuous monitoring of dissolved oxygen tension revealed that oxygen available to the submerged postconfluent monolayer was more rapidly exhausted than by comparable populations of fibroblasts.

The association between cell shape changes during transformation and the sites on the cell undersurface which are in closest proximity to the growth surface (focal adhesion sites) is being studied with mouse and human fibroblasts and mouse keratinocytes. Qualitatively, a greater number of focal adhesion sites are seen with normal, spontaneously transformed, or chemically-transformed fibroblasts than on the undersurface of either SV40-transformed fibroblasts or mouse keratinocytes. In general, the distribution of focal adhesion sites is more random in neoplastic cells. Use of computerized image analysis permits quantification of the area represented by the adhesion sites and also provides an assessment of differences in cell shape.

Growth and cytomorphic studies with UV-sensitive human fibroblasts revealed an in vitro photosensitivity, which suggests either deficiencies in DNA repair after exposure to longer wavelength fluorescent light or a heightened sensitivity to fluorescent light-generated photoproducts. Additional collaborative studies have revealed that repair of trans-diaminodichloroplatinum-induced DNA protein crosslinks need not be coupled to semiconservative DNA synthesis, but may be dependent on cell cycling (see also R. Gantt, Z01CP04977-06).

Significance to Biomedical Research and the Program of the Institute:

Most solid human tumors are carcinomas which develop in epithelium-rich tissues. These studies consider the environmental conditions for continued morphologic integrity, proliferation, or sustained expression of differentiated function as part of the development of a transformation assay. We find that calcium ion and dissolved oxygen are critical environmental variables in proliferation and function, and that epithelial cells utilize dissolved oxygen rapidly. Also, epithelial cells are more resis-

tant to fluorescent light-associated cell injury. Chronic exposure to fluorescence increases the risk of melanoma in humans and is mutagenic and carcinogenic for mouse cells in culture. Since DNA damage is caused by light-generated oxygen radicals, rapid oxygen utilization by epithelium may represent a mechanism for coping with excess oxygen and minimizing potential mutagenic and carcinogenic damage. This form of "cellular defense mechanism" would contribute to the difficulty experienced in this and other laboratories in inducing malignant transformation in human epithelial cells in culture. Studies with epidermal keratinocytes show that neither neoplastic transformation nor limiting calcium ion concentration necessarily abrogate this step in terminal differentiation. Studies with fibroblasts initiated from Group A xeroderma pigmentosum patients, who are genetically predisposed to cancer, revealed a heightened photosensitivity to low intensity fluorescent light, as compared to fibroblasts from normal donors. This may represent an exaggerated sensitivity to toxic photoproducts generated during exposure or a deficiency in repair of insults generated by wavelengths >300 nm.

Proposed Course:

1. Complete morphometric analyses of cell shape differences and quantification of focal adhesion sites in paired nonneoplastic and transformed fibroblasts and keratinocytes.

2. Extend the findings on photosensitivity of xeroderma pigmentosum Group A cells to other complementation groups, and distinguish between enhanced sensitivity to photoproducts and deficient repair of light-mediated cellular DNA damage.

3. Studies on dissolved oxygen are completed and will be phased out as manuscripts are completed.

Publications:

Price, F. M., Taylor, W. G., Camalier, R. F. and Sanford, K. K.: Approaches to enhance proliferation of human epidermal keratinocytes in mass culture. J. Natl. Cancer Inst. 70: 853-861, 1983.

Taylor, W. G.: Serum-independent modulation of hemicyst formation by dissolved oxygen in postconfluent epithelial monolayers. In Vitro. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05056-05 LCMB

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Molecular Studies of Human and Viral Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation): E. P. Reddy Visiting Scientist, LCMB, NCI

COOPERATING UNITS *(if any)*:

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The genetic changes that lead to the activation of two human oncogenes, T-24 and Hs242, have been shown to be point mutations affecting the amino acid composition of these oncogene products. Thus, the change has been localized to the 12th position in the case of T24, and 61st position in the case of Hs242.

Biochemical organization of several retroviral and human oncogenes has been studied by nucleotide sequence analysis. From the predicted amino acid sequences, antibodies have been prepared and successfully used in the identification of the transforming gene products of these onc genes.

Studies were conducted to understand the genetic changes that lead to the maintenance of the malignant state in tumors that resulted from a "hit-and-run" phenomenon. These studies provided evidence that such changes involve the rearrangement of protooncogenes which, in turn, result in the production of aberrant onc-gene transcripts that might play a crucial role in the maintenance of malignancy.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. Aaronson	Chief	LCMB, NCI
S. Tronick	Head, Recombinant DNA Unit	LCMB, NCI
A. Srinivasan	Visiting Associate	LCMB, NCI
Y. Yuasa	Visiting Fellow	LCMB, NCI
D. Swan	Expert	LCMB, NCI
R. Balachandran	Visiting Fellow	LCMB, NCI
M. Barbacid	Visiting Scientist	LCMB, NCI
F. Mushinski	Medical Director	LCBGY, NCI
M. Potter	Chief	LG, NCI
T. Papas	Chief, Carcinogenesis Regulation Section	LMV, NCI

Objectives:

Project 1. To study the biochemical mechanisms involved in the activation of human oncogenes.

Project 2. To study the biochemical organization and molecular mechansims involved in the transformation of fibroblasts and lymphoid cells by Abelson murine leukemia virus.

Project 3. To study the biochemical organization and mechanism of action of myelocytomatosis virus (MC29).

Project 4. To study the role of myc oncogenes in murine plasmacytomagenesis.

Project 5. To study the role of myb oncogene in murine leukemogenesis.

Project 6. To study the biochemical organization of simian sarcoma virus and identification of its transforming gene product.

Methods Employed:

Molecular cloning, DNA sequence analysis, site-specific mutagenesis and DNA transfection.

Major Findings:

Project 1: DNA-mediated gene transfer techniques have made it possible to identify the presence of dominant tranforming genes in a variety of human tumors. Two such genes, one from a bladder carcinoma (termed T24) and the other from a lung carcinoma (termed HS242) have been isolated by molecular cloning techniques. Both these genes exhibited high levels of transforming activity in an in vitro transformation assay and were found to be derived from a similar structure present in the normal human genome. The mechanism of activation of T24 and Hs242 oncogenes was studied by construction of several recombinants from parts of the oncogenes and their normal homologs.

Analysis of such recombinants for transforming activity and comparison of their sequences showed that the genetic changes leading to the activation of these oncogenes are point mutations which resulted in the alteration of amino acid sequences of the proteins encoded by them. Thus, it was discovered that the T24 bladder carcinoma oncogene has undergone a point mutation in the first exon, which resulted in the incorporation of valine instead of glycine as the 12th amino acid residue. On the other hand, the Hs242 oncogene was found to have undergone a point mutation in an entirely different region of the molecule, but leading to a similar activation of the genome. The genetic lesion responsible for the transforming activity of the Hs242 oncogene has been localized to the second exon, which results in the substitution of leucine for glutamine as the 61st amino acid of the protein. No changes were observed in the first exon, the region of the genome in which the T24 oncogene has undergone a change. Thus, single amino acid substitutions appear to be sufficient to confer transforming properties on the gene products of T24 and Hs242 oncogenes.

In order to further understand the structural organization of these oncogenes, the entire genome of the T24 bladder carcinoma was sequenced. These studies revealed that this gene codes for a protein of 189 amino acids with a molecular weight of approximately 22,000. Comparison of the amino acid sequence of p21 protein coded by the T24 oncogene with its viral counterparts (coded by Harvey- and BALB-MSV) showed that this gene family is highly conserved during evolution, with approximately a 1% divergence in protein sequence from mouse to man. These studies also indicated that the genome is activated by changes mostly in the amino terminal domain of the molecule. These studies, for the first time, have provided a molecular basis for the carcinogenic process.

Project 2: Nucleotide sequence analysis of the proviral genome of Abelson murine leukemia virus (A-MuLV), an acute transforming virus of murine origin, has been determined. Like other transformng viruses, A-MuLV contains sequences derived from its helper virus, Moloney murine leukemia virus (M-MuLV), and a cell-derived protooncogene (abl) insertion sequence. By comparison of the A-MuLV sequence with that of M-MuLV, it was possible to precisely localize and define sequences contributed by the host cellular DNA. From the nucleotide sequence, we have predicted the amino acid sequence of p120gag-abl, the product of the A-MuLV gag-abl hybrid gene. The amino acid sequence of the putative abl gene, when compared with the sequences of other tyrosine-specific protein kinases (src, fes, fps, and yes), revealed significant homologies, indicating that all these functionally related transforming genes are derived from divergent members of the same proto-oncogene family. In addition to the gag-abl sequence, the proviral genome was found to contain an additional open reading frame that could code for an 18-K protein, whose role is at present undetermined.

Project 3: Myelocytomatosis virus MC29 is a defective avian retrovirus with a hybrid transforming gene (Δgag-myc) consisting of a 1,358-base pair (bp) sequence from the retroviral gag gene and a 1,568-bp sequence (v-myc) shared with a cellular locus, termed c-myc. We have sequenced the entire MC29 proviral genome coding for the gag-myc hybrid protein and the amino acid sequence of this transforming protein. In addition, we

have subjected to sequence analysis 2,735 bp of the cloned c-myc gene, which includes the v-myc-related region of 1,568 bp, an intervening sequence of 971 bp, and unique flanking sequences of 45 bp and 195 bp at the 5' and 3' ends, respectively. Analysis of the genetic information and alignment of the c-myc sequence with the sequence of MC29 indicated that: (i) the two myc sequences share the same reading frame, including the translational termination signal; (ii) there are nine nucleotide changes between c-myc and v-myc that correspond to seven amino acid changes; (iii) the 971-bp intervening sequence of c-myc can be defined as an intron by consensus splice signals; (iv) the unique 5' sequence of c-myc could either extend its reading frame beyond the homology with v-myc or could be an intron because its junction with the myc region of the locus is a canonical 3' splice-acceptor site; (v) the v-myc contains 10 nucleotides at its 5' end not shared with the c-myc analyzed here nor with known gag genes, and is probably derived from an upstream exon; and (vi) the c-myc locus can generate a mRNA whose termination signals have been identified to be located 83 bp and 119 bp from the point of divergence between the v-myc and c-myc. We conclude that the gene of the c-myc locus of the chicken and the onc gene of MC29 share homologous myc regions and differ in unique 5' coding regions and we speculate, on this basis, that their protein products may have different functions. The hybrid onc gene of MC29 must have been generated from the c-myc gene by deletion of the 5' cellular coding sequence, followed by substitution with the 5' region of the viral gag gene.

Project 4: To determine whether increased expression of any of the known onc genes plays a role in the development of mouse lymphoid tumors, several onc genes were used to probe messenger-enriched RNA [poly(A)$^+$RNA] from a variety of pristane-induced BALB/c plasmacytomas, from normal tissues, from T- and B-cell lymphomas, and from other tumors. Three onc genes associated with lymphoid tumors were used as hybridization probes: c-myc from normal chicken DNA, v-myb from avian myeloblastosis virus, and v-abl from Abelson murine leukemia virus (A-MuLV). In addition, v-bas from BALB/c murine sarcoma virus was included as an example of onc genes associated with soft tissue sarcomas rather than lymphoid tumors. Only myc RNA was consistently elevated in plasmacytomas. Because chromosomal rearrangements are consistently found in pristane-induced plasmacytomas and the nonimmunoglobulin DNA sequences called NIARD, NIRD and LyR have been shown to be rearranged frequently in lymphoid tumors, studies were done to look for changes in myc gene loci at the DNA level. We observed rearrangements in myc DNA in certain plasmacytomas, and these are strikingly similar to those reported for NIARD and LyR.

The basis for increased myc gene transcription in plasmacytomas is not understood, but the evidence suggests that different mechanisms may be operating in different plasmacytomas. Apparently, neither myc gene amplification nor myc gene rearrangement is required for increased myc transcription.

Project 5: In the presence of pristane, Abelson murine leukemia virus (A-MuLV) induces three classes of tumors termed undifferentiated lymphosarcomas (ABLSs), plasmacytomas (ABPCs), and plasmacytoid lymphosarcomas (ABPLs). Most of the ABLS and ABPC tumors contain the A-MuLV proviral genome integrated in their cellular DNAs and synthesize v-abl RNA. On the other hand, most of the ABPLs lack A-MuLV genomic inserts and do not synthesize v-abl mRNA. These tumors were examined for secondary changes that led to maintenance of the malignant state in the absence of transforming virus. A detailed analysis of tumors for the expression of various oncogenes revealed that they express large amounts of c-myb RNA of unusually large size. They were also found to express excessive amounts of myc RNA. Further analysis of tumor DNAs revealed that these cells did not contain A-MuLV proviral genomes integrated into their genome, nor did they show myc gene rearrangements characteristic of mouse plasmacytomas and Burkitt's lymphomas. However, all the ABPL tumors showed extensive rearrangement in their c-myb locus. These results suggest that DNA rearrangements in protooncogenes might lead to stable transformed phenotype in cells that lack transforming virus. These studies also offer a molecular explanation of hit-and-run phenomena exhibited by several DNA and RNA tumor viruses.

Project 6: The complete nucleotide sequence of the proviral genome of simian sarcoma virus (SSV), an acute transforming retrovirus of primate origin, has been determined. Like other transforming viruses, SSV contains sequences derived from its helper virus, simian sarcoma-associated virus (SSAV), and a cell-derived (v-sis) insertion sequence. By comparison with the sequence of M-MuLV, it was possible to precisely localize and define sequences contributed by SSAV during the generation of SSV. Comparative sequence analysis of SSV and SSAV showed that SSAV provides regulatory sequences for initiation and termination of transcription of the SSV transforming gene. Moreover, coding sequences for the putative protein product of this gene appear to initiate from the amino terminus of the SSAV env gene. Antibodies to synthetic peptides derived from the carboxy and amino termini of the putative protein predicted by the open reading frame identified within v-sis specifically detect a M_r 28,000 protein, $p28^{sis}$, in SSV-transformed cells. These and other findings confirm the predicted amino acid sequence of this protein and localize it to the coding region of the SSV transforming gene.

Significance to Biomedical Research and the Program of the Institute:

The studies described have provided, for the first time, insights into the molecular mechanisms of carcinogenesis. Thus, point mutations have been shown to activate normal genes (protooncogenes) into malignant genotypes. This provides a good model to explain the mechanism of chemical and environmental carcinogenesis. Studies on hit-and-run phenomena have again demonstrated the probable involvement of oncogenes in various malignancies. Nucleotide sequence studies have not only provided a large amount of information on the biochemical organization of mammalian onc genes, but also provided approaches to isolate and characterize the transforming proteins encoded by these onc genes. These studies combined with mutagenesis studies are expected to provide further information on the mechanisms of action of various oncogene products, which in turn will lead to developing approaches toward cancer prevention.

Proposed Course:

1. The nucleotide sequence data will be utilized to carry out site-specific mutagenesis studies on the various human and viral onc genes in an effort to elucidate the molecular mechanisms involved in the transformation process.

2. Synthetic peptides will be generated from the predicted amino acid sequences of the transforming proteins and will be used to prepare antisera. These antisera will be used to study the expression of the transforming protein in naturally occurring and chemical-induced lymphoid tumors. These antisera will also be used to determine the biochemical nature of the proteins and their role in normal cell growth and differentiation.

3. A detailed structural and biological analysis of the translocated myb and myc-genes will be carried out to understand their role in naturally occurring lymphomas.

Publications:

Aaronson, S. A., Reddy., E. P., Robbins, K., Devare, S. G., Swan, D. C., Pierce, H. J. and Tronick, S. R.: Retroviruses, onc genes, and human cancer. In Harris, C. C. and Autrup, H. N. (Eds.): Human Carcinogenesis. New York, Academic Press. (In Press)

Aaronson, S. A., Storch, T. G., Balachandran, R. and Reddy, E. P.: Different hematopoietic target cells for transformation by replication-competent murine leukemia viruses. In Marchesi, V. T. and Gallo, R. C. (Eds.): Differentiation and Function of Hematopoietic Cell Surfaces. New York, Alan R. Liss, Inc., 1982, pp. 251-261.

Bernstein, K. E., Reddy, E. P., Alexander, C. B. and Mage, R. G.: A cDNA sequence encoding a rabbit heavy chain variable region of the Vμa2 allotype showing homologies with human heavy chain sequences. Nature 300: 74-76, 1982.

Boyle, W. J., Lipsick, J. S., Reddy, E. P. and Baluda, M. A.: Identification of the leukemogenic protein of avian myeloblastosis virus and of its normal cellular homologue. Proc. Natl. Acad. Sci. USA 80: 2834-2838, 1983.

Devare, S. G., Reddy, E. P., Law, J. D., Robbins, K. C. and Aaronson, S. A.: Nucleotide sequence of the simian sarcoma virus genome: Demonstration that its acquired cellular sequences encode the transforming gene product, p28sis. Proc. Natl. Acad. Sci. USA 80: 731-735, 1983.

Habara, A., Reddy, E. P. and Aaronson, S. A.: Rauscher murine leukemia virus: Molecular cloning of infectious integrated proviral DNA. J. Virol. 44: 731-735, 1982.

Kindsvogel, W. R., Reddy, E. P., Moore, J. M. and Faust, C. H., Jr.: A cloned cDNA probe for rat immunoglobulin epsilon heavy chain: Construction, identification, and DNA sequence. DNA 1: 335-343, 1982.

Mushinski, J. F., Bauer, S. R., Potter, M. and Reddy, E. P.: Increased expression of myc-related oncogene mRNA characterizes most BALB/c plasmacytomas induced by pristane or Abelson murine leukemia virus. Proc. Natl. Acad. Sci. USA 80: 1073-1077, 1983.

Mushinski, J. F., Potter, M., Bauer, S. R. and Reddy. E. P.: DNA rearrangement and altered RNA expression of the c-myb oncogene in mouse plasmacytoid lymphosarcomas. Science 220: 795-798, 1983.

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Samuel, K. P., Baluda, K. P. and Reddy, E. P.: Nucleotide sequence analysis of integrated avian myeloblastosis virus: Structural similarities to transposable elements. In Chandra, P. (Ed.): Biochemical and Biological Markers in Neoplastic Transformation. New York, Plenum Press. (In Press)

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D., Baluda, M. A. and Reddy, E. P.: Complete nucleotide sequence and biochemical organization of the transforming gene and large terminal redundancies of avian myeloblastosis virus. In Scolnick, E. M. and Levine, A. J. (Eds.): UCLA Symposium on Tumor Viruses and Differentiation. New York, Alan R. Liss, Inc. (In Press)

Papas, T. S., Rushlow, K. E., Watson, D. K., Bader, J., Ray, D. and Reddy, E. P.: The transforming gene of avian myeloblastosis virus (AMV). Nucleotide sequence analysis and identification of its translational product. In Neth, R., Gallo, R., Greaves, M. F., Moore, M. A. S. and Winkler, K. (Eds.): Molecular Trends in Human Leukemia V. Berlin/Heidelberg, Springer Verlag, 1983, pp 207-213.

Papas, T. S., Rushlow, K. E., Watson, D. K., Lautenberger, J. A., Perbal, B., Baluda, M. E. and Reddy, E. P.: Nucleotide sequence and organization of the transforming region and large terminal redundancies (LTR) of avian myeloblastosis virus (AMV). J. Cell. Biochem. 20: 95-103, 1982.

Pavirani, A., Mage, R., Jacobsen, F. W., Reddy, E. P., Bernstein, K. and Fitzmaurice, L.: Construction of a partial rabbit spleen cDNA library and identification of immunoglobulin clones. Eur. J. Immunol. 12: 854-860, 1982.

Pavirani, A., McCartney-Francis, N., Jacobsen, F., Mage, R., Reddy, E. P. and Fitzmaurice, L.: Analyses of the splenic mRNAs expressed by rabbits of different immunoglobulin kappa light chain allotypes: Conserved sequences in the 3' UT region and allotype-specific probes. Cell. (In Press)

Putman, D. L., Nayar, K. T., O'Neill, B., Reddy, E. P., Levy, J. A. and Kouri, R. E.: Genetics of xenotropic virus expression in mice. II. Expression of major virus structural proteins in crosses involving NZB/BINJ, SWR/J, and 129/J strains of mice (41634). Proc. Soc. Exp. Biol. Med. 173: 217-221, 1983.

Reddy, E. P.: Nucleotide sequence analysis of the T24 human bladder carcinoma oncogene. Science 220: 1061-1063, 1983.

Reddy, E. P., Reynolds, R. K., Santos, E. and Barbacid, M.: A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature 300: 149-152, 1982.

Reddy, E. P., Reynolds, R. K., Watson, D. K., Schultz, R. A., Lautenberger, J. and Papas, T.S.: Nucleotide sequence analysis of the proviral genome of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA 80: 2500-2504, 1983.

Reddy, E. P., Smith, M. J. and Srinivasan, A.: Nucleotide sequence of Abelson murine leukemia virus genome: Structural similarity of its transforming gene product to other onc gene products with tyrosine-specific kinase activity. Proc. Natl. Acad. Sci. USA 80: 3623-3627, 1983.

Reddy, E. S. P., Das, M. R., Reddy, E. P. and Bhargava, P. M.: Inhibition of reverse transcriptases by seminal plasmin. J. Biochem. 209: 183-188, 1983.

Robbins, K. C., Devare, S. G., Reddy, E. P. and Aaronson, S. A.: In vivo identification of the transforming gene product of simian sarcoma virus. Science 218: 1131-1133, 1982.

Santos, E., Reddy, E. P., Pulciani, S., Feldmann, R. J. and Barbacid, M.: Spontaneous activation of a human proto-oncogene. Proc. Natl. Acad. Sci. USA. (In Press)

Srinivasan, A., Dunn, C. Y., Yuasa, Y., Devare, S. G., Reddy, E. P. and Aaronson, S. A.: Abelson murine leukemia virus: Structural requirements for transforming gene function. Proc. Natl. Acad. Sci. USA 79: 5508-5512, 1982.

Swan, D. C., McBride, O. W., Robbins, K. C., Keithley, D. A., Reddy, E. P. and Aaronson, S. A.: Chromosomal mapping of the simian sarcoma virus onc gene analogue in human cells. Proc. Natl. Acad. Sci. USA 79: 4691-4695, 1982.

Watson, D.K., Reddy, E.P., Duesberg, P.H. and Papas, T.S.: Nucleotide sequence analysis of the chicken c-myc gene reveals homologous and unique coding regions by comparison with the transforming gene of avian myelocytomatosis virus MC29, Δgag-myc. Proc. Natl. Acad. Sci. USA 80: 2146-2150, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05060-05 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of Oncogenic Expressions in Animal and Human Cancers

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. S. Rhim Microbiologist, LCMB, NCI

COOPERATING UNITS *(if any)*

U. Utah, Logan, Utah; Southwest Fdn. for Res. & Educ., San Antonio, Texas; Dept. Path., USC, Los Angeles, CA; Dept. Path., Columbia U., New York, N.Y.

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Viral Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goals of this project are: (1) to establish and define a cell culture transformation system for identification of individuals predisposed to cancer genetically or by virtue of exposure to environmental carcinogens; (2) to develop human cell transformation systems, with particular emphasis on epithelial cells, in order to study host factors regulating cell transformation and the mechanisms of carcinogenesis by chemicals, viruses, hormones and x-irradiation; (3) to identify and rescue human and/or primate sarcoma (src) information; and (4) to develop and test measures to prevent and/or control cell transformation and the neoplastic event for eventual application in primates, including humans.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
Y. Yuasa	Visiting Fellow	LCMB, NCI
M. Kraus	Guest Researcher	LCMB, NCI
J. Fujita	Guest Researcher	LCMB, NCI
P. Arnstein	Veterinary Medical Officer	LCMB, NCI
K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB, NCI
S. Sieber	Deputy Director	DCCP, NCI

Objectives:

1. To develop a cell culture transformation system for identification of individuals at high risk for early cancer.

2. To develop and study human cell transformation systems, particularly epithelial cells, and the factors regulating cell transformation, to elucidate mechanisms of cellular transformation by carcinogenic agents and viruses.

3. To search for and rescue human and primate sarcoma (src) information from primate and human cancers.

4. To develop measures to prevent and/or control transformation and cancer in animals and ultimately in humans.

Methods Employed:

Biological methods include cell and virus cloning, transformation focus, cell aggregation, soft agar, genome rescue and transfection assays. Biochemical methods include reverse transcriptase assay, radioimmunoprecipitation, and I^{125} protein A assay.

Major Findings:

Inherited susceptibility to retrovirus-induced transformation of Gardner syndrome cells: A number of in vitro studies had previously indicated that skin fibroblasts can be used to distinguish individuals with Gardner syndrome (GS) and familial polyposis coli (FPC) from others in the general population. In order to establish further the accuracy of the test, coded skin samples received from Eldon Gardner and Randy Moon, University of Utah, were tested for focus formation by the Kirsten mouse sarcoma virus (Ki-MSV). Parallel specimens were also tested by Suraiya Rasheed, University of Southern California. The results indicated that, based on the higher susceptibility to retrovirus-induced transformation and chromosomal aneuploidy, the GS and FPC cells could be distinguished from those of the general population with more than 70% accuracy.

Glucocorticoids enhance retrovirus transformation of mammalian cells: Certain hormones are known to influence carcinogenesis in vitro, as well as in experimental animals. It has been reported that in mouse cells, glucocorticoids (hydrocortisone and dexamethasone) enhanced the production of certain DNA and RNA tumor viruses, but did not promote the production of murine sarcoma virus (MSV). However, the effect of these hormones on the replication of MSV in other mammalian cells has not been examined. In contrast to the reported lack of effect of steroid hormones on the MSV-induced transformation in mouse cells, we found that the glucocorticoids, hydrocortisone (1 to 10 µg/ml) and dexamethasone (5 µg/ml), enhanced transformation induced by Ki-MSV in normal rat kidney (NRK) and human cells in vitro. Indeed, hydrocortisone enhances the Ki-MSV-induced transformation in NRK and human skin fibroblasts 10- to 30-fold. On the other hand, the hormones estradiol, testosterone, and progesterone (5 µg/ml) had no effect. Individual foci appeared earlier and were larger in hydrocortisone-treated cells compared with untreated cells. This enhancing effect is further evidenced by the increased virus yield and murine leukemia virus complement fixation antigen production in the test system. Hydrocortisone did not promote the production of Ki-MSV in mouse embryo cells, as previously reported. However, individual foci in hormone-treated cells appeared earlier and the size of the foci were larger than those appearing in untreated cells. Thus, the hydrocortisone treatment offers a simple, rapid, sensitive transformation assay method for MSV in mammalian cells and may be of practical use in achieving greater production of type C virus from certain retrovirus-producing mammalian cell lines.

Neoplastic transformation of rabbit cells by murine sarcoma viruses: Rabbit cells have been widely used for isolation and replication of a variety of retroviruses, including those characterized as xenotropic. However, rabbit cells have seldom been used for transformation studies with MSV. We have previously shown that rabbit cells could be transformed morphologically by Ki-MSV and that such cells contained both infectious virus and the group-specific murine leukemia virus antigen. However, the tumorigenicity of the transformed cells was not studied. The results indicate that Ki-MSV, Ki-MSV(BaEV), and M-MSV(FeLV) induce neoplastic transformation of rabbit cells. Rabbit cells transformed by Ki-MSV and M-MSV(FeLV) were found to be virus producers, whereas those transformed by Ki-MSV(BaEV) were nonproducers. The nonproducer cells were obtained by simply infecting rabbit cells with Ki-MSV(BaEV) and subculturing the infected cells. Although the morphologically altered nonproducer cells did not produce infectious virus or murine leukemia virus antigen, they did contain a rescuable MSV genome. All of the transformed cells formed colonies in soft agar, grew to high saturation densities, and produced tumors when transplanted into nude mice. The Ki-MSV and M-MSV(FeLV)-transformed cells produced tumors in newborn WH/J rabbits, providing an important tool for studying tumor immunity.

Transformation of fetal lamb kidney cells by bovine leukemia virus: Bovine leukemia virus (BLV), a unique retrovirus, causes lymphoma or leukemia on inoculation into cattle and sheep. However, little or no successful in vitro transformation of sheep and calf cells has been reported. Induction of transformed phenotypes in sheep fibroblasts from testes following inoculation with BLV has been reported, but only after

several passages. The rapid successful transformation of fetal lamb kidney cells by BLV was obtained. Morphological transformation was observed in normal fetal lamb kidney cells (designated as BK-571 [passage 13] and BN-895 [passage 19]) within a few weeks after BLV inoculation. BLV-transformed fetal lamb kidney cells had the following properties generally associated with viral transformation: (a) altered morphology; (b) increased growth rate; (c) colony formation in soft agar medium; (d) formation of large cell aggregates and growth in this aggregate form above an agar base; and (e) tumorigenicity in nude mice. The transformed cells contained BLV-specific p24 and gp51 antigens and were virus producers. BLV was detected in transformed cells by syncytial assay. The transformed cells yielded high titered viral reverse transcriptase activity and viral particles, and contained marker chromosomes (M_1, M_2, M_3, M_4, and M_7) and a large submetacentric chromosome which were not present in control cells. Although the mechanisms of acquisition of the transforming capacity of BLV during long-term culture in vitro are not clear, it may be speculated that BLV acquired this capacity by recombination with endogenous bovine or sheep viral or cellular genes during long-term subculture in vitro. A similar phenomenon is well documented in the rodent system where it was observed that retroviruses originating from normal rodent cells were able to transform only after repeated passages.

Induction of tumors in chimpanzees with a chemical carcinogen: In collaboration with Dr. Sieber, liver tumors were induced in adolescent chimpanzees following continuous treatment with a chemical carcinogen, DENA. This is the first documentation of experimentally induced chimpanzee tumors. The tumors were hepatocellular carcinomas. Tumor lines were established and characterization studies are now in progress. Attempts to isolate an oncogene from these tumors are underway.

Viral transformation of human epithelial cells: Since most human tumors are of epithelial origin, the importance of studying human epithelial cell carcinogenesis is well recognized. However, normal epithelial cells are very difficult to culture. A new culture medium, NCTC 168, designed for human skin epithelial cells, was developed by Dr. Sanford and her associates. A viral transformation study utilizing primary cultures of human epithelial cells grown in NCTC 168 supplemented with horse serum has recently been initiated. Primary human epithelial cells infected with adeno 12-SV40 virus grew, differentiated and became established cell lines, whereas the uninfected cells did not grow. Characterization studies are in progress.

Isolation of oncogenes from human lung carcinoma-derived cell lines: Several oncogenes were isolated from 20 human lung carcinoma-derived cell lines in the LCMB using the DNA-mediated transfection assay. One of the isolates has been identified from a carcinoma-derived cell line, designated Hs242. This transforming gene, related to c-bas/has (human) was cloned in biologically active form and demonstrated a specific transforming activity of $>10^4$ ffu/ug DNA insert. Restriction endonuclease mapping revealed no detectable differences from its normal allele, indicating that relatively subtle changes were responsible for its activation

as a transforming gene. By restriction enzyme and nucleotide sequence analysis, the Hs242 oncogene was shown to be unaltered in the first exon, the region responsible for activation of the T24 and EJ human oncogenes. Thus, genetic changes at more than one site in the same protooncogene can be responsible for its acquisition of transforming properties under natural conditions. Studies were aimed at localizing the number and nature of the genetic alterations responsible for activation of the Hs242 oncogene.

Significance to Biomedical Research and the Program of the Institute:

1. The development of testing systems for identification of genetic susceptibilities to cancer could prove very useful in cancer diagnosis.

2. Definition of factors associated with cell transformation should provide important insights into the mechanisms of carcinogenesis induced by chemicals, oncogenic viruses, hormones and other environmental influences.

3. Development of primate and human cell lines for rescue and identification of primate and human cancer genes will provide the tools for eventual development of protective vaccines against cancer.

4. Development of a new model for the study of human epithelial cell carcinogenesis is important in understanding the process of neoplastic conversion in human epithelial cells.

Proposed Course:

1. Continuation of studies to elucidate the various factors (with emphasis on the effect of hormones) regulating cell transformation induced by oncogenic viruses and chemical carcinogens.

2. Further attempts to develop a system for transforming human epithelial cells by virus and chemicals in order to elucidate human epithelial cell carcinogenesis.

3. Continued characterization of adeno 12-SV40 transformed human epithelial cells, and their clonal lines in order to establish permanent lines for further genetic studies, and for understanding the mechanisms involved in cellular gene expression and tumorigenicity.

4. Further characterization of chimpanzee tumor cell lines derived from tumors induced by a chemical carcinogen.

5. Continuation of oncogene isolation studies from human tumors, with emphasis on those derived from cervical, ovarian, and brain carcinomas and hepatomas, using the DNA transfection 3T3 cell assay.

6. Search for DNA transfection-susceptible cell lines other than the NIH/3T3 mouse fibroblast line.

Publications:

Purdy, R. H., Goldzieher, J. W., LeQuesne, P. W., Abdel-Baky, S., Durocher, C. K., Moore, P. H., Jr. and Rhim, J. S.: Active intermediates and carcinogenesis. In Mariam, G. and Lipsett, M. B. (Eds.): International Workshop on Catechol Estrogens. New York, Raven Press, 1983, pp. 123-140.

Rasheed, S., Rhim, J. S. and Gardner, E. J.: Inherited susceptibility to retrovirus-induced transformation of Gardner syndrome cells. Am. J. Hum. Genet. (In Press)

Rhim, J. S.: A cell aggregation assay: A rapid means of evaluating and selecting in vitro transformed cells. Cancer Detection and Prevention. (In Press)

Rhim, J. S.: Virus as an etiological factor in Cancer. Cancer Detection and Prevention. (In Press)

Rhim, J. S., Bedigian, H. G. and Fox, R. R.: Neoplastic transformation of rabbit cells by murine sarcoma virus. Int. J. Cancer 30: 365-369, 1982.

Rhim, J. S. and Huebner, R. J.: The chimpanzee as an experimental model in cancer research. In Kalter, S. (Ed.): Viral and Immunological Diseases in Nonhuman Primates. New York, Alan R. Liss, 1983, pp. 231-232.

Rhim, J. S. and Huebner, R. J.: Neoplastic transformation induced by adeno 12-SV40 hybrid virus in skin fibroblasts from humans genetically predisposed to cancer. Cancer Detection and Prevention. (In Press)

Rhim, J. S., Huebner, R. J., Heberling, R. L. and Kalter, S. S.: Induction of chimpanzee sarcoma in an infant chimpanzee after transplantation of human osteosarcoma nonproducer cells infected with baboon endogenous virus. In Kalter, S. (Ed.): Viral and Immunological Diseases in Nonhuman Primates. New York, Alan R. Liss, 1983, pp. 233-234.

Rhim, J. S., Kraus, M. and Arnstein, P.: Neoplastic transformation of fetal lamb kidney cells by bovine leukemia virus. Int. J. Cancer. (In Press)

Rhim, J. S., Trimmer, R., Huebner, R. J., Papas, T. S. and Jay, G.: Differential susceptibility of human cells to transformation by murine and avian viruses. Proc. Soc. Exp. Biol. Med. 170: 350-358, 1982.

Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. S., Reddy, E. P. and Aaronson, S. A.: Isolation of a new c-bas/has(human) oncogene: Point mutation within different structural domains of this protooncogene can lead to its acquisition of transforming properties. Nature. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05061-05 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Study of Abelson Murine Leukemia Virus Genome

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

A. Srinivasan Visiting Associate, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The integrated Abelson murine leukemia virus (A-MuLV) genome cloned in bacteriophage vector was used to analyze the functional nature of the different segments of the viral genome. Construction of mutants having sequential deletions of the A-MuLV-specific abl gene revealed that only the proximal 40% of abl with its associated 5' helper viral sequences is required for fibroblast transformation. To define regions of long terminal repeat (LTR) required for A-MuLV transforming gene function, deletion mutants encompassing different domains were constructed and assayed following transfection of NIH/3T3 cells. Deletions involving only TATA AND CAAT sequences showed reduced transforming activity, whereas deletions extending to the end of the LTR abolished transforming activity. Removal of both 72-bp repeats completely abolished the transforming activity. Interestingly, ligation of wild type 3' LTR to the defective 5' LTR subgenomic clones restored the transforming activity. Nucleotide sequence analysis of the complete proviral genome of A-MuLV had been determined. The amino acid sequence of the abl gene, derived from the primary DNA sequence, has significant homologies to other tyrosine-specific protein kinases encoded by src, fes, fps and yes onc genes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
S. G. Devare	Visiting Associate	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI
Y. Yuasa	Visiting Fellow	LCMB, NCI
J. Pierce	Staff Fellow	LCMB, NCI
A. Eva	Visiting Associate	LCMB, NCI
S. R. Tronick	Microbiologist	LCMB, NCI
R. Narayanan	Visiting Fellow	LCMB, NCI
A. Gazit	Visiting Fellow	LCMB, NCI

Objectives:

1. Structural and functional analyses of the transforming gene of A-MuLV.

2. Dissection of A-MuLV LTR by in vitro mutagenesis and its role in viral gene expression.

Methods Employed:

1. Molecular cloning techniques, transfection assay, immunoprecipitation and SDS-PAGE.

2. Molecular cloning, DNA transfection and sequencing.

Major Findings:

1. The integrated A-MuLV genome cloned in bacteriophage λgtWES.λB was used to localize viral genetic sequences required for transforming function. Comparison of the biological activity of cloned A-MuLV genomic fragments showed that subgenomic clones that lacked the 5' LTR and adjoining sequences (300 bp downstream of the repeat) were not biologically active. In contrast, subgenomic clones that lacked the 3' LTR and as much as 1.3 kb of the A-MuLV cell-derived abl gene were as efficient as wild-type viral DNA in transformation. The A-MuLV encoded polyprotein p120 and its associated protein kinase activity were detected in transformants obtained by transfection with Cla I, Bam HI and Hind III subgenomic clones. In contrast, individual transformants obtained with subgenomic Sal I clones expressed A-MuLV protein ranging in size from 82,000 to 95,000 daltons.

   Further, to understand the nature of the v-abl gene, primary DNA sequence analysis of A-MuLV proviral genome was undertaken. Comparison of the A-MuLV sequence with that of M-MuLV revealed homology of 1775 bp at the 5' end and 793 bp at the 3' end of the genome.

The open reading frame starts at position 1067 and extends for an additional stretch of 2045, terminating with a TGA codon in the abl gene in addition to the entire sequence of p15, p12, and the first 21 codons of p30. Comparative analysis of abl with other onc genes which encode for proteins with an associated kinase activity revealed that abl shared a homology of 176, 158, 140, and 138 amino acids with yes, src, fes and fps.

2. The LTR ras sequence is homologous to the 5' noncoding and 3' noncoding regions of eukaryotic genes, which implies that these sequences may have a functional role in the expression of the viral genome. Mutants with deletions in 5' LTR were constructed utilizing the available restriction enzyme sites and also by using the BAL 31 exonuclease. The mutants characterized could be divided into three groups according to the location of deletions from the putative RNA start site. The first group consists of mutants which have suffered deletions of 76 and 179 nucleotides eliminating one or both 72-bp repeats. Deletions covering CAAT, TATA, and putative RNA start site form the second group. The third group consists of mutants with large deletions (349 and 520 bp) encompassing CAAT, TATA, cap site, tRNA binding site and sequences downstream. The specific alterations in LTR were assayed by transfecting DNA onto NIH/3T3 cells and scoring the transformed foci. Removal of both 72-bp repeats completely abolished the transforming activity. Mutants lacking CAAT and TATA sequences showed reduced levels of transforming activity, and mutants with larger deletions were not able to transform cells.

Significance to Biomedical Research and the Program of the Institute:

Using cloned infectious A-MuLV proviral DNA as a tool, we have made progress in uncovering the processes underlying transformation. Results of these experiments are providing insights and approaches to a better understanding of the biochemical basis of neoplasia.

Proposed Course:

1. Using the primary DNA sequence data available for the A-MuLV transforming gene abl, attempts will be made to construct in-frame deletion mutants to gain more information about the functional domain of the abl protein.

2. The 5' terminus of A-MuLV-specific mRNA produced in cells transformed by wild-type A-MuLV and LTR deletion mutants will be determined using the primer extension method. This would help to identify the LTR region involved in positioning the 5' terminus of A-MuLV mRNA.

Publications:

Reddy, E. P., Smith, M. J. and Srinivasan, A.: Nucleotide sequence of the Abelson murine leukemia virus genome: Structural similarity of its transforming gene product to other onc gene products with tyrosine-specific kinase activity. Proc. Natl. Acad. Sci. USA. (In Press)

Srinivasan, A., Dunn, C. Y., Yuasa, Y., Devare, S. G., Reddy, E. P. and Aaronson, S. A.: Abelson murine leukemia virus: Structural requirements for transforming gene function. Proc. Natl. Acad. Sci. USA 79: 5508-5512, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05062-05 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transforming Genes of Naturally Occurring and Chemically Induced Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
A. Eva Visiting Associate, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The development of DNA-mediated gene transfer has provided an approach for the detection of cellular transforming genes. By using the NIH/3T3 transfection assay, transforming genes have been detected in diverse human and animal tumors, including sarcomas and hematopoietic malignancies. One approach toward identification of genes that may be preferential targets for somatic mutations leading to their acquisition of transforming activity involves analysis of animal tumor cells induced by specific carcinogens. Analysis of four methylcholanthrene-induced mouse fibrosarcomas demonstrated that two of these tumors are capable of transforming NIH/3T3 fibroblasts, that the acquired transformed phenotype is serially transmissible, that the transforming gene associated with these tumors is the same and is the cellular analog, c-kis, of the transforming gene of Kirsten murine sarcoma virus. These findings establish that c-kis is activated as a transforming gene at high frequency in these tumors. By using the same NIH/3T3 cell transfection assay, we also have sought to detect and characterize transforming DNA sequences of a spectrum of human hematopoietic tumors. We have detected in both acute and chronic myelogenous leukemia cells, as well as in acute human lymphoid leukemia cells, transforming genes capable of being serially transmitted to NIH/3T3 cells. The oncogene of one intermediate T-cell leukemia was demonstrated to be the activated human allele of c-kis, whereas two additional intermediate T-cell leukemias contained a different transforming gene related to, but distinct from, c-kis. This same gene was also found to be the transforming gene of one acute and one chronic myelogenous leukemia. Thus the NIH/3T3 transfection assay commonly detects related oncogenes in human hematopoietic tumor cells. Moreover, the activation of these oncogenes appears to be independent of the specific state of cell differentiation or tumor phenotype.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
S. R. Tronick	Head, DNA Recombinant Unit	LCMB, NCI
K. C. Robbins	Expert	LCMB, NCI
M. Barbacid	Visiting Scientist	LCMB, NCI

Objectives:

1. To study which genes are involved in transformation induced by chemical carcinogens, utilizing methylcholanthrene-induced mouse tumors.

2. To investigate which transforming genes are associated with specific human leukemias and lymphomas. Studies are directed to identify and isolate these genes in order to determine by what mechanism they are able to induce the transformed phenotype.

Methods Employed:

Standard and developmental techniques in cell biology, biochemistry, and recombinant DNA.

Major Findings:

1. We have shown that DNAs of two of four methylcholanthrene-induced mouse fibrosarcomas contained transforming genes indistinguishable in their pattern of restriction endonuclease resistance to inactivation of biologic activity. This transforming gene was identified as the activated cellular oncogene, c-kis, homologous to the Kirsten murine sarcoma virus onc gene.

2. We surveyed 22 human hematopoietic tumors and tumor cell lines for sequences capable of transforming NIH/3T3 cells by DNA transfection. A primary human acute myelogenous leukemia, a chronic myelogenous leukemia cell line, and cell lines derived from three independent acute lymphocytic leukemias demonstrated oncogenes capable of conferring the transformed phenotype to NIH/3T3 cells through serial cycles of transfection. The transforming gene associated with one acute lymphocytic leukemia was identified as the activated cellular homologue of c-kis, the Kirsten murine sarcoma virus oncogene. A transforming gene which was demonstrated to be common to several myeloid and lymphoid tumor cells was shown to be distantly related to c-kis.

Significance to Biomedical Research and the Program of the Institute:

The development of the DNA-mediated gene transfer technique has recently made it possible to identify transforming genes in a variety of tumors, including malignancies of human origin. By this technique, we demonstrated that DNA isolated from either primary human tumors or human tumor cell

lines induces morphological transformation of mouse fibroblasts. The fact that the transforming genes associated with human hematopoietic tumors are related to the Kirsten murine sarcoma virus oncogene represents an advantage in our understanding of the molecular basis of some human tumors. This is due to the fact that intensive investigations of acute transforming retroviruses has led to considerable knowledge concerning their genomes and translational products, as well as their interaction with the host. Thus, our demonstration that human tumor transforming genes represent activated human cellular analogues of retroviral oncogenes should make it possible to apply this knowledge to understand the mechanisms by which human oncogenes induce transformation. Moreover, it will be important to establish when in the course of oncogenesis these genes are activated. In this respect, the detection of a common transforming gene homologue to the Kirsten murine sarcoma virus oncogene in methylcholanthrene-induced mouse tumors has the relevance of providing a defined experimental system in which we can study whether the activation of c-kis occurs early or late in chemically induced tumorigenesis, and to define its precise involvement in the progressive development of the tumors.

Proposed Course:

A large number of transforming genes derived from tumors of many different somatic cells have been shown to be related just to the ras family of oncogenes. These findings argue that the number of cellular genes that can acquire transforming activity may be rather limited. It is also possible that the transfection assay selects for these oncogenes. For these reasons, we are screening another set of human hematopoietic tumors on NIH/3T3 cells, searching for other possible transforming genes, and trying to set up new cellular systems susceptible to transfection in order to establish their ability to acquire the transformed phenotype. Attempts are also being made to clone the transforming gene of the primary human acute myelogenous leukemia in order to establish the molecular modification involved in determining the transforming activity of this gene.

Several BALB/c and NIH/Swiss mice were injected with methylcholanthrene. Tumors which developed were taken at different times of tumor growth, and are now being screened for the presence of c-kis as the active transforming gene of NIH/3T3. In this way, we hope to be able to establish the frequency of activation of c-kis in the methylcholanthrene-induced tumors, as well as the stage(s) of tumor development at which the activated c-kis is detected.

Publications:

Aaronson, S. A., Dunn, C. Y., Ellmore, N. W. and Eva, A.: Retroviral onc genes in human neoplasia. In Rauscher, F. J. and O'Connor, T. E. (Eds.): Oncogenes and Retroviruses: Evaluation of Basic Findings and Clinical Potential. New York, Alan R. Liss, Inc., pp. 207-222, 1983.

Eva, A. and Aaronson, S. A.: Frequent activation of c-kis as a transforming gene in fibrosarcomas induced by methylcholanthrene. Science 220: 955-956, 1983.

Eva, A., Tronick, S. R., Gol, R., Pierce, J. H. and Aaronson, S. A.: Transforming genes of human hematopoietic tumors: Frequent detection of ras-related oncogenes whose activation appears to be independent of tumor phenotype. Proc. Natl. Acad. Sci. USA. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05063-05 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on Epstein-Barr Virus and Herpesvirus saimiri

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

D. V. Ablashi, Coordinator of DNA Virus Studies, LCMB, NCI

COOPERATING UNITS *(if any)* G. Krueger and G. Bertram, U. Cologne, West Germany; G. Pearson, Mayo Clinic, Rochester, MN; U. Prasad, M. Yadav and K. Prathap, U. Malaya, Malaysia; S. K. Sundar and J. Menezes, U. Montreal, St. Justin Hospital, Canada; A. M. Faggioni, Inst. of General Pathology, Rome, Italy

LAB/BRANCH

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Epstein-Barr virus (EBV) immunovirology was investigated as a marker for diagnosis/prognosis in (a) nasopharyngeal carcinoma (NPC) patients and (b) populations at risk for NPC. Specifically, the role of EBV virus capsid antigen (VCA) IgA antibody and EBV nuclear antigen (EBNA) was studied. In five normal individuals of Cantonese origin, a 10-fold increase of EBV-VCA/ IgA antibody over 1.5 years was diagnostic of NPC, and the presence of EBNA in the tumor cells correlated with the diagnosis. IgA antibody detected in tissues from the fossa of Rosenmueller identified NPC in 40 cases of so-called occult primary tumors of the head and neck. EBV serology correlated in a follow-up of 71 NPC patients since 1978. In general, NPC antibody titers were useful in a follow-up of undifferentiated (UC) and nonkeratinizing carcinomas (NKC-2) of the nasopharynx. Moreover, IgA titers were inversely related to ADCC titers in UC-NPC but not in non-NPC patients. Total B and T cell counts and skin testing provided good prognostic markers for patient follow-up. Depletion of IgA from UC-NPC patients sera removed the lymphocyte inhibition (LSI) activity. However, one such serum did exhibit a 40% inhibition of LSI, suggesting the presence of other inhibiting factors besides LSI. The presence of amyloid infiltration in 12% of NPC cases had never been reported previously. Herpesvirus saimiri (HVS) monoclonal antibodies were characterized as to their biological activity against two strains of HVS and herpesvirus ateles (HVA). Monoclonal antibodies were classified into 11 groups, as determined by immunofluorescence (IF) patterns in the nucleus or cytoplasm of cells. They failed to react with two strains of HVA or with HVS early antigen (EA)-producing cells. None of the antibodies neutralized HVS or HVA.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

P. Levine	Senior Investigator	CEB, NCI
J. Dahlberg	Microbiologist	LCMB, NCI

Objectives:

1. To study the EBV immunovirology associated with NPC, with particular emphasis on the rise and fall of IgA antibody to EBV-VCA in identification of individuals at risk for NPC, identification of occult primary tumors of the head and neck as NPC, clinical evaluation of IgA antibody and EBNA as aids in NPC diagnosis and prognosis, and the mechanisms involved in specific EBV-induced LSI by IgA antibody recovered from NPC patients in active stages of the disease.

2. To reexamine the histologic types of NPC tumors in terms of squamous cell carcinoma (SCC), keratinizing and nonkeratinizing carcinoma (KC and NKC), and undifferentiated carcinoma (UC) in order to determine whether one or a combination of all histiotypes exists in a given tissue.

3. To evaluate other immunologic markers (non-EBV) as aids in the follow up of NPC patients.

4. To biologically characterize monoclonal antibodies prepared against HVS, using immunofluorescence and neutralization assays.

Methods Employed:

Primary cell cultures and continuous cell lines of human and animal origins were used for virus isolation, biological and biochemical assays. Standard biochemical and immunological procedures were applied. Human sera were obtained from patients with cancers under study, healthy donors, and individuals working with the oncogenic herpesviruses in the laboratory. Monoclonal antibodies, as well as sera from nonhuman primates with or without HVS-induced tumors, were used. Two strains of HVS (prototype and OMI) and two strains of HVA (73 and 810) were used.

Major Findings:

1. In collaboration with Profs. Prasad and Yadav, University of Malaya, 500 serum samples from Cantonese Chinese from a high risk area in Malaysia (Saba Province) were tested for EBV-IgA or EBV-EA/IgA antibodies. Thirty sera were positive for IgA to EA (>1:5) or IgA-VCA (1:10). Serial serum samples from these individuals, taken over a period of 1.5 years, showed a 10-fold or greater increase in IgA titers in five individuals who later developed NPC of the undifferentiated type (UC). Thus, a rise in IgA to EBV-VCA antibody was reliable in predicting early pretumor activity. Sera from these patients also

were positive for LSI activity, further supporting neoplastic changes. Four months after treatment, the LSI titers in five individuals dropped, coincident with remission.

2. In collaboration with Drs. Krueger and Bertram, University of Cologne, and Dr. Pearson, Mayo Clinic, 71 NPC patients diagnosed in 1978 were followed for EBV antibody titers in parallel with tumor growth. Predictions of relapse based on EBV-related serological changes, as well as predictions of prognosis based on antibody-dependent cellular cytotoxicity (ADCC) were made, and non-EBV-related skin testing in a multitest system was evaluated. The data suggested that EBV antibodies at diagnosis correlated with histologic type subgroups of NPC, while antibody titers in squamous cell carcinomas (SCC) and NKC-1 were only demonstrable at low titers. Significant levels of antibody were observed only in undifferentiated carcinomas (UC). The most significant correlation was for EBV-VCA/IgA. In general, EBV antibody titers were not useful in SCC and NKC-1, but a good correlation was observed between EBV antibody titers and the clinical course of the disease in UC and NKC-2. IgA anti-VCA titers were inversely related to ADCC levels in patients with UC-NPC, suggesting that serology was useful in UC-NPC and NKC-2, but not at all in SCC.

   Initial and follow-up titers of ADCC did not distinguish between NPC and non-NPC tumors of the head and neck region. ADCC titers decreased, however, with tumor progression in some NPC patients, while in most non-NPC patients no relationship was observed.

   Only UC type 2 of the nasopharynx showed a curve similar to that found for NPC. The total numbers of B and T cells decreased after therapy, with subsequent increase at the time of relapse. Only patients with EBV-positive NPC tumors showed an initial rise in B cells of the IgM type, followed by an increased number of IgG and IgA cells at the time of relapse. Skin testing provided a good prognostic marker. The skin test may be useful in follow-up because it was demonstrated that enhanced reactivity after therapy was associated with good prognosis (survival of more than three years), while persistent low reactivity in delayed hypersensitivity was less favorable, with death occurring within two years.

3. The use of EBV VCA/IgA antibody, detection of EBNA in cells, computerized axial tomography of the nasopharynx and histological examination of biopsy material obtained from the fossa of Rosenmueller aided in confirming 40 cases of NPC with occult primary tumors of the nasopharynx. Of these, 33 (82.5%) were Chinese, 6 (15%) were Malaysian, and one was Indian. EBV serology showed that 87.5% had IgA titers of $\geq$10; 52.5% of these had titers of $\geq$40, and only two cases had titers of $<$5. Only 12 cases were clearly EBNA-positive. The EBNA-positive cells ranged from single epithelial cells to clusters of 10-100 cells. Thus, it appears that EBV immunovirology was able to support the tentative

diagnosis of NPC. Moreover, the significance of the fossa of Rosenmueller being the site of initial development of NPC was confirmed by finding the EBNA-positive cells in these 12 cases. The CAT scan further supported the suggestion that the cells lining the fossa of Rosenmueller were the first to experience malignant changes in NPC.

4. Since we had previously found LSI activity in the serum IgA fraction of NPC patients with active or progressing tumor, we used four IgA-depleted sera to search for the removal of inhibitory activity in EBV-induced lymphocyte stimulation. One serum produced 40% inhibition, suggesting the presence of other inhibitory factors besides LSI. The IgA antibodies bind to EBV virus particles and can subsequently be removed by ultracentrifugation with consequent loss of LSI activity. Three of the four sera did not affect the response of sensitized lymphocytes to EBV antigens, since lymphocytes which were washed well after preincubation with these sera had a blastogenic response comparable to that of the control.

5. In collaboration with Prof. Prathap, University of Malaya, adequately biopsied nasopharyngeal tissue from 434 consecutive Malaysian patients with NPC were reviewed. The patients were Chinese, Malaysians and of other ethnic origins. A notable feature and one to which attention does not appear to have been drawn in the past was the presence of amyloid infiltration in 12% of cases. Biopsies from 20 patients were examined for EBNA-positive cells. The EBNA-positive cells were from a squamous cell carcinoma (SCC), and undifferentiated carcinoma (UC); no EBNA was observed in cells from nonkeratinizing carcinomas (NKC) or other tumors of the head and neck region. There were 164 cases with no clear-cut histologic classification. Fifty-three cases were SCC + NKC, 69 had SCC + UC, 31 were UC + NKC, and 11 were SCC + NKC + UC. The findings suggest SCC, NKC, and UC of the nasopharynx, as defined by the World Health Organization, are variants of a fairly homogeneous group of neoplasms in Singapore and Malaysian NPC, and may well be similar to those found in Hong Kong and China. The presence of EBNA in SCC was surprising because previous reports had suggested the presence of EBNA only in UC cells. This may very well be dependent upon the degree of cell differentiation.

6. In collaboration with Dr. Dahlberg, 22 monoclonal antibodies to HVS were characterized as to their reactivities against HVS early and late antigen by IF and neutralization using two strains of HVS (prototype and OMI). Using the tissue culture fluid as a source of antibody, six antibodies showed weak IF reactivity in the cytoplasm or nucleus of the HVS-infected OMK cells. However, when ascites fluid was used as a source of antibody, reactivity was demonstrated by strong intranuclear or cytoplasmic staining. One antibody was specific for the nucleus of OMK cells infected with the HVS prototype and OMI strains. However, it failed to react with OMK uninfected cells or with OMK cells infected with HVA-73 and HVA-810. These two H. ateles viruses possess common IF reactivities when sera from animals infected with HVS are used. The other monoclonals, which fall into 10 groups, gave strong IF reactions ranging from generalized diffuse or punctate staining of the cytoplasm.

Some monoclonals also gave marginal staining of the nuclear membrane and staining at the surface of the infected cell. All monoclonal antibodies were of the IgG class. None of these were positive for HVS-EA and also failed to react with HVA-810 and HVA-73. The reactivity of these antibodies correlated well with immunoprecipitation. None of the monoclonals neutralized either strain of HVS. However, three of the 11 monoclonal antibodies did produce some reduction in HVS-induced plaques.

Significance to Biomedical Research and the Program of the Institute:

It is significant that the use of EBV-VCA/IgA antibody titers can be used in following individuals at high risk for developing NPC, thus providing early diagnosis and increased opportunities for arresting tumor activity. Secondly, the use of EBV titers, particularly IgA, could aid in confirming the diagnosis of NPC in tumors of the head and neck, or in correcting misdiagnoses. The presence of EBNA-positive cells in the fossa of Rosenmueller supports the presumption that the cells lining the fossa are those which experience the first neoplastic changes in NPC. These results also strengthen the etiologic role of EBV in NPC. The specificity of IgA antibody in NPC was confirmed by depletion of IgA from the sera of NPC patients in active disease. This observation suggests that consideration should be given to removing serum IgA from NPC patients to improve their prognosis and perhaps to shed some light on the mechanism of LSI blocking. The total number of B and T cells in NPC patients and skin testing can thus be used as markers in therapy, because the total number of B and T cells decreased after therapy and increased in relapse. Persistent low delayed hypersensitivity was associated with a less favorable prognosis, and enhanced reactivity was predictive of good survival. The presence of amyloid infiltration in 12% of NPC cases had never before been reported and should be followed to help determine the role of amyloids in NPC. The lack of clear-cut histologic classification in 164 NPC cases from Malaysia suggested either that this group of neoplasms in the far east is extremely homogeneous, or that the histologist(s) had not carefully studied the slides.

The HVS reactivities of HVS monoclonal antibodies revealed that these could be useful in studying the strain differences and identification of new isolates, as well as in identification of viral proteins/glycoproteins. Since one monoclonal is specifically reactive by IF in the nucleus of an HVS-infected cell, it may have cellular components that give EBNA-type reactivities. Such an antibody may be important in identification of the HVS genome in tumor cells or in HVS-transformed cell lines.

Proposed Course:

These projects will be followed up, taking advantage of new findings and developments, for as long as the Principal Investigator remains in laboratory research.

Publications:

Ablashi, D. V.: Possible approaches for the prevention of nasopharyngeal carcinoma. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Ablashi, D. V., Aulakh, G. S., Luetzeler, J., Sundar, K. S., Armstrong, G. R. and Faggioni, A.: Fatal lymphoproliferative disease in a common marmoset (Calithrix jacchus) following inoculation of AG-876 strain of EB virus and a tumor promoting agent: Preliminary report. Comp. Immunol. Microbiol. & Infect. Dis. 6: 151-160, 1983.

Ablashi, D. V., Baron, S., Armstrong, G., Faggioni, A., Viza, D., Levine, P. H. and Pizza, G.: Spontaneous production of high levels of leukocyte (α) interferon by a human lymphoblastoid B cell line (LDV/7). Exp. Biol. Med. 171: 114-119, 1982.

Ablashi, D. V., Levine, P. H., Prasad, U. and Pearson, G. R.: Fourth International Symposium on Nasopharyngeal Carcinoma: Application of field and laboratory studies to the control of NPC. Cancer Res. 43: 2375-2378, 1983.

Ablashi, D. V., Prasad, U., Pearson, G., Prathap, K., Armstrong, G. R., Faggioni, A., Yadav, M., Easton, J. M., Chan, S. H. and Levine, P. H.: EBV-related studies with clinicopathological correlation in Malaysian nasopharyngeal carcinoma. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Armstrong, G. R., Longo, D., Faggioni, A., Ablashi, D., Pearson, G. and Slovin, S.: Detection and isolation of Epstein-Barr virus in lymphocytes from patients with chronic B lymphocytic leukemia (CLL). In Magrath, I. T., O'Conor, G. T. and Ramon, B. (Eds.): Role of the Environment and Pathogenesis of Leukemia and Lymphoma. New York, Raven Press. (In Press)

Bertram, G., Pearson, G. R., Faggioni, A., Krueger, G. R. F., Sesterhenn, K., Ablashi, D. V. and Levine, P. H.: A long-term study of EBV and non-EBV related tests and their correlation with the clinical course of nasopharyngeal carcinoma. In Prasad, U., Ablashi, D. V., Levine P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Cowan, F. M., Klein, D. L., Armstrong, G. R., Ablashi, D. V., Pearson, G. R. and Bensinger, W. I.: Inhibition of rat adenocarcinoma metastases by staphylococcus aureus protein A. Biomed. and Pharmacotherapy 36: 29-31, 1982.

Faggioni, A., Ablashi, D. V., Armstrong, G., Dahlberg, J., Sundar, S. K., Rice, J. M. and Donovan, P. J.: Enhancing effect of N-methyl-N-nitroso-guanidine (MNNG) on Epstein-Barr virus (EBV) replication and comparison of short term and continuous TPA treatment of nonproducer and producer cells for EBV antigen induction and/or stimulation. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Gerber, P., Ablashi, D., Magrath, I., Armstrong, G., Andersen, P. and Trach, L.: Persistence of transforming and nontransforming virus in high passage P3HR-1 cell lines. J. Natl. Cancer Inst. 69: 586-590, 1982.

Kamaraju, L. S., Levine, P H., Sundar, S. K., Ablashi, D. V., Faggioni, A., Armstrong, G. R., Bertram, G. and Krueger, G. R. F.: Epstein-Barr virus-related lymphocyte stimulation inhibition: A possible prognostic tool for undifferentiated nasopharyngeal carcinoma. J. Natl. Cancer Inst. 70: 643-647, 1983.

Kamaraju, L. S., Levine, P. H., Sundar, S. K., Ablashi, D. V., Faggioni, A., Armstrong, G. R., Bertram, G. and Krueger, G. R. F.: Epstein-Barr virus-related lymphocyte stimulation inhibition as an indicator of active disease activity in undifferentiated nasopharyngeal carcinoma. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Levine, P. H., Nkrumah, F., Ablashi, D. V., Pearson, G. R., Faggioni, A., Viza, D., Lvovsky, E. and Pizza, G.: Clinical and experimental data on the effects of antiviral agents against oncogenic herpesviruses: Implications for the treatment of nasopharyngeal carcinoma. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Pizza, G., Levine, P. H., Ablashi, D. V., Armstrong, G., Bengali, Z. and Canon, G. B.: Variation in the immune response to Herpesvirus saimiri in squirrel and rhesus monkeys. Comp. Immunol. Microbiol. Immunol. Infect. Dis. 5: 437-446, 1982.

Prathap, K., Prasad, U. and Ablashi, D. V.: The pathology of nasopharyngeal carcinoma in Malaysia. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Prasad, U., Ablashi, D. V., Prathap, K., Yadav, M., Singaram, S. P., Singh, P. and Singh, I.: Problem of occult primary in nasopharyngeal carcinoma. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Strauss, S. E., Armstrong, G. R., Seidlin, M., Horneff, J., Clark, J., Longo, D., Faggioni, A., Pearson, G. and Ablashi, D. V.: Acyclovir treatment of human herpesvirus infections: Implications for the treatment of Epstein-Barr virus (EBV)-related disorders. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press)

Sundar, S. K., Menezes, J., Levine, P. H., Ablashi, D. V., Faggioni, A., Kamaraju L. S. and Prasad, U.: Studies on the relationship of IgA to a lymphocyte stimulation inhibitor in patients with undifferentiated nasopharyngeal carcinoma. In Prasad, U., Ablashi, D. V., Levine, P. H. and Pearson, G. R. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Kuala Lumpur, University of Malaya. (In Press).

Viza, D., Boucheix, C., Cesarian, J. P., Ablashi, D. V., Armstrong, G., Levine, P. H. and Pizza, G.: Characterization of a human lymphoblastoid cell line, LDV/7, used to replicate transfer factor and immune RNA. Biol. Cell 46: 1-10, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05112-04 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Murine Leukemia Viruses: Mechanism of Leukemogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
T. Storch Research Associate, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have sought to understand the mechanisms by which murine leukemia viruses (MuLV) induce tumors by identifying: 1) preleukemic changes in the lymphocyte compartment of MuLV-infected mice using antibodies to cell surface antigens and flow cytometry; and 2) specific defects in lymphocyte ontogeny and function that confer resistance to leukemogenesis. We find that the preleukemic period for Moloney-MuLV-infected mice has three distinct phases: 1) cells with an altered expression of Thy 1 antigen appear in the thymus; 2) the number of lymphohemopoietic precursors in the spleen increases significantly; 3) the turnover of lymphohemopoietic precursors increases significantly. These preleukemic changes are delayed in CBA/N mice, which are resistant to leukemogenesis. Resistance is associated with at least two autosomal genes, one of which controls dualtropic virus replication. An X-linked resistance gene can also be demonstrated in crosses to susceptible strains.

PROJECT DESCRIPTION

Name, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. Aaronson	Chief	LCMB, NCI
P. Arnstein	Veterinary Officer	LCMB, NCI

Objectives:

To understand how murine leukemia viruses (MuLV) cause lymphoma, we will identify 1) the subpopulations of lymphoid cells in MuLV-infected animals that govern susceptibility and resistance to T- and B-cell lymphoma, and 2) the region of the MuLV genome responsible for transforming specific lymphoid cells.

Methods Employed:

Mice were infected with clonal MuLVs that transform specific lymphoid targets or MuLV recombinants that had been generated using selective tissue culture conditions. Targets for infection and preleukemic changes in composition and function within the lymphoid system were identified using fluorescent antibodies to viral and lymphoid antigens, fluorescent DNA stains and flow cytometry. Mice with genetic defects in lymphocyte ontogeny and function were screened for resistance to and alteration of the target cell for transformation.

Major Findings:

Moloney-MuLV induces a T-cell lymphoma while Rauscher-MuLV induces a pre-B-cell lymphoma and a small proportion of erythroleukemias. Thymocytes from preleukemic Moloney-MuLV-infected mice express significantly higher levels of ecotropic and xeno/dualtropic viruses than thymocytes from Rauscher-MuLV-infected mice. Spleen and bone marrow cells from Moloney- and Rauscher-MuLV-infected mice express similar levels of ecotropic virus, but expression of xeno/dualtropic virus is significantly higher in Rauscher-MuLV-infected mice.

Following the establishment of viremia, the preleukemic period for Moloney-MuLV-inoculated mice has three distinct phases. In the first phase, cells with an altered expression of Thy 1 antigen appear in the thymus. In the second, spleen size increases until it is twice that of uninfected controls. Most of this increase is due to null cells and is associated with a greater proportion of dividing cells in the spleen, but not in the thymus or marrow. In the third, the spleen size of infected mice remains constant, but the fraction of dividing spleen cells is 4-fold greater than in uninfected mice due to an increased proliferation of B-cells and null cells. Most of the null cells express determinants associated with precursors of lymphocytes and hemopoietic cells. Similar preleukemic changes occur in a variety of inbred mouse strains with different H-2 haplotypes. These preleukemic changes are delayed in Moloney-MuLV-infected CBA/N mice, which are resistant to leukemogenesis. CBA/N resistance is not due to expression of xid since

CBA/CaHN mice which do not carry xid have a similar survival time. Resistance is due to at least two autosomal genes, one of which controls dualtropic virus replication. An X-linked resistance gene can also be demonstrated.

Significance to Biomedical Research and the Program of the Institute:

Understanding the mechanisms by which murine leukemia viruses transform specific lymphoid cells may provide important insights into the alterations that lead to naturally occurring malignancies of these cell types.

Proposed Course:

Project will terminate in Fiscal Year 1983.

Publications:

Storch, T. G., Arnstein, P., Manchar, V., Leiserson, W. M. and Chused, T. M. Proliferation of infected lymphoid precursors precedes T cell lymphoma induced by Moloney leukemia virus. J. Immunol. (In Press)

Storch, T. G. and Chused, T. M. Genetic analysis of CBA/N resistance to induction of T-cell lymphoma by Moloney leukemia virus. J. Exp. Med. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05159-03 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunoglobulin and c-myc Rearrangements in MuLV-Transformed Lymphoid Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
R. Balachandran · Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A large number of tumors and cell lines of BALB/c and NFS/N strains of mice transformed by Rauscher and Moloney murine leukemia viruses (R-MuLV and M-MuLV) were established in continuous culture and tested for the presence of Fc receptors, as well as intracellular and surface IgM molecules. The preliminary results indicated that these cell lines belong to different stages of B cell development. R-MuLV appears either to induce transformation of a stem cell that undergoes further differentiation and becomes blocked at various stages of development, or transforms B-cells at various stages of differentiation. Further analysis of the nature of immunoglobulin gene rearrangements suggested that these cell lines belong to five successive stages of B cell development or B-cell lineages. Since the c-myc gene is observed to play a role in plasmacytomagenesis, R-MuLV-induced cell lines were studied for c-myc rearrangement. The 5' Eco RI and Hind III sites were rearranged in cells belonging to a particular developmental lineage.

In T cells transformed by M-MuLV, the immunoglobulin gene rearrangement is mainly restricted to the heavy chains. The c-myc rearrangement was rare in these cell lines.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI
D. Swan	Expert	LCMB, NCI

Objectives:

To study immunoglobulin and c-myc rearrangements in lymphoid cell lines transformed by murine leukemia viruses.

Methods Employed:

Tissue culturing of lymphoid cells, DNA extraction, restriction enzyme analysis of cellular DNA, hybridization studies with immunoglobulin and myc-related probes.

Major Findings:

R-MuLV transforms three categories of lymphoid cells belonging to B-cell lineage. One category completely lacked IgM expression, while a second possessed intracellular IgM heavy chains and surface μ chains, but completely lacked the κ light chains. A third category synthesized complete IgM molecules consisting of μ and κ chains. Studies on the nature of heavy and light chain rearrangements in immunoglobulin-negative cell lines provided a more precise stepwise sequence of events that might occur during B cell differentiation. Analysis of the cells for the presence of Fc receptors, IgM expression and immunoglobulin gene rearrangement has shown that the cells transformed by R-MuLV belong to stepwise five compartments of B cell differentiation. The earliest identifiable cells of the B-cell lineage are without any rearrangement of immunoglobulin genes. These cells possess Fc receptors, but they neither synthesize the IgM nor possess any characteristics of other hematopoietic cells. A second category of cells displayed rearrangements of heavy chains. These findings argue that these are highly immature cells of B-cell lineage. A third category of cells arose when pre-B cells were analyzed for immunoglobulin gene rearrangement. A small number of pre-B cells possessed rearranged light chains without light chain expression. A negligible fraction of cells transformed by R-MuLV are complete IgM-synthesizing B-cells. These cells possess heavy and light chain rearrangements.

In the T lymphoid cells transformed by M-MuLV, the immunoglobulin rearrangement is mainly restricted to heavy chain genes. Rearrangement of the c-myc gene is also rare.

Significance to Biomedical Research and the Program of the Institute:

The B- and T-lymphoid cells transformed by R-MuLV and M-MuLV detailed in this report provide a model system for continued study of the nature of immunoglobulin genes during B-cell development and rearrangement of developmentally regulated expression of cellular oncogenes.

Proposed Course:

Project is completed.

Publications:

Aaronson, S. A., Storch, T. G., Balachandran, R. and Reddy, E. P.: Different hematopoietic target cells for transformation by replication-competent murine leukemia viruses. In Marchesi, V. T. and Gallo, R. C. (Eds.): Differentiation and Function of Hematopoietic Cell Surfaces. New York, Alan R. Liss, Inc., 1982, pp. 251-261.

Balachandran, R. and Srinivasan, A.: Caffeine inhibits DNA polymerase I from Escherichia coli. Studies in vitro. Carcinogenesis 3: 151-153, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05164-03 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interaction of Hematopoietic Cells and Mammalian Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. H. Pierce Staff Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A recombinant transforming murine retrovirus which contains the src gene of the avian retrovirus, Rous sarcoma virus, was utilized in a bone marrow colony-forming assay that detects transformation of murine hematopoietic cells. This virus was shown to be capable of transforming immature murine hematopoietic cells in vitro.

Harvey and BALB murine sarcoma viruses were shown to promote the growth and differentiation of myeloid precursor cells in vitro.

The induction of leukemogenesis by Moloney murine leukemia virus was demonstrated to be a multistep process. During the first stage, animals were viremic, but no overt symptoms appeared. In the second stage, the hematopoietic system underwent a greatly enhanced proliferative response, while hematopoietic cells remained nonmalignant. Only the final stage of the disease was representative of an overtly malignant state in which spleen cells from the infected animal were capable of forming transformed colonies in soft agar and transplantable tumors in recipient animals.

Several human hematopoietic tumors of diverse phenotypes were shown to possess transmissible onc genes by utilizing DNA transfection techniques. The onc genes of many were shown to be related to the neuroblastoma gene, n-ras, while one was related to the Kirsten murine sarcoma virus gene, kis. The activation of ras-related oncogenes appeared to be independent of hematopoietic tumor phenotype.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
P. E. Reddy	Visiting Scientist	LCMB, NCI
R. Balachandran	Visiting Fellow	LCMB, NCI
A. Eva	Visiting Associate	LCMB, NCI
D. Swan	Expert	LCMB, NCI

Objectives:

1. Investigate the ability of replication-defective retroviruses other than Abelson murine leukemia virus to induce in vitro transformation or alter the growth and differentiation properties of murine hematopoietic cells utilizing in vitro soft agar colony methods.

2. To determine the mechanism by which replication-competent chronic murine leukemia viruses induce leukemogenesis in vivo.

3. Analyze human hematopoietic tumors and cell lines of diverse phenotypes for oncogenes by detection of transforming activity in NIH/3T3 transfection assays.

Methods Employed:

Standard hematopoietic cell culture techniques included an in vitro hematopoietic colony formation assay developed to detect transformation of murine lymphoid cells, establishment of transformed colonies into continuous cell lines, and cloning of established lines in soft agar.

Identification of the hematopoietic phenotype of retrovirus transformants was performed utilizing histochemical staining, immunofluorescence techniques, and biochemical assays such as radioimmunoprecipitation and enzyme assays.

A quantitative spleen cell colony assay was also developed to detect the presence of transformed hematopoietic cells after in vivo infection with chronic murine leukemia viruses. Transplantation experiments were also employed utilizing subcutaneous inoculation of inbred strains to determine the tumorigenicity of in vivo derived spleen cells.

Standard NIH/3T3 cell transfection assays were utilized to detect transforming genes from human hematopoietic tumors. Cloning of transfected foci was performed in soft agar. Characterization of human hematopoietic cell phenotypes was determined by immunofluorescence techniques.

Major Findings:

1. Avian Rous sarcoma virus (RSV) induces sarcomas in avian species, but it has not been demonstrated to have any tranforming effect on hematopoietic

cells in the avian system. A recombinant transforming murine retrovirus was constructed which contains the src gene of RSV inserted in amphotroph-murine leukemia virus, a murine replication-competent helper virus which has no transforming activity in vitro. The resulting replication-defective murine retrovirus was shown to produce the transforming gene product of RSV, $pp60^{src}$, and to induce tranformed foci on NIH/3T3 when pseudotyped with a variety of helper viruses. This virus was capable of inducing sarcomas as well as an acute leukemia in newborn and adult mice. An in vitro hematopoietic colony-forming assay was utilized to demonstrate that the recombinant src-containing murine retrovirus was capable of inducing transformed murine hematopoietic cell colonies in culture. In contrast, no colony-forming activity was observed when the same virus or wild type RSV was employed with avian bone marrow cells in a similar hematopoietic assay which detected transformation by other avian retroviruses.

Murine bone marrow colony formation was found to be sarcoma virus-dependent, followed single-hit kinetics and required the presence of mercaptoethanol in the agar medium. Cells from the colonies induced by the recombinant src-containing virus could be established as continuous cell lines which demonstrated unrestricted self-renewal and leukemogenicity in vivo. We are presently attempting to further characterize their phenotype and stage in hematopoietic cell differentiation. These results demonstrated that the target cell specificity of a particular onc gene product may be altered depending on the animal species in which the onc gene acts.

2. BALB and Harvey murine sarcoma viruses (MSVs) are part of a family of retroviruses whose mouse and rat-derived onc genes are closely related. In addition to their ability to cause solid tumors, these viruses have been shown to induce leukemia in susceptible mice. It has previously been demonstrated that these viruses are capable of inducing proliferation and differentiation of cells of the erythroid series in vitro and erythroleukemia in vivo. We have also shown that these viruses are able to transform a lymphoid progenitor cell both in culture and in vivo.

   In our present investigation, we have demonstrated that BALB- and Harvey-MSVs are also capable of affecting the growth properties of cells within the myeloid lineage. Murine bone marrow cells were infected in vitro with BALB- or Harvey-MSV and then cultured in soft agar without the addition of exogenous growth factors. Under these conditions, large diffuse colonies of myeloid cells formed in the semi-solid agar medium 6 to 8 days after infection. The presence of transforming sarcoma virus in the cells from these colonies could be detected by infectious center assay on murine NIH/3T3 cells. The induction of myeloid colonies could not be demonstrated by the murine type C helper viruses used to pseudotype either virus or by culture fluids from nonproducer cells containing the BALB- or Harvey-MSV genomes. Although the myeloid colonies were able to proliferate to a macroscopic size in agar, cells from these colonies were not capable of continued proliferation in culture. Phenotypic analysis of

the cells revealed that the majority expressed markers associated with mature macrophages, including nonspecific esterase, lysozyme production, and the ability to phagocytize latex beads. The effects of BALB- and Harvey-MSV on cells within the myeloid lineage appear to resemble the growth-promoting and differentiating action of these same viruses on erythroid precursor cells, rather than the direct transforming effect they possess on lymphoid precursors. These results demonstrate that the bas- and has-containing retroviruses are capable of altering the growth properties of target cells within all three hematopoietic lineages.

3. The induction of leukemogenesis by Moloney murine leukemia virus was investigated by utilizing a spleen cell colony-forming assay which detects transformed lymphoid cells present in infected animals. Subcutaneous transplantation studies were also employed in conjunction with the colony assay as another method for detecting tumorigenic leukemia cells. During the earliest stages of the disease (1-4 weeks), no demonstrable effects on the hematopoietic cell system were observed. Colony formation could not be detected from spleens of infected animals and these spleen cells were not transplantable in syngeneic mice.

   The second phase of the disease (4-8 weeks) was characterized by splenomegaly, thymic and lymph node enlargement. During this phase, spleen cells formed only small colonies or no colonies in soft agar. In many cases, the spleen cells were not capable of forming subcutaneous tumors but were capable of homing to the spleen and lymph nodes, inducing a rapid proliferative disease in the animal. After serial transplantation of spleens from this second phase of the disease, subcutaneous tumors eventually formed and colony formation in agar could be observed. Only during the final stage of the disease process (9-12 weeks) were spleen cells from the original animal capable of forming colonies in soft agar which were establishable as continuous cell lines in culture and were able to form subcutaneous tumors after transplantation.

   These results indicate that chronic leukemia viruses may act by inducing proliferation of a subset of hematopoietic cells which allow for the selection of a malignant cell in the advanced stage of the disease.

   We surveyed 22 human hematopoietic tumors and tumor cell lines for sequences capable of transforming NIH/3T3 cells by DNA transfection. A primary human acute myelogenous leukemia, a chronic myelogenous leukemia cell line, and cell lines derived from three independent acute lymphocytic leukemias demonstrated oncogenes capable of conferring the transformed phenotype to NIH/3T3 cells through serial cycles of transfection. One of three transforming genes associated with acute lymphocytic leukemia cells (classified as thymocyte developmental stage II) was identified as the activated cellular homologue of the Kirsten murine sarcoma virus onc gene, kis, a member of the ras family of onc genes. A transforming gene, which was demonstrated to be common to several human myeloid and lymphoid tumor cells, was shown to be a distantly related member of the ras gene family. Thus, the NIH/3T3 transfection assay commonly detects related oncogenes in human hematopoietic tumor cells. Moreover, the activation of these oncogenes appears to be independent of the specific stage of cell differentiation or tumor phenotype.

Significance to Biomedical Research and the Program of the Institute:

Investigation of the diversity of hematopoietic target cells for neoplastic transformation by a particular transforming retrovirus may provide insights into pathways by which these viruses exert their oncogenic potential and, in particular, the relationship of the differentiated state of the cell to its susceptibility to onc gene action. Analysis of human hematopoietic tumors for transmissible onc genes and the mechanism by which these onc genes act in maintaining transformation at the level of RNA and protein expression may help to determine how these genes function and are activated in human leukemogenesis.

Proposed Course:

1. We are currently analyzing the phenotypic characteristics of the hematopoietic cell lines transformed by the src-containing murine retrovirus in order to determine if in vitro functional similarities between the onc gene product of this virus and other mammalian replication-defective retroviruses may reflect a common pathway by which they exert their oncogenic potential. Other recombinant retroviruses are presently being generated in order to expand the understanding of the action of diverse onc genes under identical assay conditions.

2. We are interested in determining whether BALB- or Harvey-MSV hematopoietic cell transformants display immunoglobulin gene rearrangement in order to more precisely define their stage in the lymphoid developmental pathway. We also intend to determine whether these lymphoid progenitor cell transformants have the capacity to differentiate along either T or B cell developmental pathways following transformation by temperature-sensitive mutants or exposure to agents which promote differentiation in other systems. We have currently isolated two cell lines transformed by a temperature-sensitive mutant of Kirsten-MSV, another member of the ras gene family, and are investigating the ability of these lines to differentiate upon shift to the nonpermissive temperature. These transformants should prove a useful approach for analysis of lymphoid cell ontogeny and the possible function of TdT in normal lymphoid development. They should also help to confirm whether transformation of hematopoietic cells results from a block in differentiation caused by the action of a transforming protein.

3. Efforts are underway to more precisely define the mechanism by which hematopoietic cell transition from nonmalignant to a malignant stage occurs during the three proposed stages of chronic leukemogenesis by analyzing the clonality of spleen cells at the various stages of the disease through the use of cell surface markers and TdT enzyme assays. Transfection with DNA derived from spleen cells representing the different stages will be attempted in order to determine whether onc gene activation is involved in the transition. We also plan to analyze the expression of p53, a previously defined transformation-related protein, in the spleen cells at the various stages of Moloney-MuLV-induced disease since this protein has been implicated as playing a role in malignant transformation caused by Friend-MuLV virus.

We are attempting to develop an in vitro system in which stage 2 (premalignant) Moloney-MuLV-infected cells can be converted to stage 3 (malignant) cells, utilizing lymphoid cell growth factors or feeder layer techniques.

4. Studies are underway to determine whether expression of the p21 transforming protein is related to maintenance of the transformed state in the human hematopoietic cell lines we have studied by transfection techniques and are know to possess activated ras-related onc genes. One such cell line has been found to be inducible for differentiation along the T cell pathway by phorbol esters. The levels of p21 expression before and after induction are presently being analyzed. The ability of DNA isolated from this line to induce transformation of 3T3 cells before and after induction will also be investigated.

5. The ability of amphotroph-MuLV or primate helper virus pseudotypes of BALB-and Harvey-MSV and Abelson-MuLV to induce transformation of normal human cord blood or bone marrow in vitro will be analyzed.

6. Transfection techniques will be employed to determine whether normal murine bone marrow or factor-dependent murine hematopoietic cells are capable of being transformed by DNA-mediated gene transfer.

Publications:

Aaronson, S. A., Reddy, E. P., Robbins, K.C., Devare, S. G., Swan, D. C., Pierce, J. H. and Tronick, S. R.: Retroviruses, onc genes and human cancer. In Harris, C.C. and Autrup, H. N. (Eds.): Human Carcinogenesis. New York, Academic Press. (In Press)

Eva, A., Tronick, S., Gol, R., Pierce, J. H. and Aaronson, S. A.: Transforming genes of human hematopoietic tumors: Frequent detection of ras-related oncogenes whose activation appears to be independent of tumor phenotype. Proc. Natl. Acad. Sci. USA. (In Press)

Pierce, J. and Aaronson, S.A.: BALB- and Harvey-MSV transformation of a novel lymphoid progenitor cell. J. Exp. Med. 156: 873-887, 1982.

Pierce, J. and Aaronson, S. A.: In vitro transformation of murine pre-B lymphoid cells by Snyder-Theilen feline sarcoma virus. J. Virol. 46: 993-1002, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05166-03 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Mechanisms Involved in Transforming Activity of Human Protooncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. Pulciani Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have applied genetic engineering and gene transfer techniques to analyze cancer development. By DNA-mediated gene transfer experiments, we demonstrated the presence of transforming genes in human cell lines established from neoplastic tissues, as well as human primary tumors. The oncogenes present in carcinomas of the colon, lung, gall bladder, pancreas, urinary bladder, as well as rhabdomyosarcomas, were molecularly characterized and found to be homologous to the Kirsten-MSV transforming gene.

The transforming gene present in a bladder carcinoma cell line, T24, was molecularly cloned and shown to be a mutated allele of the human c-has/bas-1 protooncogene, the human genetic sequences related to the BALB-MSV transforming gene. A single base pair change was found to be directly responsible for the neoplastic properties of the oncogene isolated from the T24 cell line. Recently it was reported that the c-has/bas-1 protooncogene assumed transforming activity when under the transcriptional control of viral LTR, which enhances its expression levels.

We planned transfection experiments to learn whether the c-has/bas-1 human protooncogene could by itself cause transformation. NIH/3T3 cells were transfected with large amounts of the c-has/bas-1 protooncogene to provide recipient cells with multiple (>40) copies of this protooncogene integrated into their genomes; all were characterized as transformed. However, DNA from the NIH/3T3 transformants failed to transfer the transformant phenotype in successive cycles of transfections, indicating multiple copies of the c-has/bas-1 protooncogene are necessary for transformation. During characterization of the NIH/3T3 transformants containing many copies of the c-has/bas-1 protooncogene, we isolated an in vitro activated oncogene in which the acquired transforming activity was switched on by a single point mutation located in the same position as that found in the T24 oncogene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
K. C. Robbins	Expert	LCMB, NCI
S. R. Tronick	Microbiologist	LCMB, NCI
M. Barbacid	Visiting Scientist	LCMB, NCI

Objectives:

To analyze the physiological properties of the human c-has/bas-1 protooncogene.

Methods Employed:

The techniques utilized were DNA extraction and DNA transfection. The DNAs were extracted from a variety of malignant transformed cells and human tumors and then added as calcium phosphate coprecipitates to NIH/3T3 cells. The foci of transformed cells were then analyzed by Southern blotting procedures to detect the presence of newly acquired genetic information.

Major Findings:

By DNA-mediated gene transfer experiments, we have shown that human tumor cell lines, as well as primary human solid tumors, contain a dominant transforming gene. Several human tumor cell lines, established from carcinomas of the colon, lung, and gall bladder, unmanipulated tumors including carcinomas of the pancreas and rhabdomyosarcomas, possess the same oncogene. The results strongly exclude the possibility that cellular transforming genes might have been activated during in vitro manipulation of the cell cultures, and point out also that clinically different human tumors might have a similar origin, since they share a common oncogene.

Subsequent molecular characterization of the human dominant transforming genes present in the rhabdomyosarcoma and the carcinomas of the colon, lung, gall bladder, urinary bladder and pancreas revealed their homology with the oncogene of Kirsten-MSV. The human c-has/bas-1 protooncogene homologue to the BALB-MSV transforming gene (v-bas) and the normal allele of the oncogene isolated from T24, a human carcinoma bladder cell line, was shown to have transforming activity when integrated in multiple copies (>40) in the cellular genome of NIH/3T3 cells. During transfection experiments, we isolated a transforming mutated form of the c-has/bas-1 protooncogene, whose malignant acquired properties were sparked by a single base mutation, as reported for the T24 oncogene.

Significance to Biomedical Research and the Program of the Institute:

Identification of transforming genes present in human tumor cells and the characterization of the molecular mechanisms by which they are able to cause transformation are important steps toward understanding human neoplasia.

Proposed Course:

Biochemical and biological analysis of molecularly cloned human DNA analogues of retroviral onc genes will continue.

Publications:

Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Aaronson, S. A. and Barbacid, M. B.: Oncogenes in solid human tumors. Nature 300: 539-542, 1982.

Pulciani, S., Santos, E., Lauver, A., Long, L. K. and Barbacid, M.: Transforming genes in human tumors. J. Cell. Biochem. 20: 51-61, 1982.

Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Robbins, K. C. and Barbacid M.: Oncogenes in human tumor cell lines: Molecular cloning of a transforming gene from human bladder carcinoma cells. Proc. Natl. Acad. Sci. USA 79: 2845-2849, 1982.

Rossi, G. B., Affabris, E., Belardelli, F., Pulciani, S., Titti, F., Dolei, A., Capobianchi, M. R. and Peschle, A.: Possible ruolo del genoma virali nel controllo del differenziamento eritroide delle cellule eritroleuicemiche di Friend. In Piccin, A. (Ed.): I Seminario Nazionale Virus Oncogeni a RNA, 1981, pp. 332-349.

Santos, E., Pulciani, S. and Barbacid, M.: Characterization of a human transforming gene isolated from T24 bladder carcinoma cells. Fed. Proc. (In Press)

Santos, E., Reddy, E. P., Pulciani, S., Feldmann, R. J. and Barbacid M.: Spontaneous activation of a human proto-oncogene. Proc. Natl. Acad. Sci. USA. (In Press)

Santos, E., Tronick, S., Aaronson, S. A., Pulciani, S. and Barbacid, M.: The T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature 298: 343-347, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05167-03 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analysis of the Transforming Gene of Simian Sarcoma Virus

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
K. C. Robbins Expert, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The transforming gene of simian sarcoma virus (SSV), v-sis, directs the synthesis of a protein of 28,00 daltons, p28sis. Biochemical characterization of p28sis has revealed that the molecule is a membrane-associated protein which is synthesized in the endoplasmic reticulum, glycosylated, and then tranformed to the cell surface where it is cleaved into proteins of 11,000 and 20,000 daltons. The exact glycosylation site of p28sis has been identified and the site of its cleavage is known to within 10-15 amino acids.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
S. R. Tronick	Head, DNA Recombinant Unit	LCMB, NCI
D. Swan	Expert	LCMB, NCI
A. Eva-Varesio	Visiting Associate	LCMB, NCI
A. Srinivasan	Visiting Associate	LCMB, NCI
S. Devare	Visiting Associate	LCMB, NCI
P. Reddy	Visiting Scientist	LCMB, NCI
A. Gazit	Visiting Fellow	LCMB, NCI
G. Naharro	Visiting Fellow	LCMB, NCI
H. Igarashi	Visiting Fellow	LCMB, NCI

Objectives:

1. To examine the interaction of the SSV-transforming protein (p28^{sis}) with other cellular components.

2. To assess the role of *sis*-related genes in human neoplasia.

3. To determine the mechanism of SSV-induced oncogenesis and to apply this knowledge to understanding the etiology of cancers in humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of gene products; in vitro synthesis and immunoprecipitation; peptide synthesis; antibody production; fractionation of cellular components and protein purification to analyze gene products; molecular hybridization techniques to analyze genes; recombinant DNA techniques for the purification and amplification of genes; analysis of genetic structure using restriction endonuclease mapping, nucleotide sequencing, and electron microscopy techniques; DNA transfection and construction of virus mutants for analysis of transforming activity.

Major Findings:

1. We sought to identify the species of origin of the cell-derived *sis* sequences of SSV. A molecular clone comprised of *sis* DNA detected related nucleotide sequences at low copy number in normal cellular DNAs of species as diverse as humans and quail. The extent of hybridization and degree of base-pair matching with *sis* DNA were greatest with New World primate DNAs. The thermal denaturation curve midpoints of hybrids formed between *sis* and woolly monkey DNAs were indistinguishable from homologous *sis* DNA hybrids, establishing the woolly monkey *Lagothrix spp.* as the source of *sis* sequences. In comparative studies, *sis* was shown to be more conserved among mammalian species than unique-sequence woolly monkey cellular DNA. There was no detectable homology between *sis*

and the cell-derived sequences of other fibroblast-transforming retroviruses. These findings indicate that sis is likely to be a unique onc gene among transforming retroviruses.

2. To investigate the possible role of retrovirus onc-related genes in human neoplasia, we have analyzed human tumor cell lines for evidence of expression of onc-related genes. Our demonstration of SSV sis-related transcripts in human fibrosarcoma cell lines, but not in their normal fibroblast counterparts, argues that the expression of this onc gene is probably associated with the transformed state of the cells. The human c-sis gene has been cloned into a vector designed to induce c-sis RNA expression. The biological effects of this DNA molecule are being examined by transfection analysis.

3. The primate cell-derived transforming gene (v-sis) of SSV is represented as a single copy marker within cellular DNAs of mammalian species, including human. The human analogue of v-sis can be distinguished from its rodent counterparts by Southern blotting analysis of Eco RI restricted DNAs. By testing for the presence of the human v-sis-related fragment, c-sis (human), in somatic cell hybrids possessing varying numbers of human chromosomes, as well as in segregants of such hybrids, it was possible to assign c-sis to human chromosome 22. Chromosome 22 is a small acrocentric chromosome to which no cancer-associated genes have been previously assigned. However, this human chromosome is one of several in which translocations are associated with specific types of tumors. The so-called Philadelphia chromosome, found in almost all cases of chronic myelogenous leukemia (CML), involves the reciprocal translation of the distal portion of chromosome 22 (22q11) onto the long arm of chromosome 9. The Philadelphia chromosome has also been identified in some cases of multiple myeloma as well as in other B-cell neoplasms. A different translocation of chromosome 22 is found in many instances of Burkitt's lymphoma. The same region of chromosome 22 (22q11) is translocated, but in this case onto chromosome 8.

4. The sequence of the transforming region of SSV has been determined by using molecularly cloned viral DNA. This region encompassed the 1.0-kilobase pair woolly monkey cell-derived insertion sequence, v-sis, and flanking simian sarcoma-associated viral (SSAV) sequences. A 675-nucleotide-long open reading frame commenced 19 nucleotides within the SSAV sequences to the left of the v-sis helper viral junction and terminated within v-sis itself. Possible promoter and acceptor splice signals were detected in helper viral sequences upstream from this open-reading frame, and potential polyadenylation sites were identified downstream both within v-sis and in helper viral sequences beyond v-sis. The recombinational event that led to the generation of SSV occurred in the middle of two functional codons, indicating that SSAV provided the regulatory elements for transcription as well as the initiation codon for translation of SSV cell-derived transforming sequences.

5. The importance of the cell-derived gene of SSV, v-sis, in SSV transformation has been established by analysis of the transforming activity of SSV deletion mutants constructed in vitro. Such information, in

combination with knowledge that v-sis contained a long potential coding frame, provided the basis for attempts to identify the SSV transforming protein. A small peptide selected from the amino acid sequence of the v-sis gene product, predicted by nucleotide sequence analysis, was used to prepare antiserum. Antiserum against this peptide was used to identify a v-sis coded protein of 28,000 daltons, which corresponded to the size predicted from the v-sis open reading frame. Preliminary characterization indicated that p28^{sis} differs from other onc-gene products.

Significance to Biomedical Research and the Program of the Institute:

Our studies have defined and characterized the SSV transforming gene. In addition, this work has provided the knowledge required to identify the SSV-coded transforming protein. Studies are in progress to examine the interaction between this transforming protein and cellular components in order to determine the molecular mechanism involved in SSV transformation.

Proposed Course:

1. Continue characterization of the SSV transforming gene product, p28^{sis}.

2. Construct mutants from our biologically active SSV DNA clones for use in determining the properties of p28^{sis} which are important for induction and maintenance of tranformation.

3. Continue to assess the involvement of retrovirus onc-related genes in naturally occurring human malignancies.

Publications:

Devare, S. G., Reddy, E. P., Law, J. D., Robbins, K. C. and Aaronson, S. A.: Nucleotide sequence of the simian sarcoma virus genome: Demonstration that its acquired cellular sequences encode the transforming gene product p28^{sis}. Proc. Natl. Acad. Sci. USA 80: 731-735, 1983.

McBride, O. W., Swan, D. S., Robbins, K. C., Prakash, K. and Aaronson, S. A.: Chromosomal mapping of tumor virus transforming gene analogues in human cells. In Pearson, M. L. and Sternberg, N. L. (Eds.): Gene Transfer and Cancer 1982. New York, Raven Press, 1982. (In Press)

Naharro, G., Dunn, C. Y. and Robbins, K. C.: Analysis of the primary translational product and integrated DNA of a new feline sarcoma virus, GR-FeSV. Virol. 125: 502-507, 1983.

Naharro, G., Tronick, S. R., Rasheed, S., Gardner, M. B., Aaronson, S. A. and Robbins, K. C.: Molecular cloning of integrated Gardner-Rasheed feline sarcoma virus: Genetic structure of its cell-derived sequence differs from that of other tyrosine kinase coding onc genes. J. Virol. (In Press)

Robbins, K. C., Devare, S. G., Reddy, E. P. and Aaronson, S. A.: In vivo identification of the transforming gene product of simian sarcoma virus. Science 218: 1131-1133, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05168-03 LCMB

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chromosomal Localization of Human Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

D. Swan Expert, LCMB, NCI

COOPERATING UNITS *(if any)*

M. Gardner, Dept. of Path., UC Davis, School of Medicine, Davis, CA;
O.W. McBride, Lab. of Biochemistry, DCBD, NCI

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Chromosomal localization of several human onc gene analogues has been carried out using human-rodent somatic cell hybrids. The following have been mapped: N-ras, chromosome 1; Ki-ras 1, chromosome 6; Ki-ras 2 and 3, chromosome 12; myc, chromosome 8; abl, chromosome 9; bas, chromosome 11. Two rearranged myc loci from Raji Burkitt's lymphoma have been cloned and characterized.

PROJECT DESCRIPTION

Name, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. Tronick	Microbiologist	LCMB, NCI
A. Yaniv	Visiting Scientist	LCMB, NCI
P. Reddy	Visiting Scientist	LCMB, NCI
R. Balachandran	Visiting Fellow	LCMB, NCI

Objectives:

1. Chromosomal mapping of human onc genes.

2. Clone and characterize rearranged myc alleles in Burkitt's lymphoma cells.

Methods Employed:

Hybridization of cloned human immunoglobulin and human onc gene analogues to an array of human/rodent hybrid cell lines has allowed us to establish which human chromosomes carry each of these genes. Hybrid cells were formed between human and either mouse or Chinese hamster cells. These hybrids are known to segregate human chromosomes and, when harvested for DNA isolation, the human chromosome content of each hybrid cell line was established by standard isozyme analysis. Hybrid cell DNA was cut with a suitable restriction enzyme which gave a size difference between the hybridizing fragments in human and rodent DNAs. Restricted DNAs were electrophoresed in agarose gels, transferred to cellulose nitrate filters and hybridized to each of the probes. The human fragment hybridizing to each of the probes used was seen only in those cell lines which contained the human chromosome carrying the corresponding gene. Since each hybrid cell line usually contained more than one human chromosome, it was necessary to test many hybrids before unequivocal assignment could be made. In most cases it was necessary to further subclone some of the positive cell lines in order to allow additional segregation of human chromosomes.

The Raji rearranged myc alleles were identified in restriction enzyme-digested DNA which had been transferred to cellulose nitrate and hybridized to two cloned normal c-myc(human) exons. One of the restriction enzymes which showed these rearranged alleles was Eco RI. We were therefore able to clone them in the Eco RI site of the λ WES-B vector. Preliminary restriction enzyme mapping has been carried out on the two clones.

Major Findings:

The following human c-onc genes have been mapped:

N-ras	human chromosome 1
Ki-ras-1	human chromosome 6p
Kis-ras-2,3	human chromosome 12
myc	human chromosome 8q
abl	human chromosome 9
Ha-ras(bas)	human chromosome 11

C-myc has been shown to rearrange in Burkitt's lymphoma DNA. Raji cells show two rearranged myc genes--one containing two myc exons, the other containing only the 3' exon.

Significance to Biomedical Research and the Program of the Institute:

It is necessary to know the location of genes within the genome in order to understand the interaction between different genes. For example, it had been shown previously in Burkitt's lymphoma that cells containing a (2:8) translocation always expressed κ immunoglobulin light chain, whereas those containing (8:22) translocation always expressed λ. Since the identification of the genes for each of these immunoglobulin proteins on chromosomes 2 and 22, respectively, it has been postulated that the activation of the immunoglobulin loci might result in activation of the myc gene. As a consequence of these mapping studies, it has been shown subsequently that the myc gene (originally on chromosome 8) gets translocated to an immunoglobulin locus (either μ or κ or λ) in Burkitt's cells. We have shown in Raji DNA that the translocation does not always involve the complete myc locus. It remains to be seen whether transcripts from the rearrranged Burkitt's myc genes can give rise to transformation.

Proposed Course:

1. Further human onc genes will be mapped as they become available.

2. Rearranged Burkitt's myc genes will be sequenced and compared to normal myc alleles.

3. Cloned rearranged myc alleles will be tested for their ability to transform B-lymphocytes.

Publications:

Battey, J., Max, E. E., McBride, O. W., Swan, D. and Leder, P.: A processed human immunoglobulin epsilon gene has moved to chromosome 9. Proc. Natl. Acad. Sci. USA 79: 5956-5960, 1982.

McBride, O. W., Battey, J., Hollis, G. F., Swan, D. C., Siebenlist, U. and Leder, P.: Localization of human variable and constant region immunoglobulin heavy chain genes on subtelomeric band q32 of chromosome 14. Nucleic Acids Res. 10: 8155-8170, 1982.

McBride, O. W., Swan, D. C., Robbins, K. C., Prakash, K. and Aaronson, S. A.: Chromosomal mapping of tumour virus transforming gene analogues in human cells. In Pearson, M. L. and Stromberg, N. L. (Eds.): Gene Transfer and Cancer. New York, Raven Press. (In Press)

McBride, O. W., Swan, D. C., Santos, E., Barbacid, M., Tronick, S. and Aaronson, S. A.: Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome 11. Nature 300: 773-774, 1982.

Nebert, D. W., Negishi, M., Enquist, L. W. and Swan, D. C.: Use of recombinant DNA technology in the study of genetic differences in drug metabolism affecting individual risk of malignancy. In Harris, C. C. and Cerutti, P. A. (Eds.): Mechanisms of Chemical Carcinogenesis. New York, Alan R. Liss, Inc., 1982, pp. 351-362.

Prakash, K., McBride, O. W., Swan, D. C., Devare, S. G., Tronick, S. R. and Aaronson, S. A.: Molecular cloning and chromosomal mapping of a human locus related to the Moloney murine sarcoma virus transforming gene. Proc. Natl. Acad. Sci USA 79: 5210-5214, 1982.

Swan, D. C., McBride, O. W., Robbins, K. C., Keithley, D. A., Reddy, E. P. and Aaronson, S. A.: Chromosomal mapping of the simian sarcoma virus onc gene analogue in human cells. Proc. Natl. Acad. Sci. USA 79: 4691-4695, 1982.

Swan, D., Oskarsson, M., Keithley, D., Ruddle, F. H., D'Eustachio, P. and Vande Woude, G. F.: Chromosomal localization of the Moloney sarcoma virus mouse cellular (c-mos) sequence. J. Virol. 44: 752-754, 1982.

Taub, R., Kirsch, I., Morton, C., Lenoir, G., Swan, D., Tronick, S., Aaronson, S. and Leder, P.: Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt's lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. USA 79: 7837-7841, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05169-03 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of Human Transforming Genes Belonging to the ras Family

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Y. Yuasa Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A transforming gene related to c-bas/has (human) was cloned in biologically active form from a human lung carcinoma-derived cell line, Hs242. Recombinants were constructed between the Hs242 oncogene and its normal allele in order to localize the genetic lesion that led to the acquisition of transforming properties. In vitro transformation assays, coupled with primary nucleotide sequence analysis of these recombinants, revealed that the genetic change that led to the activation of the Hs242 oncogene is a point mutation in the second exon. This point mutation results in the incorporation of leucine instead of glutamine in the 61st amino acid of the predicted protein. No changes were observed in the first exon, the region of c-bas/has in which a point mutation is responsible for activation of the T24 and EJ bladder carcinoma oncogenes. Thus, single amino acid substitutions within different structural domains of the same protooncogene can independently be responsible for its malignant conversion under natural conditions.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI
J. S. Rhim	Microbiologist	LCMB, NCI
S. K. Srivastava	Visiting Fellow	LCMB, NCI

Objectives:

1. To clone transforming genes molecularly from human tumor cell DNA which transforms normal fibroblasts.

2. To characterize these genes to delineate their role in human neoplasia.

Methods Employed:

1. Transfection on normal mouse fibroblasts with DNA isolated from various human tumor cells.

2. Detection of human repetitive sequences (Alu) in the transformed cells by Southern blot hybridization.

3. Molecular cloning of transforming DNA sequences using the λ phage system.

4. Nucleotide sequence analysis of the transforming gene and its normal homologue.

Major Findings:

The transforming gene of a human lung carcinoma-derived cell line, Hs242, has been cloned in biologically active form, and identified as c-bas/has (otherwise known as c-Ha-ras). The genetic lesion responsible for the transforming activity of the Hs242 oncogene has been localized to a point mutation in the second exon which results in the substitution of leucine for glutamine as amino acid 61 of the predicted protein. No changes were observed in the first exon, the region of c-bas/has in which a point mutation is responsible for activation of the T24 and EJ bladder carcinoma oncogenes.

Oncogenes of another lung carcinoma (SW-1271) and colon carcinoma (#7060) were found to be related to the newly isolated N-ras oncogene. The N-ras oncogene has two Eco RI digestions, indicating that both fragments are essential for transformation. To examine the SW-1271 and #7060 oncogenes, we tried to clone these N-ras-specific fragments. Transfectant DNA was digested with Eco RI, purified and ligated to λgt WES.λβ arms. Several clones hybridizing to the N-ras-specific probes were obtained.

Significance to Biomedical Research and the Program of the Institute:

The application of transfection methods to the transfer of purified DNA into mammalian cells in culture has made possible a new experimental approach to investigating the genetic basis of malignant transformation. With this method and the use of recombinant DNA technology, it should be possible to identify specific DNA sequences responsible for acquisition of tumorigenic activity.

Proposed Course:

Comparison of the transforming N-ras genes and their normal homologue by nucleotide sequence analysis.

Publications:

Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. S., Reddy, E. P. and Aaronson, S. A.: Acquisition of transforming properties by alternative point mutations within c-bas/has human protooncogene. Nature. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05231-02 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Cloning and Characterization of Human Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

E. Santos Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

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NCI, NIH, Bethesda, Maryland 20205

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1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project deals with the identification and molecular characterization of dominant oncogenes present in human tumor cell lines and in naturally occurring tumors. A common oncogene, related to the Kirsten strain of murine sarcoma virus (MSV), has been detected frequently in a variety of human tumor cell lines and solid tumors including carcinomas of the lung, pancreas and an embryonal rhabdomyosarcoma. Particular findings dealing with the T24 bladder carcinoma oncogene include: (1) the T24 oncogene as an activated allele of the c-has/bas-1 human protooncogene; (2) comparative sequence analysis of both alleles shows that a single point mutation (a G to T transversion) leading to the substitution of valine for glycine as the twelfth amino acid residue of its gene product (designed p21) is reponsible for the malignant properties of the T24 oncogene; (3) the normal allele of the T24 oncogene has been mapped to human chromosome 11; (4) a spontaneous transforming mutant of the normal allele that arose during transfection experiments has also been cloned; (5) analysis of the nucleotide sequence of this mutant reveals the occurrence of a different point mutation (a G to A transition) at the same position of that found in the T24 oncogene, this time leading to substitution of aspartic acid for glycine at position 12 of the amino acid sequence. Current projects involve molecular studies dealing with the Kirsten-related oncogene frequently detected in a variety of human tumors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged in this Project:

M. Barbacid Visiting Scientist LCMB, NCI

Objectives:

The work is directed to the understanding of the molecular mechanisms underlying the development of malignant transformation in humans. We intend to use currently available probes for human oncogenes as well as antibodies directed against their protein products in studies aimed at elucidating the origin and development of naturally occurring human tumors.

Methods Employed:

Methods used include standard molecular biology and recombinant DNA techniques including growth and use of different bacterial strains for preparation of recombinant plasmids and phages, DNA and RNA isolation and purification, restriction endonuclease analysis, Southern and northern blotting and hybridization techniques, nucleotide sequencing of DNA and immunoprecipitation of proteins.

Major Findings:

1. Identification of oncogenes present in solid human tumors.

2. Identification of a point mutation responsible for the malignant properties of the T24 oncogene.

3. Mapping of the normal allele (c-has/bas gene) of the T24 oncogene to human chromosome 11.

4. Identification of a spontaneous transforming mutant of the c-has/bas human gene and determination of a point mutation responsible for its transforming properties.

Significance to Biomedical Research and the Program of the Institute:

These studies may be instrumental in understanding the mechanisms that trigger malignant transformation in human subjects and in establishing the etiology of human cancer.

Proposed Course:

1. Molecular cloning of a Kirsten-related oncogene detected in a variety of human lung tumors.

2. Study of the involvement of that oncogene in the neoplastic process in naturally occurring human tumors.

Publications:

McBride, O. W., Swan, D. C., Santos, E., Barbacid, M., Tronick, S. R. and Aaronson, S. A.: Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome 11. Nature 300: 773-774, 1982.

Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Aaronson, S. A. and Barbacid, M.: Oncogenes in solid human tumors. Nature 300: 539-542, 1982.

Pulciani, S., Santos, E., Lauver, A. V., Long, L. K. annd Barbacid, M.: Transforming genes in human tumors. J. Cell Biochem. 20: 51-61, 1982.

Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Robbins, K. C. and Barbacid, M.: Oncogenes in human tumor cell lines: Molecular cloning of a transforming gene from human bladder carcinoma cells. Proc. Natl. Acad. Sci. USA 79: 2834-2839, 1982.

Reddy, E. P., Reynolds, R. K., Santos, E. and Barbacid, M.: A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature 300: 149-152, 1982.

Santos, E., Pulciani, S. and Barbacid, M.: Characterization of a human transforming gene isolated from T24 bladder carcinoma cells. Fed. Proc. (In Press).

Santos, E., Reddy, E. P., Pulciani, S., Feldmann, R. J. and Barbacid, M.: Spontaneous activation of a human protooncogene. Proc. Natl. Acad. Sci. USA. (In Press).

Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. and Barbacid, M.: The T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature 298: 343-347, 1982

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05232-02 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Murine Epithelial Cell Lines for In Vitro Transformation Studies

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

B. Weissman Staff Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

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Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A model system for the transformation of epithelial cells has been developed. The system uses a mouse epidermal keratinocyte cell line, designated BALB/MK, which is totally dependent on the presence of epidermal growth factor (EGF) for its continued proliferation. In addition, the cell line will terminally differentiate in the presence of high levels of extracellular calcium. Transformation of the cells by either chemical carcinogens or a variety of mammalian sarcoma viruses results in the abrogation of the dependence on EGF as well as a block in calcium-induced terminal differentiation. Furthermore, different sarcoma viruses block terminal differentiation at different steps in the pathway. The recent findings that certain human tumors of epithelial origin contain activated onc genes similar to those found in mammalian sarcoma viruses emphasize the importance of this transformation system.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator engaged on this Project:

S. A. Aaronson Chief LCMB, NCI

Objectives:

1. To determine the effect(s) of retrovirus infection on the growth requirements of murine epidermal keratinocytes.

2. To determine at which specific stage of epidermal differentiation murine epidermal keratinocytes are blocked by different retroviruses.

3. To determine the effects of chemical carcinogens on the growth and differentiation of murine epidermal keratinocytes.

Methods Employed:

A permanent cell line of BALB/c mouse origin has been established for use in these studies. The cell line, designated BALB/MK, is totally dependent on the presence of epidermal growth factor (EGF) for continued proliferation and also responds to calcium-induced terminal differentiation. Cells are infected with cloned retroviruses or treated with chemical carcinogens, and transformed cells are selected for by their ability to grow in the absence of EGF. Transformed cells are subcloned and studied for various markers of epidermal differentiation. These include keratin synthesis, cornified envelope production, desmosome formation, and tumorigenicity.

Major Findings:

1. The majority of mammalian sarcoma viruses tested will transform BALB/MK cells with a titer that is 100-1000 fold less than that observed with BALB/c 3T3 cells. Only simian sarcoma virus was incapable of abrogating the EGF requirement of BALB/MK cells.

2. Transformation by BALB-, Harvey-, or Kirsten-MSV leads to a block in calcium-induced differentiation in BALB/MK cells as well as the acquisition of the ability to grow in newborn syngeneic animals.

3. Transformation by different retroviruses leads to blocks in terminal differentiation at different stages.

4. BALB/MK cells are susceptible to transformation by methylcholanthrene.

Significance to Biomedical Research and the Program of the Institute:

Recent findings have shown that transforming genes isolated from human tumors of epithelial origin are related to retroviral onc genes. Furthermore, retroviral onc genes have been shown to have specific effects on the stages of

differentiation in hematopoietic cells. The findings that different retroviruses transform epithelial cells as well as affect specific stages of epithelial differentiation generalize the role on these genes in the processes of neoplastic transformation. Transformation of the same cells by both retroviruses and chemical carcinogens will also provide insights into common or divergent mechanisms shared by these neoplastic agents. In addition, the relationship between transformation and differentiation in epithelial cells may also be elucidated.

Proposed Course:

1. Characterize the effects of chemical carcinogens on the growth and differentiation of BALB/MK cells.

2. Characterize the effects of viral transformation on the ability of BALB/MK cells to bind EGF.

3. Determine the effects on phosphorylation of tyrosine-containing proteins in BALB/MK cells after viral transformation.

4. Develop new markers of differentiation for epidermal keratinocytes using BALB/MK cells.

Publications:

Weissman, B.E. and Aaronson, S.A.: BALB and Kirsten murine sarcoma viruses alter growth and differentiation of EGF-dependent BALB/c mouse epidermal keratinocyte lines. Cell 32: 599-606, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05234-02 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Cloning and Characterization of Human Transforming Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

V. Notario Visiting Associate, LCMB, NCI

COOPERATING UNITS *(if any)*

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have cloned a 9.5-kbp fragment of an oncogene present in a human fibrosarcoma cell line (HT-1080). The cloned fragment is present in all tested HT-1080-derived transformants, and is different from another fragment of the same oncogene (14 kbp) previously isolated. When both fragments are put together, the construction shows a molecular structure similar to that of the N-ras oncogene present in human neuroblastoma cell lines.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

M. Barbacid	Visiting Scientist	LCMB, NCI
S. Pulciani	Visiting Fellow	LCMB, NCI
E. Santos	Visiting Fellow	LCMB, NCI

Objectives:

To isolate and characterize human oncogenes and to develop an animal system to study the mechanisms of oncogenesis.

Methods Employed:

Standard molecular biological techniques for nucleic acid isolation, gene enrichment, Southern transfer, molecular hybridization, recombinant DNA techniques, and restriction enzyme mapping of cloned genes.

Major Findings:

The presence of oncogenes in human tumor DNAs was established by their ability to transmit the transformed phenotype to NIH/3T3 mouse cells, as well as by the presence in the DNA of the transformants of human Alu repetitive sequences cosegregating with the transformed phenotype after several cycles of gene transfer.

The DNA from transformed NIH/3T3 cells was then digested with restriction endonucleases which do not destroy their transforming ability, ligated to cloning vectors such as cosmid pHC-79, plasmid lambda 1059 or lambda bacteriophages, and packaged into phage particles. After infection of appropriate bacterial strains, the bacterial colonies or phage plaques were screened for the presence of Alu repetitive sequences. DNA from positive clones was extracted and used to study their molecular structures and biological activities.

Three DNA fragments (17, 9.5 and 3.4 kbp) which give positive hybridization to human Alu repetive sequences have been isolated from DNA extracted from HT-1080-derived NIH/3T3 transformants. Two of the fragments (17 and 3.4 kbp) were separated by complete Bam HI cutting of a Mbo I fragment cloned in the cosmid pHC-79. The 9.5-kbp fragment was prepared by total Eco RI cutting of a Sau 3A-I fragment cloned into the same vector. Neither the 17 nor 3.4-kbp fragment could be found in any of the HT-1080-derived transformants, indicating that they are not part of the oncogene present in the human fibrosarcoma, though they may be closely linked to it in the human genome.

The human origin of the 9.5-kbp fragment was demonstrated by the presence of human Alu repetitive sequences and by its poor hybridization to mouse cellular DNA. This fragment was present in all the HT-1080-derived transformants, hybridizing to two different Eco RI fragments in two distinct families of transformants (8.2 and 10.5 kbp). These results indicated that this fragment is part of the oncogene present in the human fibrosarcoma.

The 9.5-kbp fragment did not hybridize to another fragment (14 kbp) of the same oncogene which had been previously isolated and characterized. The new cloned DNA hybridized rather weakly to Harvey-ras probes, another fragment of the same oncogene (14 kbp) previously isolated. When both fragments are put together, the construction shows a molecular structure similar to that of the N-ras oncogene present in human neuroblastoma cell lines.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of transforming genes from human tumors, and their counterparts from normal tissues, may enable us to understand the origin of certain human neoplasias.

Proposed Course:

Current work is focused on the cloning of the active forms of oncogenes present in human tumors or tumor-derived cell lines which so far cannot be included in any of the three oncogene families described to date (H-ras, K-ras and N-ras).

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05235-02 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Evolutionary Relationships among Types A, B, C and D Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Ing-Ming Chiu Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*
LTIB, NCI; LMM, NIAID; LTCB, NCI; Johns Hopkins University

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The closed circular form of the endogenous squirrel monkey type D retrovirus (SMRV) was molecularly cloned in bacteriophage λgtWES·λB and designated λSMRV. The restriction map of λSMRV was determined and was found to be identical to the parental SMRV linear DNA except for the deletion of one long terminal repeat (LTR). The biological activity of λSMRV was ascertained by DNA transfection of canine osteosarcoma D17 cells and the host range of the recovered virus was identical to that of parental SMRV. The λSMRV restriction map was oriented to the viral RNA by using gene-specific probes from other retroviruses. Consistent with earlier studies, multiple copies of SMRV sequences were detected in squirrel monkey cellular DNA. Related DNA fragments were also detected in the DNAs of other primate species, including human.

The genetic relationship between SMRV and other retroviruses was examined by Southern blotting and hybridization under low stringency. The pol regions of type A-related (M432), type B (MMTV) and avian type C (RSV and MAV) viruses were found to be related to SMRV pol by Southern hybridization and DNA sequence analysis. In contrast, these viruses did not share detectable homology with the pol genes of mammalian type C viruses. These findings establish the existence of two major pol gene families in the evolution of retroviruses. It was also possible to demonstrate the homology shared by the env genes of SMRV and mammalian type C viruses (SSAV, BaEV and R-MuLV). By heteroduplexing analysis, a 300-bp region of colinearity between the env genes of SMRV and BaEV was observed. These results indicate the genetic interaction between different retrovirus subfamilies has also played a role in the evolution of retroviruses.

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. Tronick	Research Microbiologist	LCMB, NCI
R. Narayanan	Visiting Fellow	LCMB, NCI
A. Srinivasan	Visiting Associate	LCMB, NCI
S. Aaronson	Chief	LCMB, NCI
R. Callahan	Research Microbiologist	LTIB, NCI
J. Schlom	Chief	LTIB, NCI
P. E. Steele	Medical Staff Fellow	IRP, NIAID
M. Martin	Chief	LMM, NIAID
F. Wong-Staal	Section Chief	LTCB, NCI
R. Gallo	Chief	LTCB, NCI

Objectives:

1. Determine the relationships among retroviruses.
2. Detect SMRV-related sequences in mammalian DNAs, including human.
3. Characterize retroviral-related sequences in human DNA.
4. Define the relationship of such sequences to human cancers.
5. Activation of transforming gene mos using LTRs.

Methods Employed:

To study the evolutionary pathway of retroviruses, this laboratory has developed a series of relaxed hybridization conditions varying the concentrations of formamide and washing temperatures. As demonstrated by direct sequencing analysis, the most diverse sequences detectable by hybridization required 60-65% homology. Other techniques used include restriction enzyme analysis, gel electrophoresis, library screening, gene enrichment, DNA sequencing, computer programming and heteroduplexing.

Major Findings:

1. Endogenous SMRV-related sequences are present in multiple copies in squirrel monkeys as well as in chimpanzees, Old World monkeys and humans.

2. The gene order of SMRV is found to be the same as in other types of retroviruses.

3. The SMRV LTR is about 300 bp long, which is among the smallest LTRs. This LTR is able to activate the oncogene v-mos, as demonstrated by transformation of NIH/3T3 cells.

4. The existence of two major pol gene families in the evolution of retroviruses is established. One family includes type A, type B, avian type C, and type D viruses. The other family includes mammalian type C viruses.

5. The homology shared by the env genes of SMRV and mammalian type C viruses is demonstrated by both Southern hybridization and heteroduplexing analysis.

6. Human retroviral-related sequences obtained using MMTV pol gene sequences as a probe were found to be related to type A and type D pol genes as well.

7. In contrast, human retroviral-related sequences obtained using BaEV pol gene sequences as a probe were related to mammalian type C viruses but not to other retroviruses, except for a weak homology at the gag gene with that of SMRV.

Significance to Biomedical Research and the Program of the Institute:

The establishment of the existence of two major pol gene families in the evolutionary pathway of retroviruses made it possible to characterize the human retroviral-related sequences isolated from human genomic libraries. It also makes possible a detailed analysis of the role retroviruses play in malignancies of humans. The further attempt to classify HTLV, the only infectious retrovirus isolated from humans to date, is currently underway.

Proposed Course:

1. To clone more human retroviral-related sequences using the region of SMRV pol that is conserved among the subfamily of types A, B, D and avian type C retroviruses.

2. To sequence the corresponding region of pol in SMRV and MMTV to find the conserved structures in reverse transcriptases.

3. To further identify the enhancement element in SMRV-LTR.

Publications:

Chiu, I.-M. and Marzluff, W. F.: Uncoordinate synthesis of histone H1 in cells arrested in the G1 phase. Biochim. Biophys. Acta 699: 173-182, 1982.

Chiu, I.-M., Andersen, P. R., Aaronson, S. A. and Tronick, S. R.: Molecular cloning of the unintegrated squirrel monkey retrovirus genome: Organization and distribution of related sequences in primate DNAs. J. Virol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05305-01 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analysis of Human Tumors and Normal Tissues for Oncogene Activation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Samuel W. Needleman Medical Staff Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Total genomic DNA is extracted from normal human cells (generally skin fibroblast-derived cell lines, or circulating lymphocytes), tumors, or tumor-derived cell lines according to standard techniques. This DNA is screened for transforming activity by use of the NIH/3T3 transfection assay. Briefly, the DNA is coprecipitated with calcium phosphate and added to log phase cultures of NIH/3T3 cells. Transformation is screened morphologically 14-21 days after transfection, while foci which appear not to be spontaneous are cloned with the glass cylinder method. DNA derived from these candidate foci is subjected to hybridization with a probe specific for human repetitive sequences. If no human repetitive sequences are found, the focus is felt to be spontaneous; if positive for human sequences, further hybridization with known transforming sequences is performed. To date, this work has shown a minority of human tumors to contain activated transforming genes, detectable in this assay, in agreement with the experience of others: Kaposi's sarcoma, 0/5; chronic myelogenous leukemia, 0/15; diffuse histiocytic lymphoma, 0/6; hypernephroma, 0/4; Hodgkin's lymphoma, 1/14; acute myelogenous leukemia. 1/1; rhabdomyosarcoma, 1/1. The latter two tumors were found to transform the murine target via transfer of the neuroblastoma-related *onc* gene n-*ras* (Shimizu *et al.*, *PNAS* 80: 383, 1983). Another line of investigation has been to screen normal fibroblastic DNA from patients with high cancer risk syndromes (e.g., Gardner's syndrome) to assess whether activation of cellular oncogenes occurs in normal tissues or is passed in germ line DNA. Such findings might account for familial cancer aggregation or multisite neoplasia; however, to date we have found no evidence for active *onc* genes in normal tissues using this assay. An obvious limitation of these studies is the intrinsic limitation of the sensitivity of the assay, which seems skewed toward picking up genes of the *ras* family. Future studies are being directed toward modifying the assay to be more sensitive, and thus hopefully broadening and increasing its ability to pick genes of the *ras* and other families.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this project:

Y. Yuasa	Visiting Fellow	LCMB, NCI
M. Kraus	Guest Researcher	LCMB, NCI
S. A. Aaronson	Chief	LCMB, NCI

Objectives:

To investigate the prevalence and nature of oncogene activation in human tumors and tumor-derived cell lines, as well as examining normal tissues from patients with recognized high cancer risk syndromes, such as Gardner's syndrome, strong family history of site-specific tumor aggregation, and patients with multiple neoplasms of tissues of diverse embryonic origin. These studies were undertaken to: (1) further our knowledge of the prevalence of active oncogenes in human tumors of various histopathologic subtypes; (2) identify new oncogenes in human neoplasms; (3) elucidate the mechanisms by which oncogenes identified in the transfection assay might confer the transformed phenotype to the target cell line, NIH/3T3. In addition, the availability of normal fibroblast lines from patients with high risk syndromes, characterized by a high degree of familial clustering and/or multisite neoplasia, afforded the opportunity to assess whether activation of oncogenes occurs in the germ line of such patients. This latter avenue would shed light on whether oncogenes might be involved in the mechanism of heritable and/or multisite neoplasia, as well as enhancing our understanding of the significance of in vitro transformation detected in this model. If normal germ line DNA contains active oncogenes, then obviously the model represents early, necessary, but not sufficient events for human neoplasia, since not all tissues (which receive this genetic information) become neoplastic.

Methods Employed:

The laboratory has utilized DNA transfection by the coprecipitation method, modified from previous reports (Wigler et al., Cell 14: 725, 1978). Briefly, total genomic DNA, extracted by standard procedures, is coprecipitated with calcium phosphate and layered onto 1.3×10^5 NIH/3T3 cells in log phase. Cells are maintained according to standard techniques and scanned morphologically for transformed foci at 14-21 days after transfection. Foci which do not appear spontaneous are cloned by the glass cylinder method and grown for DNA extraction. These transfectant DNA specimens are hybridized by the method of Southern (J. Mol. Biol. 98: 503, 1975) with a probe specific for human repetitive sequences. If the hybridization shows no human DNA present superimposed on the mouse background, the focus must be spontaneous. If there is human genetic information present, the DNA is transfected in subsequent cycles, as well as analyzed by hybridization with known oncogene probes.

Major Findings:

To date, after screening a number of human tumors and tumor cell lines, 4 have been found to contain active oncogenes. By histopathologic group these are as follows: Kaposi's sarcoma, 0/5; chronic myelogenous leukemia, 0/15; chronic myelogenous leukemia derived lines IM9 and Nalm-1, 1/2; diffuse histiocytic lymphoma, 0/6; hypernephroma, 0/4; lymphocytic non-Hodgkin's lymphoma, 0/10. One of 12 Hodgkin's tumors was found positive; the nature of this tumor's transforming gene remains to be established as it hybridizes with none of the probes tested so far. One of 1 tumor from a patient with acute myelogenous leukemia was found to be positive; its transforming gene is the n-ras gene described by Shimizu (PNAS 80: 383, 1983). One of 2 soft tissue sarcoma lines, RD, was also found to have the active form of n-ras and to be active in transfection.

Looking at normal cells from patients with Gardner's syndrome, tumor site-specific familial aggregation, and other high cancer risk situations, we have 0/34 lines containing genes which are active in this system.

Using Southern hybridization with a cloned fragment of c-abl, the normal cellular analog of the transforming gene of Abelson murine leukemia virus, we examined the 15 DNA preparations from chronic myelogenous leukemia tumors as well as the 2 chronic myelogenous leukemia-derived lines, IM9 and Nalm-1. Since the vast majority of patients with chronic myelogenous leukemia have a 9:22 chromosomal translocation (the so-called Philadelphia chromosome) and the c-abl gene is normally found on chromosome 9, we reasoned that this study might enable us to uncover activation of the c-abl gene if the hybridization pattern showed rearrangement and/or amplification. Of the 2 cell lines and 13 patients with stable phase of the disease studied, all showed patterns indistinguishable from normal. Of the 2 patients in blastic crisis, 1 showed an amplification of abl hybridizing sequences, but this finding was not able to be consistently reproduced. Similar studies with the probe c-sis, the cellular analog of the transforming gene of simian sarcoma virus, were negative.

Significance to Biomedical Research and the Program of the Institute:

There has been much excitement about the role of activated oncogenes in human neoplasia. Studies of the kind described above could further our understanding of human cancer and eventually lead to therapeutically exploitable manipulations, if it is indeed the case that the transfection model represents events which are germane to the genesis of clinical neoplasia. The finding that only a minority of human tumors tested score as positive might lead one to conclude that this is not the case. Alternatively, since human cancer is really a very heterogeneous group of diseases, these studies might sort out a subgroup of neoplasms where oncogene activation which is detectable in this assay is an important mechanism. Another possibility is that the system at this point is a crude and insensitive one, and only detects relatively high levels of an event which is in fact more universal than the data suggest.

The question of whether oncogene activation occurs in normal tissues is a crucial one. Oncogenes are highly conserved in the phylogeny of the biologic kingdom, suggesting a normal salubrious function. Whether the very subtle changes involved in endowing them transforming capacity (e.g., point mutation, enhanced expression, rearrangement, etc.) can be detected in normal tissue as has been reported (Muschel et al., Science 219: 853, 1983) is a crucial point. If this were the case, the events represented in the model might represent early, necessary events that have some relevance to human cancer; however, since the normal cells in this report did not seem destined to undergo transformation, it would be clear that DNA capable of transforming in the transfection assay, would not be sufficient for the development of clinical cancer. Rather, other subsequent events which are not examined by the model would be necessary.

The study of genetically predisposed patients, as well as those who suffer multiple-site neoplasia of tissues of diverse embryonic origin seemed profitable to us in this regard. If a family is unfortunately affected by the same tumor in many members, or a patient has tumors affecting tissues of diverse embryonic origin, we reasoned that the genetic information of the germ line of some patients such as these might contain activated oncogenes as an early necessary event for the development of tumors. Subsequent events would determine which family members, or which tissues would develop cancer.

The finding that none of the 34 skin fibroblast lines from such patients scored positive in the transfection assay suggests that at least for patients such as the ones studied, germ line DNA does not contain active oncogenes. These data suggest that oncogene activation occurs in the tissues destined to become tumors, and that other heritable factors must account for familial aggregations of cancer. Such other factors might include genes not detectable in the transfection assay, defects in DNA repair, carcinogen degradation, or immunosurveillance.

Proposed Course:

Currently, the primary concern, which has been alluded to above, is that the transfection system is not sensitive to transforming genes, or is too narrow in its spectrum of sensitivity, in that the ras family seems to be overrepresented in the tumors scoring as positive. Thus, studies are underway to try to increase the sensitivity of the current system by manipulating the cell cycle to try to ascertain whether there is a particular part of the cycle when NIH/3T3 cells are most sensitive. Additionally, certain pharmacological and biological manipulations are being tried. Another approach to broadening the spectrum of the transfection assay is to attempt to employ new target cell lines. Further effort in screening human tumor DNA preparations by transfection and hybridization, as well as more normal cell DNAs from patients with other high cancer risk syndromes, will continue; however, the primary effort will be to modify and improve the transfection assay.

Publications: None

Proposed Course:

1. Studies will be continued to further characterize the specificity of the c-terminal peptide antibody. The c-terminal peptide antibody was found to be positive for immunoprecipitation of p21 from Ha-MSV-transformed NRK and NIH/3T3 cell lines. This antibody wasn't efficient for Ha-MSV-transformed BALB 3T3, or BALB-MSV transformed NRK or NIH/3T3 cell lines. Therefore, immunoprecipitation of the unprocessed form of p21 (pro-p21) will be checked in these cell lines, which should help in understanding subtle differences in the p21 molecule in the above-mentioned cell lines. Studies will also be carried out on the nucleotide binding function of p21 coded for by transforming genes of cell lines derived from human tumors.

2. The Hs242 oncogene has been cloned from an NIH/3T3 transfectant and characterized in our laboratory (Yuasa et al., Nature, in press). We propose to clone this transforming gene and its normal allele directly from the Hs242-derived lung carcinoma because this cell line is comprised of predominantly stromal cells of normal appearance. With this approach we want to exclude the possibility that the Hs242 oncogene arose from a minor undetectable fraction of tumor cells in a major population of normal appearing cells. The other aspect of this study is to analyze the regulation of the transforming gene function in the cells of normal phenotype.

Publications:

Yuasa, Y., Srivastava, S. K., Dunn, C. Y., Rhim, J. S., Reddy, E. P. and Aaronson, S. A.: Acquisition of transforming properties by alternative point mutations within c-bas/has human protooncogene. Nature. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05307-01 LCMB

PERIOD COVERED
October 19, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Factors Responsible for Activation of Human Oncogenes into Transforming Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
A. Gazit Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Activation of human oncogenes: The long terminal repeat (LTR) of the Abelson murine leukemia virus (A-MuLV), in combination with other 5' terminal sequences of various lengths, were covalently linked to two human cellular oncogenes--human c-sis, and human c-bas/has. In contrast to human c-sis, which could not be activated by the LTR promotor, the human bas/has fragment was activated into a transforming efficiency of 10 ffu per µg DNA by upstream linkage to three out of the four LTR vectors. On the other hand, neither the 5' LTR nor the 3' LTR, when linked to the c-bas/has in the downstream position, conferred any transforming activation. Experiments are being performed to clarify the factors responsible for activation of the normal human bas/has oncogene.

Identification of coding sequences responsible for transformation activity: A series of recombinant plasmids containing deletions in the various exons of the transforming human c-bas/has gene are now being constructed and checked for transformation efficiency on NIH/3T3 cells. This is being performed with the aim of localizing the regions responsible for accurate expression of p21.

Retroviruses as cloning vectors for mammalian oncogenes: A retrovirus vector was constructed by inserting the activated human c-bas into an A-MuLV construct. Experiments are being performed to rescue the oncogene-containing viral particles by superinfection with replication-competent retrovirus.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. Aaronson	Chief	LCMB, NCI
A. Srinivasan	Visiting Associate	LCMB, NCI
Y. Yuasa	Visiting Fellow	LCMB, NCI
S. Tronick	Head, DNA Recombinant Unit	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI

Objectives:

1. To activate normal human oncogenes into transforming ones by switching on their expresssion through linkage to the long terminal repeat (LTR) of the Abelson murine leukemia virus (A-MuLV). Studies are directed toward clarifying the factors (elevated expression of the normal oncogene product, versus expression of an abnormal transforming oncogene product) responsible for converting the normal oncogene into a transforming one.

2. To localize the coding regions of a transforming human c-bas/has oncogene governing the accurate expression of a functional transforming protein.

3. To insert mammalian genes (as activated oncogenes) into the genome of retroviruses, thus obtaining infectious defective virions containing the mammalian gene, which are rescuable by superinfection with a replication-competent virus.

Methods Employed:

To construct recombinant plasmids, the conventional methods of recombinant DNA technology are employed, including preparation of plasmid DNAs, purification of DNA fragments, ligation and bacterial transformation. To check the biological activity of the cloned DNA fragments, DNA transfection, as well as cotransfection assays with SV-2, are carried out on NIH/3T3 cells. Presence and quantitation of the transfected DNA within cell DNA is checked by procedures including isolation of high molecular weight DNA, restriction enzyme digestion, electrophoresis and Southern filter DNA blot hybridization. RNA expression is assayed by the RNA blot technique, gel electrophoresis and the northern blotting procedures.

Major Findings:

1. Activation of human c-oncogenes by linkage to LTR of A-MuLV. Four vector constructions of A-MuLV LTR, cloned in pBR322, were used for ligation: 5' LTR linked to adjacent downstream sequences of 578 bp (designated Bal vector); 5' LTR with adjacent 213-bp sequences (Pvu vector); 5' LTR with 200 bp of adjacent sequences (Xma vector); and 5' LTR with 90-bp sequences (Bal vector). These four LTR-containing vectors were ligated to two human c-oncogenes: a) a human c-sis

of ~ 12 kb and (b) the internal Sac fragment (~ 2.8 kb) of human c-bas/has.

Human c-sis constructions. From each of the vector constructions, two clones with positive orientation (i.e., 5' LTR and one clone in the same expression orientation), and one clone with negative orientation, were scored for transforming capacity following transfection onto NIH/3T3 cells. No recombinant clone demonstrated any detectable transforming activity. Simultaneously, the recombinant clones were cotransfected with SV-2. Studies are being carried out to analyze quantitatively, as well as qualitatively, the level (if any) of c-sis expression in the cotransfected cultures.

Bas/has constructions. Previous experiments of linking A-MuLV LTR to human c-bas/has (Bam HI 6.6 kb) did not activate it into a transforming gene. In the present experiments, Ableson 5' LTRs were linked directly to the internal Sac fragment of human c-bas/has (~ 2.8 kb). Three out of the four LTR vectors (Pvu, Xma and Bal vectors) activated the Sac fragment into a transforming gene with a specific activity of 10 ffu μg DNA, in comparison to transforming efficiency of ~ 1 x 10^4 ffu per μg DNA demonstrated by the transforming human c-bas/has (T24 or Hs242). In contrast, the Sac fragment linked to the Bst vector did not show any transforming activity. These data strongly imply that the normal Sac fragment has a potential transforming capacity which can be activated through linkage to promotor sequences present in the LTR. This conclusion is based on data showing that the Sac fragment linked to the 5' LTR in the opposite orientation did not show any transforming activity. Nor was Sac fragment activated by the 3' LTR linked to its 3' end, thus eliminating a possible role for "enhancer" sequences of LTR in this transformation activation.

2. The localization of coding regions responsible for the transforming function of the activated human c-bas is now being studied by constructing a variety of deletion mutants in the various exons of a molecular clone of Hs242 or oncogene which was isolated from a human lung carcinoma-derived cell line. The deletion mutants will be assayed for their transforming capacity on NIH/3T3 cells.

3. With the aim of inserting mammalian genes into infectious retroviral virions, a transforming human c-bas (Hs242) was inserted into the Eco RI site of the A-MuLV genome. This was performed by exchanging the human c-bas fragment (~ 6.6 kb) with the Bst EII-Cla I fragment (~ 1 kb) of the A-MuLV DNA cloned in pBR322. By transfection onto NIH/3T3 cells, the recombinant clones demonstrated equivalent transforming (>10^4 ffu per μg DNA), as well as similar morphology of foci, as Hs242 transforming human c-bas. Transfection assays are now being performed in the presence of a helper virus to obtain rescuable virions containing the transforming c-bas oncogene.

Significance to Biomedical Research and the Program of the Institute:

1. The major significance of project #1 is the elucidation of the mechanisms responsible for converting a normal cellular oncogene into an active one. According to recent findings, the neoplastic activation of human c-bas consists of a single point mutation, thus resulting in a substitution of one amino acid. On the other hand, activation of a cellular oncogene by linkage to promotor sequences points to the possibility that increased expression of the normal oncogene is the essential factor in inducing oncogenic transformation. In the present study, we have neoplastically activated a fragment of human cellular c-bas by linking it to retroviral LTR. The quantitative, as well as qualitative, analyses of the activated oncogene expression in the neoplastically transformed cells, as compared to the genetically but not neoplastically transformed cells, will help in elucidating the molecular basis of neoplastic transformation in human cancer.

2. DNA sequence analysis of the activated human c-bas indicates the existence of at least four exons. While the T24 and EJ bladder carcinoma-derived oncogenes contain a single point mutation in the first exon, the Hs242 oncogene contains a single point mutation within the second exon. In the present study, by constructing deletion mutants in the various exons, we will localize the coding sequences responsible for the accurate expression of the functional transforming protein.

3. The transfer of foreign genes, free of chromosomal protein, into mammalian cells is an essential procedure in studying the mechanism of gene expression and regulation in complex eukaryotes. Although various methods for transferring DNA sequences into animal cells have been developed, one of the best methods for DNA transfer is the use of animal viruses as vectors. Among all these viral vectors, retroviruses appear the most suited in transport of foreign genes into eukaryotic cells, inasmuch as they provide a potential vehicle for high efficiency, stable integration into the cell genome, without lysing or morphologically transforming the eukaryotic cells. In the present study, we use the retrovirus system to insert cellular oncogenes into mammalian cells via virus particles. Such an approach will enable the study of the function and regulation of oncogenes within the normal cell. Also, the various steps leading to the ultimate transformed phenotype can be followed.

Proposed Course:

1. Activation of human cellular oncogenes by LTR:

   a) Determination of the correlation between the levels of integration, expression and transforming capacity;

   b) Quantitation of levels of transcripts and oncogene products in the transformed cells, in comparison to the levels in the cotransfected untransformed cells;

c. Determination of species of mRNAs and proteins, with transformed cultures, as compared to the untransformed ones; and

d. Identification of the genetic change (if any) of the "normal" oncogene fragment in the transformed cells, as opposed to the untransfected untransformed cells.

2. Localization of coding transforming regions in activated human c-bas oncogenes; the construction of a variety of deletion mutants, followed by examination of their biological activities.

3. Retroviral vectors for gene transfer:

a. Determination of the optimal conditions (both of vector and of insert) for constructing efficient retroviral vectors;

b. Identification of the molecular structure of the oncogene-containing defective virions; and

c. Identification of the various mRNAs in cells infected with oncogene-containing retroviruses.

Publications:

Gazit, A., Yaniv, A., Dvir, M., Perk, K., and Dahlberg, J. E.: Caprine arthritis-encephalitis virus is a distinct virus within the lentivirus group. Virology 124: 192-195, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05308-01 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transforming Potential of the Human c-sis Locus

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

H. Igarashi Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To investigate the possible role of retrovirus onc-related genes in human neoplasia, we have analyzed human tumor cell lines for evidence of expression of onc-related genes. Our demonstration of simian sarcoma virus (SSV) sis-related transcripts in human fibrosarcoma cell lines, but not in their normal fibroblast counterparts, argues that the expression of this onc gene is probably associated with the transformed state of the cells. The human c-sis gene has been cloned into a vector designed to induce c-sis RNA expression. The biological effects of this DNA molecule are being examined by transfection analysis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
S. R. Tronick	Head, DNA Recombinant Unit	LCMB, NCI
D. Swan	Expert	LCMB, NCI
A. Eva-Varesio	Visiting Associate	LCMB, NCI
A. Srinivasan	Visiting Associate	LCMB, NCI
S. Devare	Visiting Associate	LCMB, NCI
P. Reddy	Visiting Scientist	LCMB, NCI
A. Gazit	Visiting Fellow	LCMB, NCI
G. Naharro	Visiting Fellow	LCMB, NCI
K. Robbins	Expert	LCMB, NCI

Objectives:

1. To examine the interaction of the SSV transforming protein (p28*sis*) with other cellular components.

2. To assess the role of *sis*-related genes in human neoplasia.

3. To determine the mechanism of SSV-induced oncogenesis and to apply this knowledge to understanding the etiology of cancers in humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of gene products; in vitro synthesis and immunoprecipitation; peptide synthesis; antibody production; fractionation of cellular components and protein purification to analyze gene products; molecular hybridization techniques to analyze genes; recombinant DNA techniques for the purification and amplification of genes; analysis of genetic structure using restriction endonuclease mapping, nucleotide sequencing, and electron microscopy techniques; DNA transfection and construction of virus mutants for analysis of transforming activity.

Major Findings:

To investigate the possible role of retrovirus *onc*-related genes in human neoplasia, we have analyzed human tumor cell lines for evidence of expression of *onc*-related genes. Our demonstration of simian sarcoma virus (SSV) *sis*-related transcripts in human fibrosarcoma cell lines, but not in their normal fibroblast counterparts, argues that the expression of this *onc* gene is probably associated with the transformed state of the cells. The human c-*sis* gene has been cloned into a vector designed to induce c-*sis* RNA expression. The biological effects of this DNA molecule are being examined by transfection analysis.

Significance to Biomedical Research and the Program of the Institute:

Our studies have defined and characterized the SSV transforming gene. In addition, this work has provided the knowledge required to identify the SSV-coded transforming protein. Studies are in progress to examine the interaction between this transforming protein and cellular components in order to determine the molecular mechanism involved in SSV transformation.

Proposed Course:

1. Continue characterization of the SSV transforming gene product, p28^{sis}.

2. Construct mutants from our biologically active SSV DNA clones for use in determining the properties of p28^{sis} which are important for induction and maintenance of tranformation.

3. Continue to assess the involvement of retrovirus onc-related genes in naturally occurring human malignancies.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05309-01 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

German Naharro Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

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Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Gardner-Rasheed feline sarcoma virus (GR-FeSV) is an acute transforming retrovirus which encodes a gag-onc polyprotein possessing an associated tyrosine kinase activity. The integrated form of this virus has been isolated in the Charon 21A strain of bacteriophage λ, and demonstrated ability to transform NIH/3T3 cells at high efficiency upon transfection. Foci induced by GR-FeSV DNA contained rescuable sarcoma virus and expressed GR-P70, the major GR-FeSV translational product. Localization of long terminal repeats with the DNA clone made it possible to establish the length of the GR-FeSV at 4.6 kbp. Analysis of heteroduplexes formed between λ-FeLV and λ-GR-FeSV revealed the presence of a 1.7-kbp FeLV unrelated segment designated v-fgr. This region was sufficient in size to encode a protein with similar molecular weight of GR-P70.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

K. C. Robbins	Expert	LCMB, NCI
S. Tronick	Head, DNA Recombinant Unit	LCMB, NCI
S. A. Aaronson	Chief	LCMB, NCI

Objectives:

The study of acute transforming retroviruses has provided a potentially important approach to elucidate mechanisms involved in malignant transformation. GR-FeSV codes for a 70,000 dalton phosphoprotein (GR-P70), whose associated protein kinase activity is specific for tyrosine residues. In order to determine the detailed structure of the GR-FeSV genome and to compare it with other acute transforming retroviruses having tyrosine kinase activity, we undertook the molecular cloning of the integrated form of GR-FeSV and a detailed analysis of this clone.

Methods Employed:

The integrated proviral DNA (GR-FeSV) has been isolated from a clonal raccoon fibroblast line nonproductively transformed by GR-FeSV. The vector used was Charon 21A strain of λ phage propagated in E. coli K-T2 DP50supF, and DNA was purified from CsCl-banded phage. Transfection of NIH/3T3 cells with the molecular clone of GR-FeSV DNA was performed by the calcium phosphate precipitation technique. Immunoprecipitation techniques have been used to analyze the primary translational product of GR-FeSV. Heteroduplex analysis, as well as molecular hybridization, have been performed to define the onc gene (v-fgr) of GR-FeSV.

Major Findings:

Our results suggest that GR-FeSV contains a distinct tyrosine kinase coding onc gene which does not contain nucleotide sequences related to cloned DNA fragments representing a number of other retrovirus onc genes. GR-FeSV has been isolated in a biologically active form, demonstrating a specific transforming activity rivaling that of the most potent retrovirus transforming genes.

Significance to Biomedical Research and the Program of the Institute:

In our study, structural analysis of the GR-FeSV DNA clone led to the detection and localization of an onc sequence, v-fgr, to a 1.7-kbp region in the center of the genome. By the use of v-fgr as a probe, related sequences were detected in normal cellular DNAs of a variety of species employing the well conserved nature of the v-fgr protooncogene among vertebrates. A striking feature of the normal cellular sequences related to v-fgr was the large number of fragments demonstrated in each specie analyzed. In mouse cellular DNA, for example, the sum of individual v-fgr-related DNA fragments would correspond to

a simple gene of at least 125 kbp. Since the largest previously characterized cellular gene is around 40 kbp, it is likely that proto-fgr is less complex but present in multiple copies within the normal cell genome.

Proposed Course:

Sequence analysis of GR-FeSV onc gene (v-fgr) will give us information as to whether it can be evolutionarily linked to other tyrosine kinase associated onc genes. If that is so, the size of this family may be significantly increased.

Publications:

Naharro, G., Dunn, C. Y. and Robbins, K. C.: Analysis of the primary translational product and integrated DNA of a new feline sarcoma virus, GR-FeSV. Virology 125: 502-507, 1983.

Naharro, G., Tronick, S. R., Rasheed, S., Gardner, M. B., Aaronson, S. A. and Robbins, K. C.: Molecular cloning of integrated Gardner-Rasheed feline sarcoma virus: Genetic structure of its cell-derived sequence differs from that of other tyrosine kinase coded onc genes. J. Virol. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05310 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Translocation and Rearrangements of c-myc Gene in Burkitt's Lymphoma

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
A. Yaniv Visiting Scientist, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The 3' exon of the human c-myc oncogene was cloned in pBR322. This probe detected two Eco RI rearranged c-myc gene fragments (3.6 and 2.4 kb) in Raji (Burkitt's lymphoma) cells. The two DNA fragments were isolated from Raji cell DNA and cloned in lambda phage. Restriction enzyme analysis revealed that the 3.6-kb fragment carried the entire 5' and 3' c-myc exons, while the 2.4-kb fragment contained only part of the 3' c-myc exon. Neither DNA fragment contained immunoglobulin sequences.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

Steven Tronick	Head, Recombinant DNA Unit	LCMB, NCI
David Swan	Expert	LCMB, NCI

Objectives:

1. To analyze in detail the molecular rearrangements of the human c-myc locus that occurs in Burkitt's lymphoma.

2. To investigate whether this chromosomal translocation alters the structure and/or expression of the c-myc oncogene.

Methods Employed:

Standard molecular biological techniques for nucleic acid isolation, gene enrichment, gel electrophoresis, Southern transfer, molecular hybridization, recombinant DNA techniques for cloning and amplification of genes, and restriction enzyme mapping of cloned genes.

Major Findings:

The 1.5-kb Cla I-Eco RI fragment of the human c-myc containing the 3' exon was subcloned into plasmid pBR322. The availability of this cloned DNA as a probe enabled us to detect in the Raji (Burkitt's lymphoma) cell line, rearranged myc gene sequences, in addition to an apparently normal allele. This finding pointed to the presence of three separate myc loci within the Raji genome.

The rearranged Eco RI Raji DNA fragments were comprised of a 3.6-kb fragment that contained both the 3' and 5' c-myc exons and a 2.4-kb fragment which carries only the 3' c-myc exon. These DNA fragments were isolated from Eco RI-digested Raji DNA and cloned in phage λ Wes.B Eco RI arms. Restriction enzyme analysis of the cloned DNAs indicated that the 3.6-kb fragment contained the entire 5' and 3' c-myc exons, while the 2.4-kb DNA fragment seemed to contain only part of the normal 3' c-myc exon. Since the 5' flanking region in the 3.6-kb fragment contained an Eco RI site closer to the 5' myc exon than in the normal unrearranged c-myc, we assumed that this locus had been translocated.

Probing the cloned DNA c-myc-containing fragments with several immunoglobulin heavy chain probes revealed the absence of immunoglobulin sequences within these DNA fragments. Since both DNA clones are Eco RI fragments, we suggest that the 5' Eco RI site is located between the c-myc and the immunoglobulin loci.

Significance to Biomedical Research and the Program of the Institute:

The isolation and cloning of specific c-myc onc genes are extremely valuable for the study of how translocations, oncogenes, and transformation might be related in Burkitt's lymphoma. In addition, these procedures will facilitate studies that might reveal the role of such gene products in oncogenesis and normal cellular functions.

Proposed Course:

A detailed restriction enzyme and nucleotide sequence analysis will be performed in order to determine the genomic organization and breakage point in the translocated c-myc-containing DNA fragments.

Qualitative and quantitative examinations of the c-myc gene products in Burkitt's lymphoma, as well as in other related malignancies, will be performed.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05311-01 LCMB

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Study of Moloney Sarcoma Virus (MSV) Genome

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

R. Narayanan Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The effect of long terminal repeats (LTRs) on the transforming gene of Moloney murine sarcoma virus (MSV) genome, v-mos, was investigated. The relative transformation efficiency of the various recombinants was assayed by DNA transfection of NIH/3T3 cells. Comparison of cloned MSV genomic and subgenomic fragments revealed that subgenomic clones which lacked 3' LTR were not biologically active. Using site-specific mutagenesis, the region of 3' LTR required for v-mos gene function in vivo was localized. Deletion of cap site, TATA box, poly A signal, and CAAT box within LTR did not impair the transforming ability. Loss of one 72-bp repeat in the LTR did not affect the transforming activity. However, loss of both 72-bp repeats in the LTR completely abolished biological activity. These results localized the critical sequences within the LTR required for efficient mos gene activation to a region of no more than 100 bp encompassing one of the 72-bp repeats.

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
E. P. Reddy	Visiting Scientist	LCMB, NCI
A. Srinivasan	Visiting Associate	LCMB, NCI
S. G. Devare	Visiting Associate	LCMB, NCI

Objectives:

1. To study the structural requirements of LTR for the transforming gene function of Moloney-MSV.

2. To characterize the functional domains of MSV LTR by in vitro mutagenesis.

Methods Employed:

Molecular cloning techniques, transfection assays, and sequencing.

Major Findings:

Tandemization of Hind III permuted MSV-124 genome: The Hind III permuted form of MSV-124 genome from unintegrated circular MSV DNA containing only the 5' LTR was very poor in inducing transformation of NIH/3T3 cells. In contrast, a Xho I permuted MSV-124 genome, with two LTRs in tandem, had a focus-forming titer of 5×10^3 ffu/μg. In order to exclude the possibility of an inherent defect in the Hind III permuted clone, it was tandemized by bringing the 5' LTR of one molecule to the 3' end of another molecule, introduced by linker modification. The tandemized Hind III clone demonstrated a level of transforming activity comparable to that of the Xho I permuted MSV clone or to wild-type viral DNA cloned from its integrated state within the cellular genome.

The involvement of LTR in the activation of v-mos was further confirmed by the lack of transforming activity of MSV-124 subclones devoid of both the LTRs. Introducing the 3' LTR of Abelson-MuLV downstream of v-mos demonstrated efficient transforming activity. These results confirmed the ability of an LTR downstream of v-mos to confer efficient transforming ability to this onc gene.

Localization of the region within the 3' LTR required for v-mos transforming activity: In an attempt to localize the critical sequences within the 3' LTR responsible for efficient transformation of v-mos, progressive deletions within the 3' LTR were made. Mutants which lack the poly A signal; poly A and TATA box; poly A, TATA box + CAAT box sequences were each capable of transforming NIH/3T3 cells. These results suggested that these signals were not required for the 3' LTR to function effectively for v-mos transforming activity.

In an effort to better localize the critical sequences, a mutant was constructed which deleted MuLV sequences from the Bam HI site distal to v-mos, to the Sac I site within LTR, leaving intact only TATA and poly A signal. This mutant had no detectable level of transforming activity. These results suggested that the critical sequence is located within the U_3 region of the LTR. To more precisely identify the essential sequences, within the U_3 region, three more constructs were made with progressive deletion in the U_3 region. Results showed that loss of one 72 bp did not affect the transforming activity. However, removal of both the 72-bp repeats completely abolished the biological activity. These results localized the critical sequence within the LTR required for efficient Moloney-MSV transforming activity to a region of no more than 100 bp encompassing one of the 72-bp repeats.

Effect of LTR distance from v-mos on MSV-transforming activity: In order to determine to what extent the LTR potentiating effect on v-mos transforming activity was modulated by distance, constructs with varying distance between v-mos and the LTR were made by inserting λ DNA fragments. Results showed that increasing the distance by as much as 1.8 kbp did not diminish the MSV-transforming activity. With larger distances, transforming activity was significantly reduced.

Significance to Biomedical Research and the Program of the Institute:

Using cloned MSV-124 proviral DNA as a tool, we have made progress in understanding the basic processes underlying transformation. Results of these experiments are providing insights and approaches to a better understanding of human neoplasia.

Proposed Course:

Preliminary experiments suggested that the mos gene can be activated by different LTRs when introduced downstream. The knowledge of the complete nucleotide sequence of the LTRs will be used in determining the role of enhancer/core sequences in mos gene activation. Using site-specific mutagenesis, deletions will be made in the core sequences and the effect on transforming gene function will be studied. These results should shed light on the mechanism of onc gene activation by retroviral LTRs.

Publications:

Phillips, L. A., Kang, M. S. and Narayanan, R.: Retroviruses: Life cycle of leukemia and sarcoma viruses with particular reference to viral nucleic acids. In Phillips, L. A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker, Inc., 1983, pp. 411-438.

Phillips, L. A., Kang, M. S. and Narayanan, R.: Retroviruses: Studies at the nucleotide level to establish an experimental basis for a molecular approach to human cancer. In Phillips, L. A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker, Inc., 1983, pp. 583-607.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05312-01 LCMB

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural and Functional Analysis of MC29 Proviral Genome

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
S. Lavu Visiting Fellow, LCMB, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In an effort to understand the molecular mechanisms involved in the transformation process mediated by myelocytomatosis virus (MC29), molecular cloning of the complete proviral genome was undertaken. We have successfully cloned the proviral genome in λ Charon 21A phage. Studies are now being performed to test the transforming activity of the viral genome using various cell lines derived from mouse, rat, and avian species. In vitro mutagenesis of the MC29 proviral genome is currently being carried out to understand the molecular mechanisms involved in the transformation process mediated by this genome.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (Other than the Principal Investigator) engaged on this Project:

E. P. Reddy	Visiting Scientist	LCMB, NCI
S. A. Aaronson	Chief	LCMB, NCI

Objectives:

To test the transforming activity of the proviral genome of MC29 in various cell lines and to delineate the role of v-myc sequences in the transforming process. In vitro mutagenesis of the proviral genome to understand the molecular mechanisms involved in the transformation process mediated by the viral genome is currently being studied.

Methods Employed:

1. Restriction enzyme analysis of the DNA;

2. Gel electrophoresis and Southern blotting;

3. Enrichment of the inserted fragment by electroelution and sucrose gradient separation; and

4. Molecular cloning of the genome in λ phages.

Major Findings:

Total cellular DNA from two quail nonproducer cell lines, Q5 and Q8, transformed by myelocytomatosis virus (MC29) were analyzed for the presence of proviral genome. The DNA was cleaved with several different restriction enzymes and analyzed by Southern blot analysis and hybridized with a radiolabelled v-myc probe. Two bands, 20 kbp and 7 kbp in length, were identified in Q5 cell DNA cleaved with Hind III. Q8 cell DNA, on the other hand, yielded multiple bands under the same conditions, ranging in size from 2 to 20 kbp. Normal quail cell DNA yielded a single band of 20 kbp hybridizable with v-myc probe. Since Hind III does not cleave within the proviral genome, it was assumed that the 7 kbp present in Q5 cell DNA contained the entire proviral genome with flanking quail cellular sequences, while the 20-kbp band contained the quail c-myc sequences. These experiments also indicated that Q8 cell DNA contained multiple proviral genes, some of which represent deletions within the viral genome. Q5 cell DNA was therefore used for purification and molecular cloning of the MC29 proviral genome.

Total Q5 cell DNA was cleaved with Hind III and was enriched for MC29 proviral sequences by sucrose density gradient fractionation. The enriched DNA was ligated to the arms of bacteriophage λ Charon 21A and packaged in vitro into phage particles and plated onto E. coli BNN45. Approximately 200,000 plaques were screened with v-myc probe using the plaque filter hybridization technique. Two positive clones were identified and plaque-

purified until the number of recombinant phage in the population was greater than 95%. Recombinant phage DNA was prepared from the two clones and a restriction map was developed. These results indicated that one of the clones contained the entire proviral genome, while the second clone has undergone a deletion at the 3' end. The DNAs from the two clones are currently being analyzed for in vitro transforming activity using mouse, rat, and avian cell lines.

Significance to Biomedical Research and the Program of the Institute:

Myelocytomatosis virus (MC29) is an acute transforming retrovirus of aves. The oncogene of this virus appears to be involved in lymphomagenesis of man and other mammalian species. Delineation of the molecular mechanism of action of this oncogene will lead to a better understanding of its role in human lymphomagenesis.

Proposed Course:

Studies with the cloned proviral genome of MC29 to test its transformation potential on various cell lines and to study the molecular mechanisms involved using in vitro mutagenesis techniques will be continued.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF CHEMOPREVENTION

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

The problem of the isolation and characterization of transforming polypeptide growth factors (TGFs) continues to occupy an increasing amount of attention within our laboratory. Previously, we had shown that TGFs can be isolated from a variety of epithelial and mesenchymal tumors of murine, chicken, and human origin, caused either by chemicals or viruses, or of spontaneous origin. All of these TGFs are acid-stable, low molecular weight materials, that will be the subject of future attempts at amino acid sequencing once they are purified to homogeneity. New methods to achieve this desired purification have been developed in our laboratory during the past year, and we have completed the toal purification of TGF-beta from 3 non-neoplastic tissues within the past 12 months. These tissues are human placenta, human blood platelets, and bovine kidney. The experimental use of TGF-beta in wound healing has been a finding of major importance, and has provided a great deal of encouragement to proceed further with the entire problem of the molecular biology and molecular genetics of these growth factors.

TGFs characterized by their ability to confer a transformed phenotype on untransformed indicator cells have been isolated from all neoplastic and non-neoplastic tissues examined thus far by direct extraction with acid/ethanol. Assay of these polypeptides is based on their ability to induce normal rat kidney fibroblasts (NRK) to form colonies in soft agar. Two major subsets of the TGF family have been separated and characterized in terms of their interactions with epidermal growth factor (EGF). TGF-α competes with EGF for binding to membrane receptors while TGF-β binds to an as yet uncharacterized membrane receptor distinct from the EGF receptor. The presence of both TGF-α (or EGF) and TGF-β is required to induce transformation of NRK cells; neither TGF-α nor TGF-β acting alone can induce formation of large colonies of these cells in soft agar. This concerted action of these two distinct sub-classes of the TGF family has been demonstrated both for TGFs extracted directly from cells, and for TGFs isolated from conditioned medium of murine sarcoma virus-transformed mouse cells (often referred to as sarcoma growth factor). In addition, the effect is unchanged whether TGF-α or TGF-β is derived from a neoplastic or non-neoplastic source.

TGF-β has been purified over 200,000-fold from bovine kidney. This peptide is characterized by its ability to induce anchorage-dependent normal rat kidney cells to grow in soft agar in the presence of epidermal growth factor (EGF); TGF-β is not mitogenic for cells grown in monolayer culture. Purified TGF-β does not compete with EGF for binding to membrane receptors. The concentration of TGF-β required to elicit a half-maximal response for formation of colonies >3100 μm^2 in the soft agar assay is 2-3 picomolar (55 pg/ml) when assayed in the presence of 0.8 nanomolar EGF (5 ng/ml). The four-step purification procedures, which includes chromatography of acid-ethanol-tissue extracts on polyacrylamide sizing gels, cation exchange, and two steps of high pressure liquid chromatography, results in a 10% overall yield of colony-forming activity with a recovery of 3-4 $\mu g/kg$. Amino acid analysis of purified

TGF-β shows 16 half-cystine residues per mole. Analysis of the purified polypeptide by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels indicates that TGF-β is composed of two closely related polypeptide chains cross-linked by disulfide bonds. In the absence of β-mercaptoethanol, the colony-forming activity is associated with a single silver-staining band of molecular weight 25,000; in the presence of β-mercaptoethanol, the TGF-β is converted to an inactive species that migrates as a single band at molecular weight 12,500-13,000. Sequence analysis indicates that at least the first fifteen N-terminal amino acids of the two TGF-β subunits are identical.

Amino acid sequencing studies have also been started on TGF-β from human placenta and human platelets. In the case of the placental TGF, 29 residues from the amino terminus have been determined, while 10 residues from the amino terminus have been determined for the platelet TGF. It is of major significance that the sequences that have been determined thus far for all 3 peptides (bovine kidney, human placenta, human platelet) are identical, indicating that TGF-β is a highly conserved structure. It is hoped to complete the sequence determination of the platelet TGF within the coming year.

Another major area of TGF research that has been explored for the first time within the past year is the development of a receptor binding assay for TGF-β. Methods have been developed for the successful iodination of TGF-β, which was a difficult problem. Results of recent experiments now show that there are membrane-bound, high affinity, saturable receptors for TGF-β in NRK cells. Efforts are currently underway to characterize these receptors and to determine the physiological response that occurs after interaction of TGF-β with its receptors. The development of a routine receptor assay for TGF-β should be of definite use in diagnostic screening for the possibility of abnormal expression of high levels of this peptide in a variety of disease states characterized by abnormal cell proliferation.

The Laboratory of Chemoprevention continues to have a major interest in the role of retinoids in modifying the proliferative activity of cells, and in the ultimate clinical use of retinoids as chemopreventive agents. Collaborative studies on the use of retinoids to prevent breast and bladder cancer in rats and mice continue to be pursued, and the Laboratory Chief continues to provide advice and consultation to various clinical groups that are interested in the use of retinoids to prevent skin, breast, and bladder cancer in men and women at high risk. The Laboratory of Chemoprevention is still involved in the screening of new retinoids that hopefully will have better pharmacological properties than existing agents. New agents have been synthesized at the BASF laboratories in Germany which have greatly altered chemical structure, and the cell biology of these agents is being investigated in our laboratories in Bethesda.

Finally, a long term goal in our studies of transforming growth factors is the development of peptide antagonists of the TGFs. Such agents should have use both as chempreventive and chemotherapeutic agents. Recent reports from laboratories other than ours regarding the chemical systhesis of the EGF gene and its incorporation into bacteria suggest that it will also be possible to make anti-EGFs by such recombinant DNA techniques. With the discovery in our own laboratory that EGF and EGF-like peptides are required for the expression of TFG-activity, the possibility of using EGF antagonists to block malignant transformation now becomes more of a reality.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05051-05 LC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation of Polypeptide Transforming Factors from Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Anita B. Roberts Staff Scientist, LC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.6	2.0	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to develop methods for the extraction, isolation, and characterization of polypeptides with the property of conferring the transformed phenotype on normal indicator cells. These purified transforming growth factors (TGFs) will then form the basis for investigation of the mechanism of transformation and more specifically for the development of protein antagonists to these TGFs. Efforts have been focused on the purification of members of a specific subset of the TGF family, called TGF beta, which is characterized by a requirement for epidermal growth factor for functional activity and which, because of its presence in all tissues examined thus far, probably has an intrinsic physiological role in normal cell growth. TGF betas are being purified concurrently from murine-sarcoma virus-transformed cells grown in culture and from non-neoplastic derivation and different genomes will then be compared both with regard to biological activity and to chemical composition.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Mario A. Anzano Visiting Fellow LC, NCI

Objectives:

The ultimate goal of this research is to determine the complete amino acid sequence of transforming growth factors (TGFs) from both neoplastic and non-neoplastic tissues and then to use this as a basis for the design of antagonists to these polypeptides. In addition, investigations will be directed at elucidation of the biochemical mechanism of action of TGFs both in induction of expression of the transformed phenotype and in the processes of tissue repair and development. These mechanistic studies will include the effects of agents such as the retinoids and steroid hormones, which have been shown to modulate the transforming properties of TGFs.

Methods Employed:

Classical methods of protein purification and new methods based on reverse-phase-high pressure liquid chromatography have been employed. These include dialysis, solvent precipitation, the use of sizing gels and ion-exchange columns, as well as methods based on the electrophoretic properties of these polypeptides.

An assay for these TGFs based on their ability to cause a normal anchorage-dependent indicator cell to grow in an anchorage-independent manner (as assessed by growth in semisolid agar medium) has been set up in our laboratory and is routinely being used to monitor the purification of these TGFs. An image analysis system has been adapted to this assay to quantitate both the number and the size of the colonies formed.

Major Findings:

Polypeptides (TGFs) characterized by their ability to confer a transformed phenotype on untransformed indicator cells have been isolated from all neoplastic and non-neoplastic tissues examined thus far by direct extraction with acid-ethanol. Assay of these polypeptides is based on their ability to induce normal rat kidney fibroblasts (NRK) to form colonies in soft agar. Two major subsets of the TGF family have been separated and characterized in terms of their interactions with epidermal growth factor (EGF). TGF-α competes with EGF for binding to membrane receptors while TGF-β binds to an as yet uncharacterized membrane receptor distinct from the EGF receptor. The presence of both TGF-α (or EGF) and TGF-β is required to induce transformation of NRK cells; neither TGF-α nor TGF-β acting alone can induce formation of large colonies of these cells in soft agar. This concerted action of these two distinct subclasses of the TGF family has been demonstrated both for TGFs extracted directly from cells, and for TGFs isolated from conditioned medium of murine sarcoma virus-transformed mouse cells (often referred to as sarcoma

growth factor). In addition, the effect is unchanged whether TGF-α or TGF-β is derived from a neoplastic or non-neoplastic source.

TGF-β has been purified over 200,000-fold from bovine kidney. This peptide is characterized by its ability to induce anchorage-dependent normal rat kidney cells to grow in soft agar in the presence of epidermal growth factor (EGF); TGF-β is not mitogenic for cells grown in monolayer culture. Purified TGF-β does not compete with EGF for binding to membrane receptors. The concentration of TGF-β required to elicit a half-maximal response for formation of colonies >3100 μm^2 in the soft agar assay is 2-3 picomolar (55 pg/ml) when assayed in the presence of 0.8 nanomolar EGF (5 ng/ml). The four-step purification procedure which includes chromatography of acid-ethanol-tissue extracts on polyacrylamide sizing gels, cation exchange, and two steps of high pressure liquid chromatography, results in a 10% overall yield of colony-forming activity with a recovery of 3-4 μg/kg. Amino acid analysis of purified TGF-β shows 16 half-cystine residues per mole. Analysis of the purified polypeptide by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels indicates that TGF-β is composed of two closely related polypeptide chains cross-linked by disulfide bonds. In the absence of β-mercaptoethanol, the colony-forming activity is associated with a single silver-staining band of molecular weight 25,000; in the presence of β-mercaptoethanol, the TGF-β is converted to an inactive species that migrates as a single band at molecular weight 12,500-13,000. Sequence analysis indicates that at least the first fifteen N-terminal amino acids of the two TGF-β subunits are identical.

Preliminary data shows that TGF-β isolated from murine sarcoma virus-transformed cells (either directly from the cells or from the conditioned medium) and from non-neoplastic mouse kidneys is indistinguishable from bovine kidney TGF-β in its electrophoretic behavior on sodium dodecyl sulfate-polyacrylamide gels.

Both retinoic acid and dexamethasone affect the ability of TGF-β and EGF to promote colony formation of NRK cells in soft agar. Treatment of NRK cells with retinoic acid greatly increases the colony-forming activity of the cells in soft agar while treatment with dexamethasone inhibits the response. In monolayer culture, retinoic acid treatment enhances expression of transformed morphology in cells treated with TGF-β plus EGF while dexamethasone treatment restores a more normal morphology. These effects can be correlated with the ability of each of these agents to modulate EGF binding to the NRK cells. Retinoic acid treatment increases the binding of EGF approximately 4-6 fold, while dexamethasone reduces the binding to 50-60% of control levels. These effects suggest that colony formation of NRK cells in soft agar induced by the combined action of TGF-β and EGF is dependent, in part, on the number of EGF receptors of the indicator cell. These effects may relate to the putative action of TGF-β in embryonic development where changes in EGF receptor levels are known to accompany changes in differentiation.

Significance to Biomedical Research and the Program of the Institute:

The occurrence of TGFs in both platelets and in all tissues examined thus far, both neoplastic and non-neoplastic, suggests that TGFs must have an intrinsic physiological function apart from maintenance of the transformed phenotype. In support of this is the recent work demonstrating the cellular nature of the viral oncogenes and evidence that these transforming genes are actively transcribed in non-neoplastic cells. Thus, elucidation of the mechanism of action of TGFs in the expression of neoplastic behavior will likely also impact on our understanding of normal cellular processes. It should also be expected then that experiments using retinoids and other inhibitors of TGF action will offer opportunities to explore the cellular control mechanisms operative in both neoplastic and non-neoplastic growth. Finally, the recent finding that TGFs can promote wound healing in rats may lend itself to future therapeutic application of TGFs in clinical management of wound healing.

Proposed Course:

Future work will center around the purification to homogeneity of sufficient quantities of TGF-β from virally transformed mouse cells and from non-neoplastic mouse and bovine kidneys for the complete chemical characterization including isoelectric point, amino acid composition, and amino acid sequence. We also plan to develop a radioreceptor assay and a radioimmunoassay for TGF-β so that its role in normal physiology can be explored. Using the purified TGFs, we hope to examine cellular control mechanisms involved in the synthesis, secretion and functional activity of TGFs. In addition, investigations will be directed at exploring possible mechanistic links between control of cell growth and differentiation by retinoids, oncogenes, and transforming growth factors.

Publications:

Anzano, M. A., Roberts, A. B., Lamb, L. C., Smith, J. M. and Sporn, M. B.: Purification by reversed-phase high performance liquid chromatography of an epidermal growth factor-dependent transforming growth factor. Anal. Biochem. 125: 217-224, 1982.

Anzano, M. A., Roberts, A. B., Meyers, C. A., Komoriya, A., Lamb, L. C., Smith, J. H. , and Sporn, M. B: Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. Cancer Res. 42: 4776-4778, 1982.

Anzano, M. A., Roberts, A. B., Smith, J. M., Sporn, M. B. and De Larco, J. E.: Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type α and type β transforming growth factors. Proc. Natl. Acad. Sci. USA (In Press).

Roberts, A. B., Anzano, M. A., Frolik, C. A. and Sporn, M.B.: Transforming growth factors - characterization of two classes of factors from neoplastic and non-neoplastic tissues. In Cold Spring Harbor Conferences on Cell Proliferation: New York, Cold Spring Harbor Symposium, 1982, Vol. 9, pp. 319-332.

Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blatcher, R., Pan, Y.-C. E., Stein, S., Lehrman, S. R., Smith, J. M., Lamb, L. C. and Sporn, M. B.: Purification and properties of a type-β transforming growth factor from bovine kidney. Biochemistry (In Press).

Roberts, A. B., Frolik, C. A., Anzano, M. A., Assoian, R. K. and Sporn, M. B.: Purification of type-β transforming growth factors from non-neoplastic tissues. In Barnes, D., Sato, G. and Sirbasku, D. (Eds.): Methods in Molecular and Cell Biology. New York, Alan R. Liss, 1983. (In Press).

Roberts, A. B., Frolik, C. A., Anzano, M. A. and Sporn, M.B.: Transforming growth factors from neoplastic and non-neoplastic tissues. Fed. Proc., 42: 2621-2626, 1983.

Sporn, M. B. and Roberts, A. B.: The role of retinoids in differentiation and carcinogensis. Cancer Res. 43: 3034-3040, 1983.

Sporn, M. B., Roberts, A. B., Shull, J. H., Smith, J. M. and Ward, J. M.: Polypeptide transforming growth factors: Isolation from bovine sources and use for wound healing in vivo. Science 219: 1329-1331, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05116-04 LC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Transforming Growth Factors from Human Tissue and Cell Lines

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Charles A. Frolik Chemist, LC, NCI

COOPERATING UNITS *(if any)*

Hoffmann-LaRoche, Nutley, NJ

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	1.7	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Acid-stable polypeptides have been purified to homogeneity from human tumor cells and from normal human tissues. These proteins are able to produce a reversible morphological transformation of normal rat kidney fibroblast cells that allows these cells to grow in a soft agar medium where normal cell growth is prevented. It is the purpose of this project to characterize these transforming growth factors and to ascertain the role these factors may play in the process of carcinogenesis. Initial investigation is concentrating on the purification and characterization of these polypeptides from normal human placenta, as well as from a rhabdomyosarcoma tumor cell line (A673), using gel filtration, ion exchange and high pressure liquid chromatography. Once these proteins have been characterized, analogs will be synthesized and tested for their ability to inhibit the transforming activity of the natural peptide and therefore, possibly, inhibit the carcinogenic process itself. Finally, the mechanism whereby these factors interact with the cell to cause phenotypic cell transformation is being investigated in order to gain a greater understanding of the process of carcinogenesis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Chester A. Meyers	Expert	LC, NCI
S. Russ Lehrman	Staff Fellow	LC, NCI
Lalage Wakefield	Visiting Fellow	LC, NCI

Objectives:

The purpose of this study is to explore new methods that may be useful in the chemoprevention of cancer. Special emphasis is initially being placed on the isolation and characterization of acid stable polypeptide transforming growth factors (TGFs) found in various human tumor cells and in normal human tissue. Once characterized, TGF analogs will be synthesized and tested for their ability to block the action of TGF in causing phenotypic cell transformation.

Methods Employed:

TGF is extracted from various samples using an acid-ethanol extraction procedure. Once extracted, the peptides are purified to homogeneity by gel filtration and ion-exchange chromatography followed by reverse-phase high pressure liquid chromatography (HPLC). The purity of the samples is monitored by polyacrylamide gel electrophoresis. Transforming activity is determined by a soft agar growth assay with the number and size of the cell colonies obtained being measured using an Omnicon image analysis system. The purified factor has been characterized by amino acid analysis and sequence determination and has been iodinated for use in radioreceptor assays.

Major Findings:

1) ISOLATION AND PURIFICATION OF TGFS. TGFs have been detected in all human tissues and cell lines investigated to date. One tumor cell line (A673) has been selected for further purification of these TGFs. In addition, TGF is being isolated from normal human placenta. It was shown initially in HeLa and later confirmed in murine and bovine tissues and cell lines that there are at least two classes of TGF depending on their interaction with epidermal growth factor (EGF). One class, TGF-α, is independent of EGF for transforming activity while the second class, TGF-β, requires the presence of EGF for activity. It was later demonstrated, first in murine tissues and later in HeLa, that TGF-α is able to compete with EGF for binding to EGF membrane receptors while TGF-β does not interact with these receptors. TGF-α appears to be a component mainly of neoplastic cells while TGF-β is found in both neoplastic and non-neoplastic cells and tissue. TGF-β from human placenta and from the A673 cell line, has been purified to homogeneity by chromatography of the residue obtained from an acid-ethanol extract of the tissue or cells on a Bio-Gel P-30 gel filtration column followed by chromatography on a cation-exchange column and finally two different reverse-phase HPLC columns. The

TGF-β obtained from normal placenta appears to be identical to that isolated from the A673 tumor cell line in regards to biological activity, amino acid composition, and migration rate on HPLC columns and on gel electrophoresis systems. Both factors are approximately 25,000 molecular weight and appear to be composed of two identical peptide chains of approximately 13,000 molecular weight held together by disulfide linkages. The first 29 amino acids from the N-terminus have been sequenced by Dr. S. Stein's group at Hoffmann-LaRoche using a gas-phase sequencer.

2) CONTROL OF TGF ACTIVITY. An inhibitor of cell proliferation has been detected in the acid-ethanol extract of normal human placenta and bovine kidney and lung. The inhibitory factor is non-protein in nature as judged by its resistance to a variety of proteases and to boiling in 6M HCl. The factor has an apparent molecular weight of 2000 on gel permeation columns and has been purified to homogeneity by chromatography on an ion-exchange column and on a reverse-phase HPLC column. The inhibitor has been identified as spermine by mass spectroscopy and nuclear magnetic resonance analysis. In order for spermine to inhibit TGF induced cell proliferation, it must first be oxidized by plasma amine oxidase. Because the physiological significance of this inhibition is uncertain, the role of spermine in the regulation of TGF activity will not receive major emphasis in the near future.

3) MECHANISM OF ACTION OF TGF-β. The initial interaction of TGF-β with the cell membrane is currently under investigation. Early results indicate that there are membrane bound, high affinity, saturable receptors for TGF-β. Efforts are currently underway to further, characterize these receptors and to determine the physiological response that occurs after interaction of TGF-β with its receptors.

Significance to Biomedical Research and the Program of the Institute:

Through a knowledge of the physical structure of TGF, its mode of action in causing phenotypic transformation and its mechanism of control in normal tissues, it is anticipated that a sound approach to the chemoprevention of carcinogenesis will be able to be developed.

Proposed Course:

In the future, work will center around the purification and characterization of the TGFs from a variety of normal and neoplastic sources. In addition, compounds which enhance or inhibit the action of TGF-β in causing a reversible, morphological transformation of normal cells will be sought. Finally, investigations will continue into the mode of action of TGF-β, especially in regard to the initial interaction of TGF-β with a membrane receptor.

Publications:

Frolik, C. A.: Metabolism of retinoids. In Goodman, D. S., Roberts, A. B. and Sporn, M. B. (Eds.): The Retinoids. New York, Academic Press, 1983. (In Press).

Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M. and Sporn, M. B.: Purification and initial characterization of a type-β transforming growth factor from human placenta. Proc. Natl. Acad. Sci. USA 80. 1983. (In Press).

Frolik, C. A., Dart, L. L., and Sporn, M. B.: Variables in the high-pressure cation-exchange chromatography of proteins. Anal. Biochem. 125: 203-209, 1982.

Frolik, C. A. and Olson, J. A.: Extraction, separation, and chemical analysis of retinoids. In Goodman, D. S., Roberts, A. B. and Sporn, M. B. (Eds.): The Retinoids. New York, Academic Press, 1983. (In Press).

Roberts, A. B., Frolik, C. A., Anzano, M. A., Assoian, R. K. and Sporn, M. B.: Purification of type-β transforming growth factors from non-neoplastic tissue. In Barnes, D., Sato, G. and Sirbasku, D. (Eds.): Methods in Mol. and Cell. Biol. New York, Alan R. Liss, 1983. (In Press).

Roberts, A. B., Frolik, C. A., Anzano, M. A. and Sporn, M. B.: Transforming growth factors from neoplastic and non-neoplastic tissues. Fed. Proc. 42: 2621-2626, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05209-03 LC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synthetic and Biological Studies on Epidermal and Transforming Growth Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Chester A. Meyers Expert, LC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.75	1.75

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A group of transforming growth factors (TGF's) have been identified in both normal and transformed cells from a variety of tissue types. These factors, which are effectors of malignant phenotypic transformation, are peptides closely associated with epidermal growth factor (EGF). EGF enhances the activity of some TGF's, (TGF-beta) while others bind to the EGF receptor. This study aims to define structure-function relationships of EGF and, ultimately, TGF molecules through synthetic and chemical modification techniques, leading primarily to the rational design of effective inhibitors. Our continuing work with EGF has led to identification of a localized region in which the information for receptor binding and early biological responses resides. In the past year, TGF-beta from three tissue types in two species (bovine and human) have been purified, and the amino acid composition and partial sequence have been determined using picomole quantities of material. Synthesis of peptide fragments have begun, and efforts to raise antibodies to specific regions of the molecule are underway.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this project:

Akira Komoriya	Staff Fellow	LC, NCI
S. Russ Lehrman	Staff Fellow	LC, NCI

Objectives:

The project will continue to focus on the newly identified localized region of EGF responsible for receptor binding and biological activity. Chemical modifications directed towards this region are planned to define still smaller critical sequences, and possibly lead to development of antagonists to the native molecule. In addition, the purification of minute amounts of TGFB by others in this laboratory make further chemical characterization, synthesis of fragments, and antibody production primary objectives of the project. These tools may eventually be useful in the diagnosis, prevention, or treatment of cancer and other proliferative diseases.

Methods Employed and Major Findings:

The project embodies a broad program of chemical synthesis, modification, sequencing, analysis, chromatographic separations, antibody production, and biological testing. As a part of preparative scale isolation of TGFs, large amounts of purified EGF are collected and used by us for studies in chemical modification, synthesis, and biological assays. In addition to the standard assays for general mitogens and specific binding assays for EGF, we are able to routinely test the peptides in a biological assay where EGF specifically enhances the transforming capability of TGF.

Peptide synthesis is primArily done by state-of-the-art solid-phase methodology and we are also developing methods for semisynthetic fragment condensation of synthetic and native peptides. Solution synthesis of several partially protected C-terminal EGF peptides has been accomplished for this purpose. Purification of synthetic peptides is accomplished by a combination of gel filtration, ion exchange, partition, and reversed-phase high-performance liquid chromatographic techniques. The modified EGF studies have resulted in one derivative, S. Aureus V8 protease-treated EGF, with apparently enhanced receptor binding affinity compared with native EGF. This study also produced a variety of peptides with a range of binding affinities useful for clarifying the role of EGF receptor binding to the biological activity of the TGFs.

The synthetic program has produced cyclic and linear fragments of individual and overlapping regions of the EGF molecule. Results from biological testing of these peptides reveal a pattern which suggests that the primary receptor binding information may be localized in the middle region of the molecule (residues 20-31). This view is consistent with a computer model of EGF tertiary structure derived from completely different information. Chemical characterization studies of TGF and related peptides, particularly

by quantitative amino acid analysis in the low picomole range, is now routinely used by our laboratory.

Significance to Biomedical Research and the Program of the Institute:

This work is expected to produce a major tool, namely, an antagonist to EGF, which can be used to probe the mechanisms by which cellular proliferation and transformation occur. This should broaden our understanding of cancer and several other proliferative diseases. In addition, these molecules, and related TGF analogues, and their antibodies have potential as clinical agents in the diagnoses,treatment or prevention of such diseases.

Proposed Course:

To continue as described above, with emphasis on the modification of native EGF and development of antibodies to TGF.

Publications:

Anzano, M. A., Komoriya, A., Lamb, L. C., Meyers, C. A., Roberts, A. B., Smith, J. H. and Sporn, M. B.: Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. Cancer Res. 42: 4776-4778, 1982.

Assoian, R. K., Komoriya, A., Meyers, C. A. and Sporn, M. B.: Transforming growth factor-β in human platelets: Identification of a major storage site, purification and characterization. J. Biol. Chem. 278: 7155-7160, 1983.

Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M. and Sporn, M. B.: Purification and initial characterization of a type-β transforming growth factor from human placenta. Proc. Natl. Acad. Sci. U.S.A. (In Press)

Lehrman, S. R. and Meyers, C. A.: PTH-Amino acid identification by multi-component analysis of first and second derivative ultraviolet spectra. In Hruby, V. (Ed.): Peptides. Proceedings of the 8th American Peptide Symposium. (In Press)

Mortsch, M., Kanety, H., Komoriya, A., Meyers, C. A. and Schlessinger, J.: Synthetic epidermal growth factor fragments induce phosphorylation of endogenous membrane proteins. In Hruby, V. (Ed.): Peptides. Proceedings of the 8th American Peptide Symposium. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05266-02 LC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synthesis and Biological Activity of EGF Fragments and Analogs

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Akira Komoriya Senior Staff Fellow, LC, NCI

COOPERATING UNITS *(if any)*

Birkbeck College, Univ. of London, U.K.; National Measurements Laboratory, National Bureau of Standards; Laboratory of Biophysics, NIADDK, NIH

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.00	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have synthesized a series of overlapping fragments of mouse EGF. Biological assays of these fragments suggest that the 20-31 region of EGF is most important for binding. Using a series of synthetic fragments of mouse EGF, the antigenic determinants of mouse EGF were determined. Further work on pseudoamino acid synthesis is being carried out. Preparation of monoclonal anti-EGF is also in progress. Enzymatic resynthesis of proteins is being investigated with the object of synthesizing EGF analogs by enzymatic condensation of an appropriate peptide with an EGF fragment obtained from enzymatic degration of native EGF. In collaboration with the units listed above, we are undertaking x-ray structural studies of EGF. Using computer graphics, a modeled EGF tertiary structure was derived.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Mark Smith Senior Staff Fellow LC, NCI

Objectives:

The purpose of this work is to identify regions of the EGF molecule which are important for binding and for mitogenic activity. Identification of these regions is important for designing and synthesis of an EGF inhibitor.

Methods Employed:

Syntheses and modifications have involved solution chemistry, solid phase synthesis, and enzymatic reactions. Synthetic peptides have been purified by gel chromatography and by high pressure liquid chromatography. Using computer graphics, a possible tertiary structure of EGF was derived.

Major Findings:

Biological assays of synthetic fragments have indicated that the 20-31 region of EGF is the most important region for binding activity. The properties of chemically modified EGFs support the modeled tertiary structure and the modeled EGF 3° structure provided a new direction for designing EGF analogues. Major receptor binding residues identified as the residues 20 to 31 were found to be also the residues that consititute a major antigenic determinant region of mEGF. The receptor binding and receptor activating residues do not appear to be separate, and they reside in the sequence 20 to 31.

Significance to Biomedical Research and the Program of the Institute:

Several growth factors have been implicated as being involved in cell transformation and proliferation. An understanding of the mechanism of action of these polypeptides would be important for the understanding of transformation and would provide us also a possible way to modify the actions of these growth factors. Development of inhibitors for the growth factors (specifically EGF) would be valuable for our efforts to modulate biological activity of EGF and TGFs. Preparation of antibodies against these growth factors that would inhibit their binding to receptors would not only be useful for mechanistic studies but also for screening tumor cells.

Proposed Course:

The molecular structure of native EGF will be studied in detail by x-ray diffraction and NMR. Total synthesis and semi-synthesis of mEGF will be attempted for finding means to introduce amino acid sequence modifications in the region defined by the residues 20 to 31.

Publications:

Acton, N. and Komoriya, A.: Synthesis of pseudopeptides. Org. Prep. and Proc. Int. 14: 381-392, 1982.

Anzano, M. A., Roberts, A. B., Meyers, C. A., Komoriya, A., Lamb, L. C., Smith, J. M. and Sporn, M. B.: Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. Cancer Res. 42: 4776-4779, 1982.

Assoian, R. K., Komoriya, A., Meyers, C. A. and Sporn, M. B.: Transforming growth factor-β in human platelets: Identification of a major storage site, purification and characterization. J. Biol. Chem. 278: 7155-7160, 1983.

Mortsch, M., Kanety, H., Schlessinger, J., Komoriya, A. and Meyers, C. A.: Synthetic epidermal growth factor fragments induce phosphorylation of endogenous membrane proteins. In Hruby, V. (Ed): Peptides. Proceedings of the 8th American Peptide Symposium. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05267-02 LC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Identification of Platelet-derived Transforming Growth Factor-beta

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Richard K. Assoian Guest Worker, LC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human platelets were extracted with acid-ethanol and platelet-derived TGF-beta was purified from the extract by a two-column procedure using sequential gel filtration in the absence and then presence of urea. Purified TGF-beta is a protein of 25,000-daltons, and it is comprised of two 12,500-dalton subunits held together by disulfide bonds. The purified factor elicits its biological activity at concentrations less than 4pM. Comparative studies showed that platelets contain 100 times more TGF-beta than do other non-neoplastic tissues.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

None

Objectives:

To examine the roles of bioactive peptides in modulating normal and neoplastic cell growth. Emphasis will be placed on 1) the isolation of a transforming growth factor (TGF) from platelets and 2) the mechanism by which platelet-derived TGF elicits a transformed phenotype in NRK-fibroblasts and smooth muscle cells.

Methods:

Clinically outdated human platelets are extracted with acid-ethanol and the soluble peptides are precipitated with ether. The extract is purified by gel filtration, high pressure liquid chromatography and preparative gel electrophoresis. Biological activity is localized by use of an anchorage-independent growth assay with NRK-fibroblasts. Peptides are chemically localized by polyacrylamide gel electrophoresis in conjunction with silver staining and analytical radioiodination.

Major Findings:

Platelets are the major non-neoplastic source of TGF-β. The platelet-derived factor has been purified to homogeneity and shown to be a protein of 25,000 daltons comprised of two 12,500 dalton subunits. Disulfide bonds are involved in maintaining the subunits in association. The amino acid analysis and structure of platelet-derived TGF-β shows similarities to PDGF, but biologically the two proteins are distinct. TGF-β is not a strong mitogen, but it does induce cells to grow in soft agar. Purified PDGF is highly mitogenic, but it has no transforming activity.

Significance to Biomedical Research and the Program of the Institute:

The presence of a transforming growth factor in platelets (a non-neoplastic cell fragment) indicates that, in the non-neoplastic situation, stringent controls are operative in limiting the biological effects of this peptide. Comparative studies with non-neoplastic and neoplastic tissues will likely aid in defining these control systems.

Proposed Course:

The mechanism of action and biochemical effects of platelet derived TGF-β will be examined with cell cultures of NRK-fibroblasts and smooth muscle.

Publications:

Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M. and Sporn, M. B.: J. Biol. Chem. 278: 7155-7160, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05268-02 LC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemical Studies of Transforming Growth Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. Russ Lehrman Staff Fellow, LC, NCI

COOPERATING UNITS *(if any)*

Hoffmann La-Roche, Nutley, New Jersey

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Polypeptides capable of transforming normal cells in vitro have been isolated from neoplastic and non-plastic cells. Several of these transforming growth factors (TGFs) are available in the quantity and purity necessary for structural investigations. We are defining the primary structure of these compounds using non-routine amino acid analysis and automated microsequencing studies of the intact molecule and fragments obtained after enzyme digestion. Amino acid analyses have been obtained on TGFs isolated from bovine kidney and human platelet placenta. Sequence analysis of TGF isolated from human placenta has produced assignments for 27 of 29 residues. These results are consistent with the amino acid analysis performed on this compound. The immediate goal is to determine the primary sequence of TGF to facilitate the development of a TGF-beta antagonist and to assist others in the study of TGF-beta molecular biology.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Chester A. Meyers	Expert	LC, NCI
Richard K. Assoian	Guest Researcher	LC, NCI

Objectives:

Polypeptides known as transforming growth factors (TGFs) confer a transformed phenotype on normal rat kidney (NRK) cells grown in vitro. These growth factors have been purified extensively and are available in quantities sufficient for structural studies. Presently we are studying the primary structure of intracellular TGFs. Later, we will define other secondary and tertiary structural features of these compounds. This information is a prerequisite to the chemical synthesis of TGF fragments which would help define those regions of TGF necessary for receptor binding and biological activity. In addition, a peptide antagonist capable of interfering with undesirable cellular proliferation is a viable long-range goal.

Methods Employed:

Determination of the primary structure of TGFs requires amino acid and microsequence analysis. The former can be done most sensitively using fluorescence detection after o-phthalaldehyde derivatization of amino acids as they elute from an ion exchange column. Proline, tryptophan and cysteine require special chemical techniques for determination. The primary sequences of reduced, carboxymethylated TGF-β from human platelet placenta and bovine kidney are presently being studied using both gas phase and spinning cup sequenators with HPLC identification of the Pth-amino acids obtained. Later work will require CNBr and enzymatic digestion for the generation of internal fragments.

Major Findings:

To date, amino acid analysis has shown that the structure of TGF-β's from the sources listed above are quite similar. All 3 compounds have a high cysteine content and low methionine content (2 residues). Twenty-seven of the first 29 amino acids of human placenta have been identified. N-terminal sequence analyses of human platelet (9 residues) and bovine kidney TGF-β (15 residues) indicate that these proteins are highly homologous.

Significance to Biomedical Research and the Program of the Institute:

Antagonists of TGF activity could be important clinical agents in the treatment of cancer and other proliferative diseases. They would also facilitate studies of the molecular events necessary for normal and neoplastic cell division. Such knowledge is essential to the rational development of anticancer drugs, which are more potent and less toxic than those currently available.

Proposed Course:

Our immediate goal is to continue studies of the primary sequence of several TGFs; conformational analysis will be used to define the 3 dimensional structure of these growth factors. The latter is essential for a detailed understanding of TGF-α TGF-β receptor interaction and is useful for the design of TGF antagonists.

Publications:

Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blacher, R., Pan, Y-C. E., Stein, S., Lehrman, S. R., Smith, J. M., Lamb, L. C. and Sporn, M. B.: Purification and properties of a type-β transforming growth factor from bovine kidney. Biochemistry (In Press).

ANNUAL REPORT OF

THE LABORATORY OF COMPARATIVE CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

The Laboratory of Comparative Carcinogenesis plans, develops and implements a research program in experimental carcinogenesis. The Laboratory (1) compares effects of chemical carcinogens in rodents and nonhuman primates to identify determinants of susceptibility and of resistance to carcinogenesis; (2) identifies, describes, and investigates mechanisms of interspecies differences and of cell and organ specificity in carcinogenesis; (3) investigates the roles of nutrition, metabolism, the perinatal age period and pregnancy in modifying susceptibility to chemical carcinogens; and (4) conducts biological and morphologic studies on the pathogenesis of naturally occurring and induced tumors in experimental animals.

Organizational Changes: During this fiscal year, the Developmental Biology and Biochemistry Section was established in LCC. This program of this group, formerly the Experimental Ontogeny Section, LCMB, complements that of the Perinatal Carcinogenesis Section and broadens the scope of investigations within LCC on susceptibility factors in carcinogenesis during early life and pregnancy.

Summary Report: The Laboratory of Comparative Carcinogenesis provides a major focus within the Carcinogenesis Intramural Program for studies on the mechanisms of chemical carcinogenesis that involve primary neoplasia in animals as experimental endpoints. An increasing volume of evidence continues to support the hypothesis that for many, if not most tissues, transient exposure to chemical carcinogens may be necessary but is not sufficient to elicit tumor development. The widely differing patterns of organ specificity that frequently occur in experimental carcinogenesis in different species, even in studies with direct-acting agents that are independent of cellular metabolism, are in many cases not explicable on the basis of toxicodynamics, nor on the basis of differential capacity to repair damage in different tissues. These findings complicate efforts at human risk assessment based on the extent of reaction or persistence of binding products of carcinogens in known animal and putative human target tissues. In addition, more and more agents are being identified that cause tumors in experimental rodents but do not react chemically with cellular constituents including DNA, i.e., are not genotoxic. The fact that potential tumor cells may remain latent for large fractions of a lifetime in experimental animals, and that increasing numbers of nongenotoxic agents are being discovered which promote proliferation of such latent cells to form preneoplastic lesions that progress to neoplasia suggest that the phenomenon of tumor promotion may be of great significance for the genesis of human cancer.

There is, at present, no unifying hypothesis for the general mechanism of action of tumor promoters other than the ill-defined concept that such agents reduce intercellular communication. Furthermore, most experimental studies on tumor promotion in specific tissues or organ systems have focused on one, or at most two species, and the empirical data base from which mechanistic hypotheses of tumor promotion will eventually emerge remains very narrow. Accordingly, there

is no certainty at present that agents identified as promoters in rodent tissues will have similar effects in other species, including man. In order to expand the limited data base on organ specificity and interspecies correlations in tumor promotion, a major coordinated program has been established in this laboratory to identify previously unsuspected promoting agents; to establish rigorously the limits of cellular specificity for tumor promotion by specific agents; to compare dose/effect relationships from one species to another, including both rodent and nonhuman primate species; and to utilize these data in mechanistic investigations on the phenomena of tumor promotion.

Several especially important findings have emerged from studies on specific compounds. The commonly used plasticiser, di(2-ethylhexyl)phthalate (DEHP) has been shown by the Tumor Pathology and Pathogenesis Section selectively to promote hepatocellular tumors in mice, but at extremely high dosage levels. The dose/effect curve is extremely steep, and encourages the speculation that there may be a true no-effect level of exposure to non-genotoxic agents such as DEHP, thus providing a strategy for effective prevention by reducing exposure within measurable limits. Promotion of hepatocellular neoplasia by DEHP differs significantly from that by phenobarbital in regard to the biologic and histochemical properties of the promoted neoplastic cells. Intrinsically higher malignant potential is characteristic of neoplastic hepatocytes promoted by DEHP, illustrating that more than one path exists for tumor promotion in this, and presumably other, cell types. Phenobarbital, the classic promoter for liver cells, has now been shown in this and other laboratories to promote carcinogenesis in the rat thyroid as well, and becomes the first well-defined promoter clearly to act on different unrelated tissues in vivo.

The importance of dietary deficiencies in carcinogenesis has been shown clearly to relate to tumor promotion. Many carcinogens of the non-genotoxic type are known to affect the thyroid, and those that do so are frequently goitrogenic. The Tumor Pathology and Pathogenesis Section has shown that simple iodine deficiency, the classic cause of diffuse goitrous thyroid hyperplasia, efficiently promotes thyroid carcinogenesis in rats. This not only identifies a promoting influence significant in human populations, but provides an experimental approach to investigation of purely hyperplasiogenic stimuli in tumor promotion, in the absence of any critical cell/agent interaction step corresponding to the "conversion" stage of promotion, and to investigating the comparative mechanisms of action of phenobarbital on the thyroid and on the liver. Diets devoid of the lipotropes methionine or choline have been shown by the Nutrition and Metabolism Section to promote hepatocarcinogenesis in nitrosamine-initiated rats, and to elicit hepatocarcinoma development when given alone in the absence of pre-exposure to a genotoxic carcinogen. Conversely, high dietary levels of the physiological methyl donor methionine suppressed the carcinogenic activity of phenobarbital in mice. These dietary modifications have obvious, testable implications for man. Further, since normal bioregulatory methylation of cytosine in nucleic acids in the rat liver is disturbed by dietary methyl deprivation, the possible relation of nucleic acid methylation to mechanisms of tumorigenesis and tumor promotion can be investigated by dietary manipulation.

Transplacental carcinogenesis studies in nonhuman primates, carried out by the Perinatal Carcinogenesis Section and the Office of the Chief, have shown that phenobarbital, at dosage levels comparable to pediatric anticonvulsive therapy in man, promotes liver carcinogenesis initiated by direct or transplacental exposure to diethylnitrosamine. This suggests that long term exposure to

barbiturates may be a significant carcinogenic risk in man, and should be investigated. The observation that risk of gestational choriocarcinoma in postpartum females is correlated with risk of neoplasia in the offspring in patas monkeys given ethylnitrosourea during pregnancy suggests an epidemiologically testable hypothesis that in regions of the world where gestational choriocarcinoma is reported to be great, an environmental carcinogen may be involved that may also affect children born in these geographic regions. The possible role of aflatoxins as condidate agents will be investigated.

Research programs on naturally occurring neoplasms in experimental animals; carcinogenesis by salts of heavy metals; biochemical determinants of prenatal and organ-specific carcinogenesis by organic carcinogens and tumor promoters; placental growth factors; and related studies in comparative carcinogenesis also continue and are described in the following summary reports of each research group.

The Office of the Chief (1) organizes comparative research on mechanisms of chemical carcinogenesis in susceptible and resistant species of experimental animals; (2) arranges and fosters collaborative approaches to specific research projects involving several Sections within and independent investigators outside the Laboratory; and (3) provides general support and direction to the intramural research program of the Laboratory.

Results during the past year have provided additional confirmatory evidence that susceptibility to transplacental carcinogenesis by the direct-acting alkylating agent, ethylnitrosourea (ENU), is greatest early in gestation in the patas monkey, and that fetal susceptibility exceeds that of adults by at least one decimal order of magnitude. The induction of rapidly fatal gestational choriocarcinoma in pregnant patas given ENU suggests a correlation between choriocarcinoma and pediatric neoplasia which may exist in human populations if environmental carcinogens play a role in causation of these tumors in man and which may be testable by epidemiologic techniques. The occurrence of myelomonocytic leukemia at the age of 69 months--well into adult life--in a patas monkey subjected to ENU solely by transplacental exposure demonstrates that in this nonhuman primate, as in rodents, neoplasms of adult life may result from prenatal exposure to carcinogens and that the consequences of prenatal exposure to chemical carcinogens are not limited to pediatric neoplasia. Recent identification of primary epithelial tumors of the peripheral lung in patas monkeys prenatally exposed to ENU and surviving to adulthood without further treatment is further evidence that neoplasms of lining epithelia--characteristically the predominant forms of human cancer--can result from transient prenatal initiation by chemical carcinogens.

The development of hepatocellular neoplasia in a patas monkey given diethylnitrosamine (DEN) transplacentally, followed by phenobarbital beginning 4 years after birth, provisionally suggests that DEN, like ENU, is a transplacental carcinogen in this nonhuman primate; that the transformed hepatocytes which result from prenatal exposure to DEN may persist, latent, for years after exposure to the carcinogen has ceased; and that phenobarbital may be a promoter of hepatocellular neoplasia in this species as it is in rats and mice. This implies the potential importance of tumor promotion generally in carcinogenesis in primates, including man, and provides a basis for systematic experimental study of the phenomenon of tumor promotion in liver and other epithelial tissues in representative nonhuman primates.

The Nutrition and Metabolism Section (1) investigates the effects of dietary constituents on target tissue susceptibility to various classes of chemical carcinogens; (2) studies mechanisms by which dietary constituents such as methyl donors and metals or their metabolites alter carcinogenic processes; and (3) correlates chemical and toxicological data for the identification of chemical carcinogens.

The Section has focused its interest on the role of the lipotropes, methionine, choline, vitamin B_{12} and folic acid in chemical carcinogenesis. The chronic administration of diets devoid of methionine and/or choline has been shown to promote the formation of hepatocellular carcinomas in the livers of rats initiated with diethylnitrosamine. Diets devoid of both methionine and choline enhanced tumor formation more than did the diets singly devoid of either methionine or choline. Methionine deficiency appeared to exert a greater tumor promoting effect than did choline deficiency. Administration of diets devoid of both methionine and choline led to the formation of metastatic hepatocellular carcinomas in rats, even in the absence of any further treatment with hepatocarcinogens. Chronic feeding of diets devoid of both methionine and choline, or containing the carcinogenic methionine antagonist ethionine decreased the hepatic levels of both S-adenosylmethionine and of 5-methyldeoxycytidine in DNA. These results are consistent with the hypothesis that in at least rat liver methyl insufficiency can play a major role in carcinogenesis. Further evidence that methyl insufficiency exerts a causative role in hepatocarcinogenesis is obtained with mice. Ethionine has demonstrated hepatocarcinogenic activity in 3 strains of mice, while tumor promotion and causation by phenobarbital in C3H mice is inhibited by methionine. These results provide reasonable evidence that a physiological insufficiency of methyl donors, possibly acting via hypomethylated DNA, contributes significantly to hepatocarcinogenesis in rodents. Results from this and other laboratories have shown clear associations between methyl insufficiency, undermethylation and tumor formation, even in humans. Establishment of a causal relation in a variety of tissues and cell types would be of major significance in understanding the etiology of cancer.

The inhibition of sarcoma production in rats treated with a single s.c. dose of cadmium chloride afforded by the simultaneous administration of magnesium acetate was accompanied by decreased accumulation of cadmium at the injection site. The simultaneous injection of calcium acetate had no significant effect either on cadmium retention at the injection site or on cadmium induction of sarcomas. Treatment of strain A mice with magnesium acetate or with calcium acetate, each of which inhibits lung tumor formation by nickel acetate had no significant effect on total nickel accumulation in this target organ. However, the injection of magnesium acetate into nickel acetate-treated mice did delay the accumulation of nickel in the lungs. These results indicate that, although the physiological divalent metals may inhibit the tumorigenic activities of toxic divalent metals, such antagonism is not a general one, and is caused only in part by altered metabolism of the carcinogenic metal. In general, the area of metals carcinogenesis can be described as lacking (1) sufficient members of research groups investigating the problem; (2) adequate model biological systems in which the effects of organic and inorganic carcinogens may be compared; and (3) reasonable evidence regarding the critical site(s) of attack of the carcinogen in the target tissue. In view of the widespread occurrence of metal carcinogens and of the great activity of same, investigations on their mode of action are important. The use of physiological metal antagonists may be expected to help specify the cellular sites of activity of the carcinogenic metals.

The Perinatal Carcinogenesis Section (1) investigates the induction of cancer in experimental animals before birth and during infancy; (2) evaluates perinatal exposures to chemical carcinogens, inducers of xenobiotic metabolism, and tumor promoters as causative factors in pediatric and adult forms of human cancer; (3) studies the effects of exposure to carcinogens during pregnancy; and (4) investigates the relation of cellular differentiation to perinatal susceptibility to chemical carcinogens and to the consequent development of neoplasia.

Prenatal exposure to chemical carcinogens, followed by postnatal application of tumor promoters, can result in tumor formation at sites where no tumor would occur in the absence of promotion. Organ specificity in transplacental carcinogenesis may therefore be more apparent than real, as latent tumor cells may persist in many tissues. To investigate this hypothesis, two major biological projects initiated last year have been continued and expanded. Mutation assays for resistance to thioguanine, ouabain, and diphtheria toxin have been successfully applied to primary cell cultures from whole embryos and from specific organs, from conceptuses of several rodent species exposed to metabolism-dependent or direct-acting carcinogens at various precisely defined periods during gestation. Mutant recovery has been high enough to allow quantitative comparisons of the mutagenic effects of a given agent in different organs or tissues and of sequential changes in susceptibility of an organ or tissue during prenatal development. This program will allow comparison of unequivocal genotoxic effects (mutation) with organ-specific carcinogenesis and should serve to identify organs where mutagenesis occurs, but where tumors do not develop. A second approach then is to expose animals given carcinogens prenatally to tumor promoters during postnatal life as one way to reveal the presence of potentially latent neoplastic cells in apparently resistant tissues. Studies on promoting agents for hepatocytes, bladder urothelium, and intestinal mucosa are being applied in long-term in vivo experiments to mice, rats, and Syrian hamsters in these studies.

The role of DNA repair in modulating susceptibility to transplacental carcinogenesis within an organ system has been advanced by development of a sensitive indirect assay for apurinic endonuclease (APE), an enzyme involved in several known enzymic pathways for DNA repair. APE has been demonstrated exclusively in the nuclei of fetal rat cells, and is significantly higher in liver, a resistant organ, than in brain, an organ in the fetal rat that is extremely susceptible to carcinogenesis. Interspecies and further interorgan studies are now in progress. In collaboration with the Office of the Chief, alkyl acceptor protein (AAP) is being assayed in different organs and separated cell populations in rats, mice, and non-human primates, to determine whether this DNA repair-associated protein is inducible in target or non-target tissues of non-human primates exposed to alkylating carcinogens, to document its appearance in different organ systems during perinatal life, and to correlate levels of AAP with differing cellular susceptibility to carcinogens in different species.

Two new projects have been initiated, on special sensitivity factors in carcinogenesis including the effects of inducers of oxidation metabolism, and on metabolic determinants of susceptibility to transplacental carcinogenesis. Athymic mice have been found to develop both squamous papillomas and sebaceous adenomas preferentially after transplacental exposure to ethylnitrosourea, illustrating a striking difference from normal Nu/+ littermates and other strains of mice that may prove most useful in investigating cellular specificity for 1- and 2-stage carcinogenesis in the skin, which will be actively investigated during the coming year.

The Tumor Pathology and Pathogenesis Section (1) characterizes the biology and pathology of naturally occurring and experimentally induced preneoplastic and neoplastic lesions of laboratory animals; (2) uses morphologic, histochemical and ultrastructural methods to define the pathogenesis of experimental tumors; (3) develops animal models to aid in understanding causes, pathogenesis and pathology of human cancers; and (4) provides guidance, consultation and collaboration in tumor and laboratory animal pathology to investigators and scientists, in the National Cancer Institute and other U.S. Federal Government agencies.

The pathology and biology of naturally occurring and experimentally induced tumors are characterized and compared. Naturally occurring tumors of F344 rats were transplanted in the mammary fat pads of weanling F344 rats. Both benign and malignant tumors were readily transplanted, demonstrating that transplantation is an indicator of neoplasia rather than malignancy. Transplanted malignant tumors, however, grew faster and killed the host more often than did transplants of histologically benign tumors. Hepatocellular carcinomas were induced in rats by methapyrilene, a non-genotoxic carcinogen, that were easily transplanted, metastasized in transplant recipients, and produced alpha-fetoprotein (AFP). Lack of genotoxicity of the carcinogen had no negative correlation with tumor malignancy or ability to produce AFP.

Iodine deficiency was found to be a potent promoter of thyroid adenomas and carcinomas induced by nitrosomethylurea. Tumors were also deficient in the differentiation antigen Thy 1.1, a new marker for use in carcinogenesis experiments, while normal follicular epithelium was not. The role of thyroid hyperplasia and hyperplasia of pituitary throtrophs in the promotion of these tumors is under investigation.

Di(2-ethylhexyl)phthalate (DEHP), a common plasticizer, and phenobarbital (PB), were found to be potent promoters of liver tumors initiated by diethylnitrosamine (DEN) in male B6C3F1 mice. A quantitative study of the development of hepatocellular hyperplastic foci, adenomas and carcinomas revealed that foci increased in size and number from 2 to 6 months after DEN injection. Foci appeared to progress to adenoma and adenomas progressed to carcinomas. DEHP, however, induced basophilic foci and tumors which appeared to grow faster than the eosinophilic foci and tumors induced by PB. Histochemically, the hepatocytes in the 2 types of foci were similar, however, since both contained glycogen, produced alpha-fetoprotein and were deficient in glucose-6-phosphatase. Phenobarbital also induced eosinophilic foci and adenomas which contained gamma-glutamyl transpeptidase (GGT) in aging F344 rats while GGT-negative basophilic foci and adenomas were seen in control F344 rats. Thus, these experiments provided additional evidence for the hypothesis that morphologic and biologic characteristics of a given neoplasm may vary with the carcinogen or promoter causally associated with its development.

The avidin-biotin-peroxidase complex (ABC) immunocytochemical technique was applied to localization of a variety of antigens in several carcinogenesis experiments. Proper fixatives had to be determined for each antigen. The large granular lymphocyte (LGL), the effector cell of natural killer (NK) cell activity, was localized to various types of epithelium and lymphoid tissues in athymic nude rats using monoclonal antibodies to a cell surface glycoprotein

produced by hybridoma clone OX-8. The LGL was also found in the stroma of induced and transplanted tumors and inflammatory lesions. OX-8 was also a marker for the diagnosis of LGL leukemia in aging F344 rats.

The LGL leukemias were transplanted and characterized for study of NK lineage and function.

The ABC immunocytochemical technique was also used to identify many other antigens in tissue sections including alpha-fetoprotein, immunoglobulins, P21 transforming protein, Sendai virus, a new papovavirus of nude rats, hormones, cell surface antigens of lymphocytes and epithelium and epithelial proteins. Refinement and development of this technique has allowed us to use it routinely as an adjunct to the many carcinogenesis experiments by investigators in the laboratory.

The TPPS provides guidance in pathology to other scientists at NCI and U.S. federal agencies. Aid has been provided to investigators in the Laboratory of Cellular Carcinogenesis and Tumor Promotion, Laboratory of Chemoprevention, Biological Response Modifiers Program, the National Toxicology Program, the Food and Drug Administration, OSHA, EPA and NIOSH.

The Ultrastructural Studies Section uses techniques of transmission and scanning electron microscopy to investigate 1) the histogenesis of experimental tumors; 2) early toxic changes as precursors of neoplasia in cells exposed to chemical carcinogens; and 3) differentiation of potentially neoplastic cells and its relation to phenotypic expression of the neoplastic genotype.

During the past year, after completing the construction of new laboratory space, the Section has established a research program in the framework of the Laboratory and, on a collaborative basis, support is provided to members of the Laboratory and other institutions with regard to various aspects of the biology of neoplastic transformation. Within the context of understanding the biology of cell transformation, the Section has concentrated, during the last year, on the following research projects: 1) evaluation of morphological and functional changes following chemical transformation of epithelial liver cells, 2) characterization of the thymic microenvironment during T-cell lymphoma development and 3) ultrastructural evaluation of natural killer cell activity.

DL-Ethionine, the ethyl analog of methionine, is known to be a specific liver carcinogen in rats and mice. Ethionine undergoes sulfur activation via ATP with the subsequent formation of S-adenosyl-ethionine (SAE). SAE serves as a substrate and a competitive inhibitor in a number of S-adenosyl-methionine requiring methylation reactions, including the methylation of ribosomal RNA which is required for final stages of rRNA processing. The formation and accumulation of SAE also results in a drain on ATP, which is required for protein and RNA synthesis. In the Nutrition and Metabolism Section of the Laboratory, several cell lines originating from rat liver tissue, some untransformed and some transformed by ethionine, have been established and serve as models for the study of different parameters in the course of transformation. The Ultrastructural Studies Section has initiated and is continuing a program directing special emphasis towards the investigation of morphologically definable transformation features, i.e., phenotypic changes, as these are as yet not well understood in epithelial cells. However, knowledge of these characteristics and the ways they change following tumorigenic transformation is important

generally to the study of the mechanisms of carcinogenesis and particularly to study the mechanisms by which an epigenetic chemical carcinogen induces transformation. In a preliminary investigation, characterization of control and ethionine transformed, liver-derived cell lines has been achieved and, by using cytological and histochemical methods, evidence has been presented for 1) the epithelial nature of these cells; 2) their origin from liver epithelium; and 3) the identification of cellular alterations in nucleus and cytoplasm that resulted specifically from ethionine treatment and subsequent transformation. For the understanding of genetically stable modifications in morphology due to transformation, cytoskeletal arrangements and adhesion characteristics of the transformed cells are now compared to those of the nontransformed controls using immunocytochemistry, reflection contrast and phase contrast microscopy, and electron microscopy. Results obtained thus far indicate, in cultures of transformed cells, that changed adhesion patterns may reflect progressive deficiencies in cell contact interactions.

The Section Head has been invited to continue a joined research project at the University of Cologne, Germany, and has spent part of a sabbatical as guest professor at the Institute of Pathology of that University. The research project encompasses studies of the thymic microenvironment, and is especially concerned with the regulatory mechanisms provided by the thymic epithelium. These mechanisms are necessary for the maturation and proliferation of prethymic T-progenitor cells to mature lymphocytes of the T-cell type. The aim of the ongoing study is to elucidate the mechanism of the intrathymic differentiation block of prethymic lymphoid stem cells that gives rise to systemic malignant lymphoma of the Thy$^-$ cell type. In vivo experiments, using the Moloney virus-induced lymphoma in the BALB/c mouse as a model, have been performed and the phenotype and distribution of the major thymic cell populations have been characterized at different stages of tumorigenesis by light and electron microscopy. Immunofluorescence studies for the presence of thymopoietin II and serum thymus factor were carried out to determine the functional state of the epithelial cells. The results show that the reticular epithelial cells of the thymus, which provide a microenvironment necessary for the differentiation of prethymic stem cells to lymphocytes of the T-lineage, are a prime target for retrovirus infection: these cells are infected by the lymphoma-producing virus, undergo phenotypic changes and are rendered functionally defective prior to lymphoma development. It is concluded from the results obtained thus far, that functionally incompetent epithelial cells fail to stimulate the maturation of T-progenitors, causing the progressive accumulation of T-cell precursors, and initiating a dysregulative lymphoma. This work will be continued as a study in comparative carcinogenesis to evaluate the thymic microenvironment in mice expressing varying capability for the induction of lymphoma. The production of monospecific antibodies against a variety of epithelial cell proteins that may serve as markers in the characterization of functionally defective thymic epithelium is in the planning stage.

As a continuing project in the characterization of carcinogenic processes at the cellular and subcellular level, with emphasis on experimental systems under investigation in this and other Sections with the Laboratory, a continuing effort is underway to characterize cell-cell interactions. Currently, in collaborating with the Tumor Pathology and Pathogenesis Section of this Laboratory (Z01CP05303-02 LCC), a study has been initiated to develop a marker system for identifying natural killer cells in intact tissue at the ultrastructural level with the aim to elucidate interactions between natural killer cells and tumor

cells. Understanding of such interactions will further knowledge of natural defense mechanisms against cancer. Thus far, using a monoclonal antibody which is specific for a cell surface antigen or natural killer cells and cytotoxic/suppressor T-lymphocytes, specific membrane staining of a subpopulation of lymphocytes in nude rats has been achieved. These cells could be identified as large granular lymphocytes. Following establishment of a preparation technique protocol that will improve ultrastructural preservation, interactions between natural killer cells will be examined ultrastructurally to further understanding of the mechanisms by which natural killer cells accomplish their cytocidal activity.

The Developmental Biology and Biochemistry Section (1) isolates, identifies and characterizes bioregulators of cellular growth and differentiation; (2) develops and applies model systems to evaluate and compare the functional activities and interactions of cellular growth factors during embryogenesis and experimental carcinogenesis; and (3) explores the use of growth factors as markers of tumor development and control.

The Developmental Biology and Biochemistry Section, formerly the Experimental Ontogeny Section, was transferred October 1, 1982, from the Laboratory of Cellular and Molecular Biology to the Laboratory of Comparative Carcinogenesis. Necessitated by the reorganization was a two-stage relocation of the Section into space contiguous with the Laboratory of Comparative Carcinogenesis in Bldg. 538. Occupancy of the permanent quarters took place in January 1983. During the course of the transfer, a realignment of the Section's research program was initiated, within the framework of existing expertise, to center it around the comparative analysis of embryo-fetal and tumor-associated growth factors as determinants in spontaneous and experimental carcinogenesis.

The Section has initially focused its efforts on the identification, isolation and characterization of placentally derived growth regulators. The placenta, which harbors a small population of potentially malignant cells, shares numerous properties with cancers and provides a unique model system for investigating the intricate balance between normal and neoplastic growth control. To date, studies have demonstrated the presence of mitogenic stimulating factors, colony stimulating factors and interferon-like activity in mouse placental extracts taken after 13 days of gestation. These investigations have led to the isolation and characterization of a new class of interferon (MuIFN-Pl) which is distinguishable from classical mouse interferon alpha, beta and gamma by serological differences and unusual decay kinetics. Furthermore, a series of comparative studies have verified the presence of this atypical interferon in placental extracts from all mouse strains examined and, thus, provisionally support the notion of its general presence in all pregnant mice. MuIFN-Pl is classified as an interferon on the basis of its transcriptionally dependent, species specific antiviral activity. Like classical interferons, MuIFN-Pl also induces the enzyme 2-5A synthetase--considered an interferon specific event--and inhibits growth factor mediated mitogenesis. MuIFN-Pl activity is highest in the placenta at term and is composed of two distinct species that function synergistically in their induction of the antiviral state. Both species are biologically and antigenically distinct from classical interferons. Though the function of MuIFN-Pl remains unclear, it does not appear to be antiviral in situ and all data collected are consistent with its classification as a feto-maternal cellular biological response modifier. The detection of an antiviral activity in extracts from rat, hamster, monkey and human placentas also supports this hypothesis.

An examination of the interaction between partially purified mouse placental-associated growth factor (PlGF) and interferons, including MuIFN-Pl, has been undertaken utilizing EGF, PDGF and other commerically available growth factors for control purposes when applicable. In conjunction with these studies, the interaction of interferons with a highly purified growth factor isolated from tumor cells (TGF, provided by Dr. D. R. Twardzik) has been undertaken. Mouse type I interferon has been found to inhibit the induction of mitogenesis by PlGF and by TGF in mouse BALB/c 3T3 cells, although it was ineffective in abrogating TGF-induced mitogenesis in bovine cells (MDBK). In comparison, TGF-induced mitogenesis in MDBK cells, a cell line sensitive to the antiviral effects of human interferon alpha, was strongly inhibited by this interferon, as well as recombinant human alpha A/D interferon. As examined in vitro, optimal interferon effects occur upon exposure of the cells to interferon prior to growth factor exposure. More recently, two MuIFN-Pl components partially purified by exclusion chromatography were found to block mitogenesis induced by EGF in mouse cells, but not in hamster cells, suggesting that the effect is specific for the species from which the interferon originated.

Technology for an in vitro induction system for antibody forming precursor cells has been developed for the rapid generation of monoclonal immune reagents directed against natural products. Frequently present in low concentration, natural products (growth factors, interferons, hormones, etc.) are often difficult to purify and are cost prohibitive to produce in quantities sufficient for multiple in vivo immunizations. The procedure involves culturing spleen cells with femtogram to nanogram quantities of antigen (as opposed to milligrams required for the usual successful in vivo immunizations) to induce antibody producing cells within 4 days (as opposed to weeks or months in vivo). After harvest, the cultured cells are fused to mouse or rat myeloma cells using well established monoclonal antibody techniques for hybridoma production. Antibody containing supernatants are usually available for testing within 4 weeks after the exposure of spleen lymphocytes to antigen. Utilizing this technology, several IgM producing hybridomas specific for a partially purified tumor growth factor have been produced. The ready availability of specific immune reagents will radically improve our ability to purify and investigate cell products formerly thought too low in concentrations for practical study. These techniques, established to meet primary section objectives, are now also available to the laboratory as a whole.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP04580-09 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Role of Lipotropes in Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
L. A. Poirier Chief, NMS, LCC, NCI

COOPERATING UNITS *(if any)*
Sloan-Kettering Memorial Institute, New York, NY; Litton Bionetics, Inc., Rockville, MD

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanisms behind the alteration of chemical carcinogenesis by the dietary lipotropes, choline, methionine, folic acid and vitamin B-12 have been studied. The metabolism and carcinogenic activity of ethionine in different species is being compared. The tissue levels of S-adenosylmethionine, S-adenosylhomocysteine, and 5-methylcytosine in animals treated with carcinogens, liver tumor promoters and methyl-deficient diets are being determined. The effects of dietary deprivation of methionine and choline on carcinogenesis in liver and other organs are under investigation using standard bioassays. The effects of methylase inhibitors on the methylation of macromolecules, on cell transformation in vitro and on carcinogenesis in vivo are determined.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Mary J. Wilson	Chemist	LCC, NCI
Narayan Shivapurkar	Visiting Fellow	LCC, NCI
Karen Hoover	Staff Fellow	LCC, NCI

Objectives:

To determine the mechanism by which physiological methyl deprivation produces liver carcinomas. To determine the extent to which methyl deprivation contributes to carcinogenesis in extrahepatic tissues.

Methods Employed:

The effects on carcinogenesis in rodents of dietary regimes altering the in vivo bioavailability of the chief physiological methyl donor S-adenosylmethionine are investigated. The dietary components varied include the methyl compounds methionine and choline and the methionine antagonist ethionine. Tissue levels of S-adenosylmethionine, S-adenosylethionine, S-adenosylhomocysteine and 5-methyldeoxycytidine in DNA are determined using appropriation combinations of HPLC, thin layer chromatography, spectrophotometry and radioisotopes.

Major Findings:

The chronic administration of amino acid-defined, methyl-deficient diets to diethylnitrosamine-initiated rats markedly increased formation of hepatocellular carcinomas. The liver tumor promoting effects were most marked in animals receiving a diet devoid of both methionine and choline, but were also noted in rats fed diets singly deficient in either methionine or choline. Diets deficient in both methionine and choline cause liver cancer in rats even without carcinogen treatment. Ethionine administered in the diet at levels as low as 0.05-0.10% is a liver carcinogen in BALB/c, Swiss, and C3H mice. The simultaneous administration of high dietary levels of methionine and choline markedly inhibited both the formation and promotion by phenobarbital of hepatocellular carcinomas in C3H mice. The chronic administration of methionine- and choline-deficient diets to rats lowers the hepatic contents of S-adenosylmethionine, increases such levels of S-adenosylhomocysteine, and diminishes the proportion of 5-methylcytosine in hepatic DNA. The chronic feeding of ethionine to rats similarly decreases the hepatic levels of S-adenosylmethionine and of 5-methyldeoxycytidine. These results provide good evidence that dietary methyl insufficiency results in hypomethylated DNA and, at least under some conditions, plays a major role in hepatocarcinogenesis.

Significance to Biomedical Research and the Program of the Institute:

One of the basic aims of the National Cancer Institute is the prevention of cancer by a delineation of the mechanism by which cancers are induced. The

reasons for studying the role of physiological methyl donors in carcinogenesis are both practical and theoretical. In practice, several physiological conditions associated with an elevated risk of cancer formation in humans are also accompanied by an abnormal stress on the body's pool of methyl donors. These include: 1) High fat intake. High fat diets increase the metabolic requirements for methionine and choline (hence the term lipotropes). 2) Familial polyposis. Biologically normal fibroblasts from colon cancer patients with this disease have an increased demand for methionine compared to the fibroblasts from their disease-free relatives. 3) Tyrosinemia. Patients born with this genetic disease have high serum levels of alpha-fetoprotein, develop a high incidence of liver carcinoma, and have a defective biosynthesis of S-adenosylmethionine. 4) Liver cancer in certain African populations. This disease has been associated with an elevated aflatoxin intake, hepatitis and a low protein, and thus low methionine, intake. The theoretical reasons for studying methyl deprivation in carcinogenesis are centered on indirect evidence implicating hypomethylation, particularly of DNA, in cancer causation. This includes the observations that 1) The chronic administration of several hepatocarcinogens and liver tumor promoters decreases the hepatic levels of S-adenosylmethionine in rats. 2) Specific genes from several human and experimental tumors are hypomethylated compared to the same genes in the corresponding normal tissues. 3) Azacytidine, an inhibitor of DNA methylation, is tumorigenic in several rodent tissues. 4) S-Adenosylethionine, a major metabolite of the hepatocarcinogen ethionine and an effective inhibitor of DNA methylation, is a cell transformant. The present studies provide good evidence that physiological methyl insufficiency under specific conditions plays a major role in hepatocarcinogenesis in rodents. Successful generalization of the hypothesis that methyl insufficiency or hypomethylation is a major contributing factor to carcinogenesis would provide 1) screening methods for populations at risk for specific types of cancer; 2) the prospect of early intervention to minimize such risks; and 3) an alternate mechanism to the common model systems of chemical carcinogenesis based upon the direct alkylation of DNA by an electrophilic metabolite of an exogenous compound.

Proposed Course:

The effects of physiological methyl donors on carcinogenesis will be extended to include: other dietary modifications known to alter the availability of S-adenosylmethionine in vivo, other carcinogens and antagonists of methionine, other tissues and species. In particular, more direct evidence for the involvement of S-adenosylmethionine and of 5-methylcytosine in carcinogenesis will be sought. Dose-response studies on liver tumor formation in mice and rats as a function of the levels of methionine and choline in the diet will be performed. Reversal of early biochemical and biological markers of hepatocarcinogenesis by increasing the bioavailability of S-adenosylmethionine will be undertaken.

Publications

Linnell, J. C., Wilson, M. J., Mikol, Y. B. and Poirier, L. A.: Tissue distribution of methylcobalamin in rats fed amino acid-defined, methyl-deficient diets. J. Nutr. 113: 124-130, 1983.

Poirier, L. A., Shivapurkar, N., Hyde, C. L. and Mikol, Y. B.: The effect of the chronic administration of liver carcinogens and tumor promoters on the hepatic levels of S-adenosylmethionine in rats. In Usdin, E., Borchardt, R. T. and Creveling, C. R. (Eds.): Biochemistry of S-Adenosylmethionine and Related Compounds. London, Macmillan Press Ltd., 1982, pp. 283-286.

Shivapurkar, N. and Poirier, L. A.: Decreased levels of S-adenosylmethionine in the livers of rats fed phenobarbital and DDT. Carcinogenesis 3: 589-591, 1982.

Shivapurkar, N. and Poirier, L. A.: Tissue levels of S-adenosylmethionine and S-adenosylhomocysteine in rats fed methyl-deficient, amino acid-defined diets for one to five weeks. Carcinogenesis (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP04582-08 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure Activity Correlations in Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
L. A. Poirier Chief, NMS, LCC, NCI

COOPERATING UNITS *(if any)*
Litton Bionetics, Inc., Rockville, Maryland

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The antagonism between the essential divalent metals calcium and magnesium and the divalent metal carcinogens, lead, nickel and cadmium are under investigation in metabolic and in carcinogenicity studies. A single s.c. injection of cadmium chloride was shown to enhance pancreatic acinar carcinoma formation in male Wistar rats. The inhibition by magnesium acetate of cadmium-induced injection site sarcomas was associated with a decreased accumulation of cadmium at the injection site. Similarly, the injection of magnesium acetate to nickel acetate-treated Strain A mice delayed the accumulation of nickel in the lungs of such animals. Thus, it appears that at least part of the protection afforded by magnesium against the tumorigenic activity of cadmium and nickel is due to diminished accumulation of the carcinogenic metal at the target site. Extension of the possible antagonistic effects of the physiologically essential divalent metals against tumor formation by nickel and lead in other target sites will be explored.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Kazimierz Kasprzak	Visiting Scientist	LCC, NCI
Karen Hoover	Staff Fellow	LCC, NCI

Objectives:

The accumulation of evidence indicates that the activated form of most organic carcinogens consists of a reactive electrophile. Possible mechanisms by which the metal carcinogens exert their activity remain relatively unexplored. The hypothesis that they act via an antagonism of the physiologically essential metals is being tested.

Methods Employed:

The carcinogenic activities of previously tested metals are inhibited using standard protocols. These include lung adenoma production in strain A mice and long-term feeding, injection and intubation of the suspect compound into rats followed by examination at necropsy for tumors. Thymidine incorporation into DNA and the metabolism of radioactive metals in vivo and in cell culture are determined by standard radioisotopic, cell culture and ultracentrifugation techniques.

Major Findings:

The previously described inhibition by magnesium of cadmium-induced sarcomas in Wistar rats and of nickel-induced lung adenomas in strain A mice was associated with a decreased or delayed accumulation of the carcinogenic metal in the target tissue. Thus, magnesium administration appeared to diminish the total effective dose of the divalent metal carcinogen reaching the target tissues. In addition to its production of testicular carcinomas and injection site sarcomas in male Wistar rats, cadmium injection was shown to significantly increase the formation of pancreatic acinar carcinomas in these animals. In studies conducted as part of her official duties with the Bioassay Program, Dr. K. Hoover found that N-hydroxy-2-acetylaminofluorene exerted a hypertrophic effect on the thyroid glands of fish and described the tumorigenic effects of selenium sulfide in rodents.

Significance to Biomedical Research and the Program of the Institute:

The aim of these studies is to increase the base of theoretical knowledge by which the potential carcinogenic hazards to man of carcinogenic metals can be diminished. Metals constitute one of the largest and broadest categories of chemical carcinogens to which humans are exposed. One metal, cadmium, is among the most active carcinogens known. As a class of carcinogen they are relatively underinvestigated. The biochemical similarity of their mode of action, if any, to the organic carcinogens remains obscure. The evidence accumulated to date indicates that an antagonism to the divalent cations, calcium or magnesium,

may constitute part of the mechanism by which the divalent metal carcinogens exert their activity. Successful demonstration of the molecular locus of antagonism between the physiological metals and the divalent carcinogens could help identify the intracellular targets of metal carcinogenesis.

Proposed Course:

The carcinogenicity of inorganic carcinogens will continue to be examined. Attempts will be made to extend the observed antagonism between carcinogenic divalent metals and calcium and magnesium. These studies will include attempts to inhibit the formation of injection site sarcomas by nickel and of renal carcinomas by lead in rats using magnesium and calcium salts as protective agents. The effects of magnesium and calcium salts on the binding of metal carcinogens to macromolecules will also be investigated. The intracellular binding sites of cadmium in target tissues will be examined. Biochemical similarities between organic and metal carcinogens will be sought.

Publications

Hoover, K. L.: Carcinogenicity of selenium sulfide in Fischer 344 rats and B6C3F1 mice. J. Environ. Pathol. Toxicol. Oncol. (In Press)

Hoover, K. L.: Hyperplastic thyroid gland lesions in fish. JNCI (In Press)

Poirier, L. A., Kasprzak, K. S., Hoover, K. L. and Wenk, M. L.: Effects of calcium and magnesium acetates on the carcinogenicity of cadmium chloride in Wistar rats. Cancer Res. (In Press)

CONTRACT IN SUPPORT OF PROJECT NUMBERS

Z01CP04582-08 LCC
Z01CP04580-09 LCC
Z01CP05353-01 LCC

LITTON-BIONETICS, INC. (NO1 CP 01039)

Title: Holding Facility for Small Laboratory Animals

Current Annual Level: $198,000

Man Years: 2.5

Objectives:

The purpose of the contract is to provide animal holding facilities for rats, mice and hamsters treated with a variety of organic and inorganic chemical carcinogens and fed several different diets, together with technical support for administration of chemicals and necropsy of experimental animals. Most carcinogenesis studies are conducted to test the major hypotheses (1) that physiological methyl deprivation promotes hepatocarcinogenesis; and (2) that the carcinogenic divalent metals exert their activity via an antagonism with the physiologically essential divalent metals. Housing is also provided for research animals of genetic backgrounds not available at FCRF, and during this year has supported a project on dose/effect relationships for carcinogenesis in strain A mice at low levels of exposure to nitrosamines.

Major Contributions:

The chronic administration of the methionine antagonist ethionine produced liver carcinomas in both male and female C3H and BALB/c mice and in Swiss female mice. Female mice developed such tumors with doses of ethionine as low as 0.05 - 0.1 per cent in the diet. A similar low level of ethionine in chow diet produced liver tumors in F344 rats. The livers of rats fed high dietary levels of selenium compounds, including sodium selenite and selenium subsulfide, showed no evidence of methyl deficiency in vivo; their hepatic levels of S-adenosylmethionine were normal. Four other major carcinogenesis studies are currently in progress.

Proposed Course:

The animal holding facilities will continue to be used to investigate: 1) the role of physiological methyl insufficiency in carcinogenesis; and 2) the antagonistic effects of the physiological metals on metal carcinogenesis. Current investigations on the potential inhibition of liver tumor promotion by phenobarbital, of renal carcinogenesis by lead and of sarcomagenesis by nickel will be completed. Additional investigations will be undertaken on carcinogenesis by metals and by methyl antagonists. In 2 years this activity will have been transferred to the NCI intramural laboratories at FCRF.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP04680-13 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development and Application of In Vitro Systems Involving Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
M. J. Wilson Chemist, LCC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Nutrition and Metabolism Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Epithelial cells derived from livers of 10-day old Fischer 344 rats were utilized in transformation and metabolism studies involving compounds known to interfere with methyl metabolism. The cells were characterized by morphological and cytochemical means in order to confirm their epithelial nature. The importance of nutrients in the culture medium to the transformation process is being assessed.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Lionel A. Poirier	Chief, NMS	LCC, NCI
Ursula Heine	Chief, USS	LCC, NCI

Objectives:

Studies involving the successful culture of epithelial cells derived from livers of 8 to 10 day old Fischer strain 344 rats and malignant transformation of such cultured cells by a variety of carcinogens continue in this laboratory. Our primary aims in current investigations have been to: 1) characterize the cultures morphologically and cytochemically and 2) establish liver cells in culture as a model system for studying the effects of compounds known or thought to interfere with normal cellular methylation reactions. Methylation of cellular macromolecules, particularly DNA, appears to be important to the process of neoplastic transformation.

Methods Employed:

Methods for culturing and cloning rat liver cells continued to be developed in this laboratory. In addition, histochemical and morphological methods were used to characterize the cells. Cells were assayed histochemically for glycogen accumulation, acid and alkaline phosphatase, adenosine triphosphatase and gamma glutamyl transpeptidase activities. The chromosome number of each of the cell lines was determined following exposure of the cultures to colchicine. Nucleolar and nuclear area determinations were made using an automated image analyzer. Morphologically, the cell cultures were examined by TEM and SEM. Markers of transformation were tumor production following injection of the cells into a syngeneic host and growth in soft agar.

Major Findings:

Studies in this laboratory revealed that liver cells in culture, like hepatocytes in vivo, were susceptible to transformation by ethionine. Furthermore, S-adenosylethionine, administered at a dose equimolar to that of ethionine, resulted in the cells becoming tumorigenic faster, indicating that S-adenosylethionine is a proximate carcinogenic metabolite of ethionine.

The liver cell line transformed by ethionine and the corresponding nontumorigenic control cell line were examined histochemically and morphologically in order to confirm the epithelial nature of the cells and to identify specific cellular alterations induced by ethionine treatment and subsequent transformation. Histochemically, the control and ethionine-transformed cells were shown to store glycogen and possess detectable glucose-6-phosphatase activity, both indicative of their origin from liver epithelium. Morphologically, analysis with an automated image analyzer revealed an increase in the number of nucleoli/nucleus as well as in the nucleolar/nuclear ratio in ethionine-transformed

cells. Ultrastructural studies revealed a decrease in the length and frequency of cellular tight junctions and hyperplasia of the inner nuclear membrane in the transformed cells. Nucleolar perichromatin granules, apparently indicative of defective rRNA processing, were also observed in ethionine transformed cells. Ethionine is known to inhibit rRNA methylation, which is necessary for correct processing, and formation of perichromatin granules has been observed in other laboratories during ethionine administration.

Liver epithelial cells have also been used in this laboratory to investigate the reported dependence of neoplastic fibroblasts on exogenous methionine. Nontumorigenic liver cells, like normal fibroblasts, grew well in medium in which methionine was replaced by homocysteine while spontaneously and chemically transformed liver cells, like many other tumorigenic rodent cell lines, showed a dependence on exogenously supplied methionine to attain maximum growth. Assays of the methionine synthesizing enzyme, methionine synthetase, revealed that a linear correlation exists for nontumorigenic and tumorigenic cell lines between their growth on homocysteine and corresponding level of methionine synthetase.

Significance to Biomedical Research and the Program of the Institute:

The availability of liver epithelial cells in culture susceptible to transformation by ethionine provides a unique model system for studying the mechanism of ethionine carcinogenesis.

Proposed Course:

Studies will be initiated to effect the transformation of liver cells with compounds known to block methyl metabolism. S-adenosylmethionine and S-adenosylethionine metabolism will be examined in vitro. Expansion of in vivo studies currently in progress in this laboratory, concerning the effect of methyl-deficient diets and ethionine administration on levels of DNA methylation, to the available in vitro system will be undertaken.

Publications

Brown, J. D., Wilson, M. J. and Poirier, L. A.: Neoplastic conversion of rat liver cells in culture by ethionine and S-adenosylethionine. Carcinogenesis 4: 173-177, 1983.

Wilson, M. J., Myasishcheva, N. V., Stoner, G. D. and Poirier, L. A.: Formation and utilization of methionine by rat liver cells in culture. In Vitro 19: 134-140, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP04807-13 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Comparative Analysis of Fetal and Neoplastic Growth Regulating Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
A.K. Fowler Chief, DBBS, LCC, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick Cancer Research Facility, Frederick, MD; Microbiology and Immunology Program, Wright State University School of Medicine, Dayton, OH

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Developmental Biology and Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The expression and control of bioregulatory macromolecules in embryo-fetal development and the effects of their interactions on cellular proliferation, differentiation and immunocompetence are investigated and compared to related growth factors of tumor-cell origin. Comparative examination of an antiviral activity in placental extracts from several inbred strains of mice demonstrated the presence of an interferon (MuIFN-Pl) exhibiting common characteristics in the placentas of all seven strains examined. MuIFN-Pl exhibits a species specific antiviral activity requiring a cellular transcriptional event and has antiproliferative characteristics. The kinetics of development and decay of the MuIFN-Pl induced antiviral state is time-dependent. MuIFN-Pl activity in placentas is maximal at term and is composed of two distinct species that function synergistically in their induction of the antiviral state in vitro. Both species, however, are biologically and serologically distinguishable from classical mouse inteferons alpha, beta and gamma. Trypsin-collagenase dispersed placental cells concentrating at the 20-30 and 30-40 per cent Percoll gradient interfaces are the major producers of interferon and represent less than 2 per cent of the total cell population.

Application of an in vitro induction system for antibody precursor cells has been used to generate a series of IgM producing hybridomas against purified TGF that directly abrogate TGF-induced mitogenesis in mouse cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Patton T. Allen	Microbiologist	LCC, NCI
Daniel R. Twardzik	Research Chemist	LVC, NCI

Objectives:

To develop and examine model systems identifying and characterizing fetal and tumor-associated bioregulatory macromolecules and to assess and compare their interactions in cellular recognition and growth regulatory processes. To develop immunological reagents to examine and contrast markers of growth regulation during normal proliferative processes and tumorigenesis.

Methods Employed:

Interferons (IFN) are assayed by their antiviral activity on appropriate indicator cells using plaque reduction procedures (PR50), inhibition of cytopathogenic effect (CPE), virus yield reduction and inhibition of viral specific RNA synthesis. Cellular mitogenesis is measured by the incorporation of radiolabeled thymidine into cellular DNA of serum depleted quiescent cells. Colony stimulating activity is quantitated by the induction of BALB/3T3 and NRK cell growth in soft agar.

Major Findings:

The comparative examination of the antiviral activity in placental extracts from several inbred strains of mice demonstrated the presence of an interferon (MuIFN-Pl) exhibiting common characteristics in the placentas of all mouse strains (NZB/N, DBA/2N, AKR/N, C3H/HeN, C57Bl/6N, BALB/cAnN and N.NIH(S)) investigated. MuIFN-Pl activity, as measured by the reduction of virus-induced plaque formation (PR50) and/or cytopathogenic effect (CPE) in L929 cells, was detected in all placental specimens examined after 13 days of gestation. Mean MuIFN-Pl levels were generally similar at equivalent stages of gestation for all mouse strains compared and were consistently highest (1,000 to 2,500 units/ml) near term. The kinetics of the development and decay of the MuIFN-Pl induced antiviral state was time-dependent with cells exhibiting 50 per cent of maximal resistance to virus infection within 5 hr of treatment and full resistance after 12-18 hr. Decay of the antiviral state was comparatively slow, relative to classical mouse type I and type II IFNs, with full protection persisting up to 24 hr after removal of the interferon and moderate protection (more than 50 per cent) evident after 48 to 72 hr. MuIFN-Pl activity of all strains also exhibited similar sensitivity (more than 90 per cent) to heat (56°C/60 min) and trypsin (0.02 per cent/90 min) treatments, was relatively labile (more than 75 per cent) at pH 2 (24 hr/4°C) and was partially neutralized (approximately 50 per cent) by high concentrations of anti-mouse type I serum, but not type II serum. These results indicate that MuIFN-Pl, which induces a species-specific antiviral state requiring a cellular transcriptional event,

is both biologically and immunologically distinguishable from classical mouse type I and type II IFNs. More recently, the MuIFN-Pl activity in extracts prepared from each of the mouse strains (n=4) examined have been shown by gel filtration to comprise two components. These components differ in apparent molecular weight (greater than 65,000 and 27,000) and are also distinguishable by their serological reactivity to anti-mouse type I IFN, as well as by the persistence of their induced antiviral state in vitro. The antiviral activity of the larger molecular weight species is partially neutralized by high concentrations of anti-mouse type I serum and its induced antiviral state decays more rapidly, paralleling the decay rate observed for classical type I IFN. In comparison, the smaller and predominant species is not neutralized by antisera to classical mouse IFNs and its antiviral activity in L929 cells is significantly more persistent. Preliminary evidence also indicates the two placental interferons function synergistically in their induction of the antiviral state, with a one-to-one mixture of the components potentiating the antiviral activity 5-fold over expected additive effects.

Previous studies that demonstrated no detectable circulating interferon (less than 10 units/ml) in pregnant mice and that also revealed the addition of placental cells to L929 monolayer cultures conferred zonal regions of protection against virus infection within the monolayer suggested the placenta was actively, rather than passively, involved in MuIFN-Pl synthesis and concentration. To pursue this question, procedures for placental cell dispersion, separation and culture were established. In initial studies, short term cultures of unfractionated placental cells spontaneously synthesized and secreted interferon. The pooled cellular and extracellular interferon levels were maximal after 24 to 48 hr of incubation and were consistently 4- to 6-fold higher than paired cultures examined immediately after seeding (2 hr). More recently, discontinuous Percoll gradient (5 to 70 per cent) separation techniques have been employed to enrich the interferon producing placental cells. Cells banding at the 20-30 and 30-40 per cent. Percoll interfaces are the major producers of interferon and represent less than 2 per cent of the total cell population. Interferon levels of the fractionated cell cultures were similar to the unfractionated placental cell cultures and were highest after 24 to 48 hr of incubation. Studies are now in progress to identify the specific cell types present in these subpopulations and to characterize the type of interferon produced.

The use of an in vitro induction system for antibody forming precursor cells coupled to monoclonal antibody technology has been developed for the generation of antibody-producing hybridomas specific for tumor growth factor (TGF, prepared from a nonproducer-sarcoma virus transformed rat cell line--provided by Dr. D. R. Twardzik, LVC, NCI) and selected placental growth regulatory factors (MuIFN-Pl). Utilizing this procedure a series of IgM producing hybridomas (6 out of 90) have been produced against TGF that directly abrogate TGF-induced mitogenesis in mouse cells. To date, a similar approach to produce monoclonal antibodies against biologically active crude and partially purified MuIFN-Pl have not been successful. Since interferons, in general, are known to inhibit the sensitizing and proliferative pathways of the immune response, attempts are currently in progress using as an immunogen, inactivated MuIFN-Pl.

Significance to Biomedical Research and the Program of the Institute:

The role played by colony stimulating and other mitogenic growth factors in carcinogenesis is not understood. In addition, the mechanisms which govern control of mitogenesis by natural regulators of cell proliferation, such as the interferons, remain unknown. The placenta is a useful organ for examining the interaction of host factors controlling the proliferation, differentiation, expansion and invasiveness of potentially malignant cells. This organ is a rich source of hormones, mitogens, colony stimulating factors and at least one known inhibitor of cell proliferation and immune modulation: placental interferon. The placenta also harbors a small cadre of potentially malignant cell types (i.e., cytotrophoblast) and a variety of poorly defined factors which permit these and other cells of fetal origin to escape maternal immune detection and elimination. These factors function to effectively maintain a stable feto-maternal relationship on the one hand, but mimic the tumor-host relationship on the other. Identifying and understanding these events in the context of maternal interaction with the fetus may provide clues to critical points of cellular control disrupted by carcinogens and/or tumor promotors in neoplastic disease. More importantly, the identificiation of new biological response modifiers (e.g., IFN-Pl) and their use in modified treatment regimens might be possible should a clearer understanding of these phenomena emerge.

Proposed Course:

Efforts to identify and purify bioregulatory macromolecules (MSF, CSF and IFN) in embryonal, fetal, and neoplastic tissues in adequate quantities to permit further characterization and study will be continued. Emphasis will be placed on the development of monoclonal antibody reagents against selected placental and tumor-associated growth factors. Once generated these reagents will be utilized to examine the antigenic relatedness of growth regulatory factors, the expression and interactions of these factors in normal and neoplastic growth and their usefulness diagnostically to detect markers of tumorgenesis. Model in vitro systems will be developed to assess and compare growth factors and their interactions on cellular proliferation, differentiation and immunocompetence.

Publications:

Reed, C. D. and Fowler, A. K.: A rapid bioassay to monitor murine leukemia virus infection in mice using cellular GP71 binding. J. Virol. Methods 4: 209-217, 1982.

Weislow, O. S., Kiser, R., Allen, P. T. and Fowler, A. K.: Partial purification of a placental interferon with atypical characteristics. J. Interferon Res. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04812-15 LCC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Interactions During Transformation of Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

U. I. Heine Chief, USS, LCC, NCI

COOPERATING UNITS *(if any)*

Department of Histology, Karolinska Institutet, Stockholm, Sweden

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Ultrastructural Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21710

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

DL-Ethionine, the ethyl analog of methionine, is known to be a specific liver carcinogen in rats and mice. A cell line derived from rat liver (established in the Nutrition and Metabolism Section of this Laboratory) and tumorigenically transformed by exposure of the cells in culture to DL-ethionine serves as a model for the study of different parameters in the course of chemical transformation. Special emphasis is directed towards the investigation of morphologically definable transformation features; i.e., phenotypic changes, as these are as yet not well understood in epithelial cells. In a preliminary investigation, characterization of the cell lines (nontransformed and ethionine-transformed) has been achieved and, by using cytological and histochemical methods, evidence has been presented for 1) the epithelial nature of these cells (presence of junctional complexes and prekeratin); 2) their origin from liver epithelium (presence of glycogen and G-6-Pase) and 3) the identification of cellular alterations in nucleus and cytoplasm that resulted specifically from ethionine treatment and subsequent transformation. For the understanding of genetically stable modifications in morphology due to transformation, cytoskeletal arrangements and adhesion characteristics of the transformed cells are now compared to those of the nontransformed controls using immunocytochemistry, reflection contrast and phase contrast microscopy, and electron microscopy. Results obtained thus far indicate, in cultures of transformed cells, a loss of cell-cell adhesion via intermediate junctions which appears to be compensated by an increase in cell-substrate adhesion via focal contacts. Concomitantly, the transformed cells exhibit an increase in fibronectin, further suggesting enhanced cell-substrate adhesion. The changed adhesion patterns in the transformed cell cultures may be defined as progressive deficiencies in cell contact interactions.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

James L. Junker	Staff Fellow	LCC, NCI
Mary J. Wilson	Chemist	LCC, NCI
Cecil H. Fox	Sr. Scientist	LB, NCI

Objectives:

To establish the epithelial nature and to characterize by standard cytological and histochemical methods a nontransformed, rat liver-derived cell line and its counterpart that is tumorigenically transformed by the epigenetic carcinogen DL-ethionine. To study and compare the cytoskeletal arrangement and adhesion characteristics of these cell lines in order to determine genetically stable modifications of the phenotype accompanying tumorigenicity. To determine, by expanding the study to include other cell lines, markers of tumorigenic transformation which may be common among liver cell lines and among epithelial cell lines in general. To determine if the cell lines established in this Laboratory have properties which would make them useful as models for studying the general mechanisms of cell-cell and cell-substrate adhesion.

Methods Employed:

The cell line TRL 1215, established in the Carcinogen Metabolism and Toxicology Branch, NCI, from rat liver cell isolates, is the model used in this study. Low passage controls, high passage controls, and cells made tumorigenic by exposure to DL-ethionine are compared. In order to characterize the cell lines, they are subjected to cytological examination using standard procedures for light microscopy and for transmission and scanning electron microscopy. Chromosome number is established and cells are assayed for glycogen accumulation, acid and alkaline phosphatases, adenosine triphosphatase, and glucose-6-phosphatase. Nuclear and nucleolar area determinations are made using an automated image analyzer. In order to examine the cellular arrangements of cytoskeletal and cytoskeletalinteractive proteins such as tubulin, actin, keratin, and fibronectin, light microscopic immunocytochemistry is employed using commercially available primary and secondary antibodies. Cell-substrate adhesions are identified by observing focal contacts with reflection contrast microscopy using either two different illuminating numerical apertures or two different illuminating wavelengths to distinguish reflection from interference phenomena. Phase contrast microscopy is performed on cells examined by reflection contrast. Cell-cell adhesions are identified by electron microscopy.

Major Findings:

The characterization of nontransformed and DL-ethionine transformed rat liver-derived cell lines of the same passage level by morphological and histochemical means to ascertain their epithelial nature and to establish their origin from liver epithelium has been achieved. The presence of intermediate junctions, bile canaliculi-like structures, prekeratin, glycogen accumulation, and glucose-6-phosphatase activity confirm the origin from epithelial cells of the liver. Persistent modifications of the phenotype resulting from ethionine transformation are variations in cell shape and size, focal multilayered growth, increase in the nucleolar/nuclear ratio and reduction in the number of cells displaying a primary cilium. Hyperplasia of the inner nuclear membrane, observed in approximately 40 per cent of the cells, elongation and branching of mitochondria and a reduction in length and frequency of cell junctions are also characteristic of the transformed cells.

Recent studies have stressed the importance of the cytoskeleton (microtubules, actin cables, intermediate filaments) and extracellular attachment points (fibronectin, focal contacts) for the preservation and regulation of cellular configuration in interphase cells and during mitosis. Changes in phenotype, as exemplified in our model system, are used as one criterion for cell transformation. Our electron microscopic studies reveal that major factors for phenotypic differences between tumorigenic and control cells are in the area of cell-cell and cell-substrate adhesion. Decreased levels of intermediate junctions and an increase in intercellular spaces was observed only in ethionine-transformed cultures; however, cell adhesion as determined by the number of focal contacts and the amount of lamellar cytoplasm per cell appears to be higher in the tumorigenic cultures. This finding of increased adhesion is in contrast to the frequent observations in both fibroblast and liver cell cultures that tumorigenic cells are smaller and less adherent than controls.

Cytoskeletal arrangements of actin, tubulin, and prekeratin are found to vary more among cultures with different degrees of confluence and passage level than between tumorigenic and control cultures.

Significance to Biomedical Research and the Program of the Institute:

Most malignant tumors of man are carcinomas; yet, most of the experimental models developed for the study of transformation and malignancy consist of cells derived from mesenchyme. The establishment in this Laboratory of nontransformed and chemically transformed, liver-derived epithelial cell cultures provides a basis for the investigation of functional changes during transformation of epithelial cells. Examination of cytoskeletal and adhesion characteristics are part of the evaluation of the new epithelial cell lines. Knowledge of these characteristics and the ways they change following tumorigenic transformation is important generally to the study of the mechanisms of carcinogenesis and particularly to study the mechanisms by which an epigenetic chemical carcinogen induces transformation. Decreased cell-cell and cell-substrate adhesion are commonly associated with tumorigenic transformation and, because of similar arrangements of cytoskeletal proteins in both phenomena, are thought to be similar mechanistically. If our observation of decreased cell-cell contact associated with increased cell-substrate adhesion holds true after quantitative analysis, then these cell

lines could prove to be a valuable model for studying the differences between cell-cell and cell-substrate adhesion.

Proposed Course:

Quantitative analysis of our preliminary results is essential to confirm the initial observations. The evaluation will be performed at different culture times and with both high and low passage level controls to insure that changes due only to length of time in culture, degree of confluence, and passage level are not attributed to ethionine-induced transformation.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP04967-08 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Genetic and Physiological Factors in Ontogeny and Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
P. T. Allen Microbiologist, LCC, NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick Cancer Research Facility, Frederick, MD; Genentech, Inc., San Franciso, CA; Dept. of Ob/Gyn, University of Louisville, TN; Uniformed Services University of Health Sciences, Bethesda, MD

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Developmental Biology and Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The properties of bioregulatory proteins found in the near-term mouse placenta are under investigation. Included are growth factor (PlGF) proteins which induce or stimulate mitogenesis and colony growth in soft agar, and interferons (IFN-Pl) which inhibit cell and viral proliferation. IFN-Pl is believed to be a unique interferon, and is distinguishable from the more classical alpha, beta, and gamma interferons by serology and by the decay kinetics of its activity. It is, however, functionally similar to the classical interferons in many aspects including the induction of 2-5 A synthetase, and the inhibition of growth factor mediated mitogenesis. Mouse PlGF is unable to compete with radiolabeled-EGF for binding to cell receptors. Mouse PlGF readily absorbs at low ionic strength to agarose charged with Green A dye-ligand and is eluted by KCl. A growth factor resembling mouse PlGF is present in the human placenta at a concentration analogous to that of mouse PlGF.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Arnold K. Fowler	Chief, Developmental Biology and Biochemistry Section	LCC, NCI
Alan O. Perantoni	Microbiologist	LCC, NCI
Daniel R. Twardzik	Research Chemist	LVC, NCI

Objectives:

To develop and study model systems for evaluating the role of physiological and genetic factors, as they relate to carcinogenesis, through investigation of the growth regulatory factors associated with prenatal ontogeny and neoplastic development.

To establish in vitro bioassay systems to examine growth regulator interactions in normal and pathological growth control.

Methods Employed:

Mitogenesis--A microtray assay using quiescent cell cultures arrested at the G1/GO phase was employed. Addition of growth factor or serum stimulates the incorporation of radiolabeled-thymidine into an acid-insoluble form in such cells.

Receptor binding--Radio-labeled EGF was purchased commercially. Materials to be tested for competing activity were added to microtray cultures of cells just prior to addition of radio-labeled EGF. After incubation for receptor interaction to occur unbound radio-labeled EGF was washed away and that remaining cell-associated was solubilized with detergent and determined by scintillation spectroscopy.

Interferon assay--Mouse interferon concentrations were determined by the induction of antiviral activity in micro tray cultures of confluent L929 cells. Cultures appropriately exposed to serially diluted interferon specimens were challenged by inoculation with MM virus. Cultures were scored for virus resistance relative to unprotected controls using a dye-binding assay via an EIA micro tray reader.

2-5 A synthetase assay--Synthetase from detergent extracts of cells was isolated by affinity chromatography on poly rI-poly rC agarose beads. After incubation with radiolabeled ATP the radioactivity incorporated into 2',5'-(Ap)nA oligonucleotide was determined.

Major Findings:

Placenta-associated growth regulatory macromolecules have been under investigation within this project over the past 2 years. Mouse placental homogenate contains a protein growth factor (PlGF) which induces mitogenesis and anchorage

independent cell growth in vitro. Interferon activity (IFN-Pl) is also present in placental homogenate. Serological and biophysical data have indicated that such homogenate contains two molecular species of IFN-Pl unique from the classical alpha, beta, and gamma mouse interferons utilized as immunogens in the preparation of serological reagents. Intensive investigation of the IFN-Pl has continued with a view toward determining the role of this factor in growth regulation during differentiation and ontogeny. Multiple approaches have been applied in this pursuit.

The use of high performance liquid chromatography (HPLC) has been applied to the purification and characterization of IFN-Pl. HPLC conditions employed in this investigation are necessarily restricted by the fragility of IFN-Pl activity, by the requirement to maintain this biological activity, and by the low initial concentration of the activity in placental extracts. The use of reverse phase resins with acetonitrile gradient elution was proscribed by conflicting pH requirements. Initial studies utilized gradients of a volatile salt, triethylammonium acetate (TEAA), for elution of the activity from a weakly anionic resin. Subsequent lyophilization eliminated the salt and concentrated the eluate fractions. A mixture of murine interferons alpha and beta, MuIFN-alpha, beta was selected as a model for initial studies. The mixture was resolved into two biologically active components utilizing 0.1M to 1.0M TEAA gradients. Despite success in resolving the two interferon activities from these mixtures, overall recovery of activity was considered inadequate for use with IFN-Pl, due to the low titer of this interferon in the initial preparations of tissue homogenate. The recent development of a polycationic resin, Poly Cat A, with an extremely high protein-binding capacity offered an attractive alternate HPLC procedure. Using a NaCl gradient IFN-Pl has been eluted from this column at an ionic strength of about 0.6M and is resolved from greater than 95 per cent of the protein of the initial homogenate loaded. This approach shows promise in the purification of IFN-Pl, and is currently being pursued.

In addition to antiviral effects, the interferons are known to exhibit a variety of other features. One of these is the induction of the enzyme 2-5 A synthetase, which catalyzes the synthesis of 2'-5' oligoadenylate from ATP. Such induction is currently considered a characteristic of all interferons, and is not known to occur through a pathway not mediated by interferon. In view of the apparent uniqueness of IFN-Pl it was considered critical to determine that the antiviral agent exhibited the capability to induce this enzyme. In a collaborative study with David Krause and Robert Silverman at USUHS, it was noted that IFN-Pl treatment of mouse L929 cells caused a greater than 200-fold increase in 2-5 A synthetase activity. The induction was time-dependent, starting from a low constitutive level, increasing rapidly for 24 hr, with a continuing, but slower increase in activity over an additional 24-hr period. These results confirm IFN-Pl's functional similarity to other interferons, despite its unique features.

The ability of interferons to inhibit cell proliferation has been known for two decades, though the mechanism by which this aspect of interferon activity occurs is still largely speculative. Several reports of antagonistic activity between interferons and protein growth factors, including platelet derived growth factor (PDGF) and epidermal growth factor (EGF), have recently appeared. An examination of the interaction between the placenta-associated growth factor (PlGF) and interferons, including IFN-Pl, has been undertaken utilizing EGF,

PDGF and other commercially available growth factors for control purposes when applicable. In conjunction with this study, the interaction of interferons with a growth factor isolated from tumor cell sources (TGF) has been undertaken. Mouse type I interferon has been found to inhibit the induction of mitogenesis by TGF and by PlGF in BALB/c mouse 3T3 cells, although it was ineffective in abrogating TGF-induced mitogenesis in bovine cells (MDBK). In comparison, TGF-induced mitogenesis in MDBK cells, a cell line sensitive to the antiviral effects of human interferon alpha, was inhibited by this interferon, as well as by recombinant human alpha A/D interferon. As examined in vitro, optimal interferon effect occurs upon exposure of the cells to interferon prior to growth factor exposure. More recently, two IFN-Pl components partially purified from mouse placental extracts by molecular exclusion chromatography on polyacrylamide beads (Bio-Gel P-100) were found to block mitogenesis induced by EGF in mouse cells, but not in hamster cells, suggesting that the effect is specific for the species from which the interferon originated. The examination of placental growth factors will continue, with emphasis on the application of the experimentally induced (ENU) gestational choriocarcinoma in patas monkeys as a model system for elucidating their interactions in controlled and uncontrolled cellular growth (cf. project Z01CP05092).

In collaboration with C. V. Rao, University of Louisville, human placentas were examined for growth factor activity using the procedures employed to demonstrate mouse PlGF. All preparations of crude human placental homogenate elicited mitogenic response in BALB/c 3T3 cells. The response was dose dependent, and the concentration of human PlGF was similar to that of mouse PlGF in analogous preparations. Biochemical characteristics of the human PlGF were similar to those of mouse PlGF: complete stability on dialysis against phosphate buffered saline, partial stability to 10 mM dithiothreitol, destroyed by 1M acetic acid or 80°C treatment. These features distinguish human PlGF from EGF and TGF.

Affinity chromatography of mouse PlGF on agarose-bound dye-ligand media is under investigation. While PlGF fails to interact with the dye-ligands Orange A and Blue B, it does bind to Blue A, Red A and Green A. KCl gradient elution on Agarose-Green A provides a single step purification factor of approximately 50X. The activity fails to elute efficently from either Blue A or Red A with KCl at concentrations up to 1.5M.

Mouse PlGF was examined for interaction with cellular EGF receptors using a binding competition procedure. Under the assay conditions employed, unlabeled EGF at 2.5 ng/ml blocked attachment of radio-labeled EGF by 50 per cent, but PlGF failed to block radio-labeled EGF binding detectably, even when tested at 1 mg/ml. These results indicate that PlGF does not interact with cells via the EGF receptors. Reports from other laboratories indicate that TGF-like growth factors that compete for EGF receptors are present in the human placenta. These are found in concentrated extracts of acid-treated placental homogenate. Acid labile PlGF appears to be present in the placenta at higher concentration than the TGF-like factors. More recently, mitogenic stimulating activity as well as colony stimulating activities have also been identified in rat, hamster and monkey placenta. Biological and biochemical characterization studies are planned for these activities and efforts will be made to determine their cellular localization in the gravid uterus.

Significance to Biomedical Research and the Program of the Institute:

The importance of exogenous growth factors on the proliferation and differentiation of tissues under experimental conditions has been recognized for many years. More recently EGF has been detected in fetal tissues, and receptors for EGF have been reported on placental membranes. These observations lend credence to the notion that PlGF is a component in a complex system of growth promotors critically balanced to achieve precisely defined tissue differentiation. The precise role that any of these factors play in ontogeny or in oncogenesis remains speculative. The goal of this project is to investigate the occurrence and interaction of bioregulatory macromolecules. At the present time both growth stimulator (PlGF) and growth antagonists (IFN-Pl) have been identified in placental tissues. Progress toward characterizing the factors and their occurrence during ontogeny has been made. With continued study the role of these factors in the control of cell proliferation and differentiation may be elucidated.

Proposed Course:

Major emphasis will be placed on the intensified use of HPLC technology for the preparation of purified placental growth factor and placental interferons for use as reagents. A study of the possible potentiation between placental interferons and the classical interferons as well as between the different molecular species of placental interferons will be performed. A more detailed examination of the interaction between placental growth factors and interferons will be carried out, as well as an examination of the interaction between placental growth factor, tumor growth factor, and other growth stimulators.

Publications

Strickland, J. E., Hennings, H., Jetten, A. M., Yuspa, S. H., Allen, P. T., Hellman, K. B. and Strickland, A. G.: Susceptibility determinants for mouse epidermal carcinogenesis. In Bartsch, H. and Armstrong, B. (Eds.): Host Factors in Human Carcinogenesis. Lyon, France, International Agency for Research on Cancer, 1982, IARC Scientific Publication No. 39, pp. 259-268.

Weislow, O. S., Kiser, R., Allen, P. T. and Fowler, A. K.: Partial purification of a placental interferon with atypical characteristics. J. Interferon Res. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05092-05 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transplacental Carcinogenesis and Tumor Promotion in Nonhuman Primates

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
A. E. Palmer Research Veterinarian, LCC, NCI

COOPERATING UNITS *(if any)*
Meloy Laboratories, Inc., Rockville, MD

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	2.0	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Nonhuman primates of the species Erythrocebus patas (patas), Macaca mulatta (rhesus), and Macaca fascicularis (cynomolgus) are subjected to direct-acting and metabolism-dependent chemical carcinogens by transplacental or direct exposure. In some cases the carcinogen-treated animals are subsequently exposed to chemicals that promote the development of neoplasms in rodents. Mechanisms of organ and species differences in the effects of chemical carcinogens and tumor promoters among rodent and nonhuman primate species are investigated. Induced tumors are evaluated by light microscopy using standard staining procedures, histochemical techniques and electron microscopy and are assayed for in vitro cultivability and transplantability to rodents.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Jerry M. Rice	Laboratory Chief	LCC, NCI
Jerrold M. Ward	Chief, TPPS	LCC, NCI
Lucy M. Anderson	Expert	LCC, NCI
Paul Donovan	Chemist	LCC, NCI

Objectives:

To study and characterize the variable sensitivities of different organ systems in rodents and nonhuman primates to carcinogens which act directly or require in vivo metabolism for carcinogenic activity during the prenatal and postnatal period. To precisely characterize neoplastic and selected nonneoplastic lesions from treated animals by light and electron microscopy, histochemistry, explantation to cell or organ culture, transplantation and by other procedures which may be appropriate. To attempt to demonstrate the phenomenon of tumor promotion in nonhuman primates and to determine whether cell and tissue specificies of tumor-promoting chemicals demonstrated in rodents are similar in the nonhuman primate models.

Methods Employed:

Carcinogenic chemicals specifically selected or designed for a given study are purified after purchase, or synthesized de novo and characterized thoroughly by chromatographic and spectroscopic procedures. Radiolabeled compounds are similarly prepared as required. The carcinogens are administered in precise doses to nonpregnant or exactly timed-pregnant rodents (mouse or rat), or nonhuman primate (patas, rhesus, cebus or cynomolgus monkeys) and the treated animals and their offspring are followed carefully for the development of the tumor. Agents which previously have been demonstrated to promote tumors in rodents are similarly purchased, purified or synthesized de-novo and administered to primates after completion of an initiating regimen of exposure to organ-specific carcinogens.

Tumor-bearing nonhuman primates are intensely monitored to study tumor growth, body weight and clinical pathological changes. Selected animals are evaluated for tumor markers such as alpha-fetoprotein or carcinoembryonic antigen. Selected tumors which may cause suffering are carefully evaluated on an individual basis, and may be surgically removed to reduce suffering and to prolong life. When tumors are judged to be inoperable, and to be causing suffering or threatening life, animals are killed by euthanasia. Thorough gross postmortem examinations are performed and all gross lesions plus major organs are evaluated by light microscopy. Selected neoplasms are evaluated by electron microscopy and/or are cultured by cell or explant culture in vitro, then transplanted to athymic (Nu/Nu) mice. Tumors are evaluated by histochemistry as appropriate.

Major Findings:

Studies on carcinogenesis by ethylnitrosourea (ENU) and diethylnitrosamine (DEN) in the Old World monkey, Erythrocebus patas, have been continued and expanded. Additional cases of both mesenchymal and epithelial tumors were observed in the offspring of monkeys given ENU intravenously during pregnancy, especially when treatment was begun during the first month of gestation. These additional findings confirm the tentative conclusions previously drawn that, like rodents, this representative primate species is quantitatively more susceptible to the direct-acting alkylating agent ENU during prenatal life, with animals exposed in utero exhibiting a higher incidence of tumors after a shorter latency than juvenile or adult animals given the same dose directly.

Evidence has accumulated of a biphasic tumorigenic response of patas lung after transplacental exposure to ENU. Rapidly growing, lethal primary sarcomas of the lung have been seen in juvenile patas after transplacental ENU, and pulmonary blastomas with an identical clinical course have developed in comparably exposed rhesus monkeys. Patas exposed transplacentally to ENU that survived to adulthood have in several cases developed multiple bronchiolo-alveolar adenomas of the lung, suggesting a tendency to prolonged latency for purely epithelial pulmonary tumors that might be susceptible to promotion by agents such as asbestos.

Careful morphologic evaluation of tissues from female patas monkeys which died of a widely disseminated, hemorrhagic malignancy within several months of the beginning of exposure to ENU during pregnancy has confirmed the provisional diagnosis of gestational choriocarcinoma, marked by replacement of endometrial stroma by tumor cells, the occlusion of numerous blood vessels in the uterine myometrium by masses of tumor cells morphologically indistinguishable from intravascular trophoblast, and numerous distant metastases, principally to the lungs. The presence of tumor tissue within the placenta in one case constitutes additional evidence for the trophoblastic origin of the neoplastic cells. This observation provides the only animal model of systemically inducible trophoblastic malignancy. Ultrastructural and biochemical studies have been initiated to further characterize this neoplasm and to confirm its postulated trophoblastic origin.

Patas monkeys given DEN during pregnancy, and their offspring, have been observed for 4 years--until the offspring became sexually mature--without evidence of neoplasia. At that time, 50 percent of both adult females and offspring were given phenobarbital, a promoting agent for hepatocellular neoplasia in rodents, in drinking water at dosages that maintained blood levels in recipients at approximately those necessary for therapeutic efficacy (suppression of convulsions) in man. After 1.5 years of phenobarbital exposure, one male offspring exhibited rising levels of alpha-fetoprotein and at laparotomy was found to have multiple nodular masses in the liver, which on biopsy were identified as hepatocellular neoplasms. While preliminary, this observation, if confirmed, indicates that (1) DEN is an effective transplacental carcinogen in nonhuman primates; (2) latent neoplastic cells may persist for years in primate liver without proliferating; (3) these cells can be stimulated by phenobarbital to proliferate to form a tumor, indicating that at therapeutic doses this barbiturate is a tumor promoter in the patas monkey and that its promoting effects are comparable in regard to target organ and target cell

in both rodents and this primate species. Three additional animals, 2 females treated with DEN during pregnancy and 1 offspring treated transplacentally, which are receiving phenobarbital now have liver masses which are compatable with neoplastic development.

It has been discovered that systemic strongyloidiasis occurs in and is readily transmissible among patas monkeys, in which the infestation can be fatal. This parasitic infestation is a zoonosis, transmissible to man, and constitutes an avoidable hazard for personnel handling these animals.

Significance to Biomedical Research and the Program of the Institute:

Research on animal models of human childhood neoplasms should provide an insight into the types of causative agents and modes of exposures responsible for childhood cancer. It is to be expected that natural selection would tend to eliminate genotypes in the human population which predispose individuals to the development of fetal neoplasms before attaining reproductive age, yet the incidence of embryonal neoplasia in childhood is relatively constant. Epidemiological studies have pointed to the occurrence of childhood neoplasms in association with certain types of congenital malformation which are non-inherited and suggest that environmental agents, alone or in combination, may play a role in the induction of such neoplasms. The inducibility of tumors very similar to the pediatric tumors of man by chemical carcinogens in laboratory rodents and primates further supports this view.

Most tumors induced transplacentally in rodents are of adult types and appear during adult life in individuals exposed in utero, resembling human experience with diethylstilbestrol. The ENU studies in monkeys have provided experimental data indicating that both adult and pediatric tumor types develop in at least one species of primate in response to carcinogenic exposure in utero and suggest that chemical carcinogens may be involved in the prenatal genesis of pediatric and possibly certain adult types of tumors in man. The demonstration of the inducibility of uterine choriocarcinoma by chemical carcinogens, at low exposure levels, further illustrates the importance of preventing human exposure to carcinogenic chemicals during pregnancy in either the workplace or environment.

The phenomenon of tumor promotion, while well established in rodents, is based on very limited data from which to extrapolate to man. The generality of the phenomenon and the extent to which organ-specific effects can be predicted in one species on the basis of bioassays conducted in another remain to be established. There is for tumor promotion, as yet, no unifying conceptual hypothesis exploitable for interspecies comparison, comparable to the role of primary damage to DNA in mutagenesis and probably in neoplastic transformation by genotoxic chemicals. It appears from experiments in rodents that promotion, unlike tumor initiation, is not persistent and that the underlying toxic effects thus are not cumulative. If, as seems likely, tumor promotion plays a significant role in the development of human cancer, the requirement for continual exposure to the promoting agent may provide prevention strategies that are much more readily applicable than in the case of persistent and cumulative genetic toxicity.

Proposed Course:

Monkeys exposed to ENU or DEN either transplacentally or directly (after weaning) should continue to be observed for the development of tumors. In vivo studies should emphasize further refinement of definition of periods of maximal prenatal susceptibility to direct-acting versus enzyme activated transplacental carcinogens; and the extent to which the enzymes, which activate different classes of metabolism-dependent carcinogen, can be induced by either the carcinogens or other agents in fetal, maternal and placental tissues at different stages of prenatal development. The possibility that the phenomenon of tumor promotion can be demonstrated in primates prenatally exposed to ENU, either by increasing the incidence of hepatocellular tumors by postnatal exposure to phenobarbital, DDT, etc., will be further explored. DNA repair phenomena, especially alkyl-acceptor protein-mediated repair and its possible induction by exposure to alkylating carcinogens, will be studied in organs and separated cell types at different stages of prenatal and postnatal development to evaluate the possible roles of DNA repair phenomena in cell- and organ specific carcinogenesis, especially as these vary among primate and available rodent species in response to metabolism-independent alkylating agents. The possible role of aflatoxins, environmental carcinogens prevalent in regions of the world notable for high incidence of gestational choriocarcinoma, as causative agents for human choriocarcinoma will be explored by direct testing in pregnant patas monkeys.

Publications

Harper, J. S.III, Rice, J. M., London, W. T., Sly, D. L. and Middleton, C.: Disseminated strongyloidiasis in Erythrocebus patas. Am. J. Primatol. 3: 89-98, 1982.

Rice, J. M.: Exposure to chemical carcinogens during pregnancy: Consequences for mother and conceptus. Proc. First World Conf. Trophoblast Neoplasms. New York, Plenum Press, 1983, (In Press)

Sly, D. L., Harbaugh, S. W., London, W. T. and Rice, J. M.: Reproductive performance of a laboratory breeding colony of patas monkeys (Erythrocebus patas). Am. J. Primatol. 4: 23-32, 1983.

CONTRACT IN SUPPORT OF THIS PROJECT

MELOY LABORATORIES, INC. (N01 CP 15766)

Title: Resources for Transplacental Carcinogenesis in Primates

Current Annual Level: $229,993

Man Years: 3.2

Objectives: This project is designed to demonstrate and characterize transplacental carcinogenesis in nonhuman primates, especially the Erythrocebus patas, an Old-World monkey. Additionally, related phenomena are studied, including the increased risk of carcinogenesis in adult females exposed to chemicals during pregnancy, tumor promotion, and mechanisms of cell and organ specificity and of species differences in the effects from both chemical carcinogens and tumor promoters.

Major Contributions: Ethylnitrosourea (ENU) has been shown to be a potent carcinogen in the rhesus (Macaca mulatta) and patas monkeys. In both species the fetus is more susceptible than is the adult, and this susceptibility is more pronounced during the first and early second trimester of pregnancy. However, the kinds of tumors seen in the two species differ in their characteristics and distribution.

Diethylnitrosamine (DEN) given to pregnant patas monkeys during gestation did not cause tumors in the offspring or mothers after four years of observation. However, after 24 to 30 months of daily doses of phenobarbital comparable to therapeutic anticonvulsant levels in man, one offspring developed hepatocellular carcinoma, and another has clinically evident hepatic masses. In addition, two of the phenobarbital treated mothers also have liver masses. None of the controls have developed abnormalities.

Except for the association between in utero exposure to diethylstilbesterol and the increased risk of vaginal adenocarcinoma during early adulthood there is little known concerning the effect of chemicals on the human fetus. Transplacental chemical carcinogenesis studies have been limited to rodent species which differ greatly from man. Most significant is the more rapid rate of fetal and neonatal growth and maturation in rodents. Nonhuman primates also have shorter gestations and mature more rapidly than do humans, but they are more similar to man in fetal growth, placentation and early development than are rodents. Tumors induced to date in rhesus and patas monkeys by transplacental exposure resemble some congenital tumors or tumors of infancy and childhood seen in man, suggesting that prenatal exposure of humans to chemicals may be a factor in tumor incidence. The demonstration of tumor promotion in nonhuman primates provides significant evidence of the importance of this phenomenon to man.

Proposed Course: Animals previously exposed to carcinogens will continue to be closely monitored for tumor development, and all tumors will be intensively studied. In addition, studies to demonstrate more precisely the varying

sensitivity of the fetus during gestation are underway. Limited numbers of animals will be treated with agents known to be promotive in rodents, after limited transplacental exposure to carcinogens. The transplacental effects of chemicals other than ENU and DEN will be explored.

CONTRACT IN SUPPORT OF THIS PROJECT

MELOY LABORATORIES, INC. (N01 CP 25613)

Title: Tumor Promotion in Cynomolgus Monkeys (Macaca fascicularis)

Current Annual Level: $116,793

Man Years: 1.5

Objectives:

This project is intended to demonstrate the phenomenon of tumor promotion in cynomolgus monkeys and to explore the promotive activity in this species of several chemicals known to promote tumors in rodents. The liver model was chosen because diethylnitrosamine (DEN) has been studied extensively and shown to be a predictable hepatocarcinogen in this species. Preliminary findings suggest that DEN initiates patas monkey liver when given intravenously or transplacentally. The effect of promoter compounds in vivo on liver metabolism, morphology and enzyme induction will be studied.

Major Contributions:

The project is in its initial year and thus, has not yet provided major findings. Preliminary studies have shown major differences in the in vivo response of cynomolgus liver as compared to Fisher rat liver to several chemicals known to promote liver tumors in rats.

A closed colony of young cynomolgus monkeys are on hand and appropriate groups are receiving DEN in an initiation regimen. At the end of the initiator treatment, selected groups will be treated with promoter compounds.

Proposed Course:

Animals receiving DEN will be placed on chronic exposure regimens of several promoter compounds at appropriate intervals after treatment with the DEN. These animals will then be closely monitored for evidence of tumor development. Tumors may be surgically excised in selected cases to determine the effect of this procedure on developing tumors and to study the pathogenesis of the process. Whenever tumor-load is determined to be causing suffering or is life threatening, the animal will be killed by euthanasia. Tumors will be studied histologically and histochemically by light and electron microscopy; by cell or organ culture for growth characteristics and by transplantation into athymic mice.

Studies to determine the impact of chemical liver tumor promoters on the induction of liver enzymes in the cynomolgus monkey liver and to compare this behavior to both that of nonpromoters and with the effect of these chemicals on rodent liver are planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05093-05 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
In Vitro Studies on Organ Specificity in Transplacental Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
J. M. Rice Laboratory Chief, LCC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	2.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The roles of morphogenetic differentiation in controlling the phenotypic expression of neoplastic transformation, the degree of malignancy of tumors, and the susceptibility of developing organs to carcinogenesis are studied using organ culture and tissue transplantation techniques, with current emphasis on the kidney. The ability of transplacentally administered carcinogens to induce genotoxic damage in cells of embryos or fetuses exposed at different stages of gestation was determined for different species. Cells were isolated from exposed embryos and gene mutations at 3 loci were assayed in vitro with simultaneous determination of survival ability. Organ specificity of induced gene mutation is being determined in embryonal cells isolated from organs of various species exposed in utero at comparable stages of gestation. Quantitative dose curves for transplacentally induced mutation were also obtained for selected carcinogens. Quantitative determination of transplacentally induced transformation is currently in progress. The above parameters, once completed, will be compared with in vivo transplacental tumorigenesis results.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Paul Donovan	Chemist	LCC, NCI
Alan Perantoni	Microbiologist	LCC, NCI

Objectives:

To identify and characterize those aspects of morphogenetic differentiation which modify the consequences of prenatal exposure to chemical carcinogens, especially in the nervous and genitourinary systems. The ultimate objective is to elucidate the control of expression of the neoplastic phenotype in transformed cells. To devise and apply improved quantitative selective mutation systems to embryonal and fetal primary cells in culture, previously treated in utero with chemical carcinogens. To determine the time course of maximum sensitivity to induced gene mutation of cells from embryos or fetuses treated transplacentally at different stages of gestation with ENU; also to simultaneously determine survival ability. To determine quantitative dose curves of transplacentally induced gene mutation and survival by select carcinogens in one species. To determine the sensitivity of various species to one metabolism independent transplacental chemical carcinogen, ENU, and to determine inter and intra litter variations in response to ENU. To determine the organ specificity in various species of gene mutation transplacentally induced by ENU. To utilize and attempt to improve the current in vitro systems of assaying transformation in primary cells, the colony assay and the soft-agar assay. To apply in vitro transformation assays to cells isolated from embryos of different species treated transplacentally with chemical carcinogens. To correlate the above in vitro quantitatively determined parameters with transplacental tumorigenesis data.

Methods Employed:

Transplacental treatment: Gravid animals are injected with different doses of agents at precise periods of gestation. Primaries are made from either the whole carcass or selected organs of different species. Mutation frequencies are determined on the cells so isolated. With experimentally determined optimum expression times, cell numbers, and selective conditions, cells are treated with the selective agent for several markers. Cells are also seeded for colony formation to determine survival curves.

Transformation assays are currently being tested for use with transplacentally treated cells of different species. These include Pienta's colony assay and the soft-agar assay. Improvements are desirable and are being sought.

Short- and long-term cell and organ culture techniques are developed and the features of tissue rudiments maintained therein characterized by histochemical, light microscopic, and ultrastructural techniques. Cultures of both normal fetal organ rudiments and selected tumors are utilized to explore the effects of morphogenetic differentiation and its induction on the behavior of tumors of undifferentiated cell type, including nephroblastic tumors of the kidney.

Tumors are induced transplacentally or by direct treatment in experimental animals to provide suitable material for study and are transplanted serially in appropriate recipients to develop standard, manipulatable models for studies in vitro. Surgical procedures relating to tissue transplantation are adapted as necessary to study the capacity of various recipient sites to modify differentiation of selected transplantable tumors. Substances and tissues known to influence differentiation are combined with tumors and normal undifferentiated inducing tissues and interactive events in the differentiation of normal and neoplastic tissues are characterized. All studies are performed in more than one species, selecting species (such as the rat and mouse) which can be readily manipulated in the laboratory and in which responses of selected organ systems (such as the kidney and brain) to chemical carcinogens during fetal life vary, extremely, both in quantitative responses to chemical carcinogens and in the nature of tumors induced.

Major Findings:

The methodology of mutation selection has been improved resulting in increased recovery of induced mutants. It was, therefore, possible to obtain quantitative dose response curves of transplacentally induced mutation by various chemical carcinogens, as reported previously. To use this method as an possible indicator of potential transplacental carcinogenic activity concomitant with induced genotoxic damage requires data from several more model carcinogens and non-carcinogens.

The sensitivity to transplacentally induced mutation at different stages of gestation has been determined for the Syrian hamster. In vivo studies are currently underway to determine dose responses and age dependence of transplacental carcinogenesis in this species. The time of maximum sensitivity to transplacental mutation induction is presently being determined in the mouse and rat.

Our efforts to understand the differences in the spectrum of renal tumors observed in the rat versus the mouse following transplacental treatment of carcinogens has been expanded. The observation that mouse kidneys develop only adult type tumors despite their age at exposure while rats often manifest embryonal renal tumors supports the idea that differences in the developmental processes in these two animals may determine the pattern of neoplastic expression. Our approach to this problem has required expertise in microdissection of fetal metanephric rudiments. We now can routinely reproduce the classic separations of ureteric bud from metanephrogenic mesenchyme performed by Grobstein. In addition, we have found the appearance of GGT activity in mouse and rat fetal kidneys to be an extremely useful marker for primitive renal proximal tubular epithelia.

The acquisition of these techniques now permits us to study those factors important in the induction and normal differentiation of fetal metanephric rudiments and also to investigate these factors which prevent completion of the normal process, resulting in expression of the nephroblastoma. Preliminary attempts to promote differentiation in rat nephroblastomas using homotypic or heterotypic inducers have been unsuccessful, but media conditions in these organ culture studies have not yet been maximized. The separated ureteric bud,

for example, deteriorates rapidly in our organ culture media; however, the supplementation of media with several hormones stabilizes cellular integrity. Further experimentation will therefore be necessary to determine if the failure to induce nephroblastoma differentiation is simply a problem of culture conditions or instead an inability of tumor tissue to respond to the normal signals of differentiation.

In the mouse, efforts are underway to establish a nephroblastoma model. Separation and transplantation of metanephric mesenchyme exposed to carcinogens transplacentally or in organ culture may allow expression of the embryonal tumor type in initiated cells when an inductive stimulus is absent.

Since considerable biological material is necessary for biochemical studies of those cell surface factors important to differentiation, culture growth of fetal kidney tissues could prove to be an important resource. For this reason, intensive efforts have been directed towards the propagation in culture of normal mouse and rat fetal metanephric tissues. Mesenchyme from both animals is adaptable to growth in standard culture media; however, growth of the bud appears to require an enriched medium. Rat bud growth has been sustained in a hormone-supplemented defined medium for several weeks, but mouse bud deteriorates rapidly even under these enriched culture conditions. Additional media supplements other than hormones are presently being considered.

Significance to Biomedical Research and the Program of the Institute:

Studies in rodents have shown that a fetus may be as much as two orders of magnitude more susceptible to carcinogens than an adult of the same species, strain, and sex. The precise reasons for this enhanced vulnerability are not clearly understood, and the fact remains unexplained that, in rodents, carcinogens acting on differentiating fetal tissues principally induce tumors of adult epithelial morphology. Many tumors which develop as a consequence of transplacental exposure to carcinogens are morphologically identical to those inducible in adults. In the mouse kidney, for example, only adenomas and a few carcinomas originating from proximal convoluted tubules develop after prenatal exposure to carcinogens when the kidney is mostly undifferentiated. This suggests that the fundamental genetic damage inflicted on undifferentiated fetal cells does not preclude subsequent programmed differentiation. The fact that differentiation overrides expression of neoplastic transformation in a given organ system (e.g., the kidney) of certain species such as the mouse, but does not do so in others such as the rat, provides a route to exploration of the basic nature of cellular differentiation to the control of neoplastic growth in the context of prenatal susceptibility to carcinogens.

The ability to determine experimentally in vitro, the relative potency of transplacental carcinogens to induce mutation in the somatic cells of the fetus is an important methodological advance. Since susceptibility to carcinogens during this time period is greatly increased relative to adults, prenatal testing of putative carcinogens has sometimes been advocated. However, the cost and difficulties of transplacental carcinogenesis experiments would be prohibitive except in some cases. This in vivo/in vitro method would partially fulfill this need.

Second, there is at present no clear explanation of the vast differences in susceptibility to transplacental carcinogens among different organs of different species. Also, vulnerability is specifically time-dependent, being nil in periods before organogenesis and rising to a maximum just before birth. The fundamental question posed by both observations is whether the resulting transplacental tumor incidence is proportional to genetic damage as measured by mutation frequency. One of the alternative explanations is that the genetic damage inflicted by the mutagen initiates, but the controlling factor in tumorigenesis is the process of differentiation and anything that influences that process.

Proposed Course:

Rat renal "blastemal-cell" tumors will continue to be studied in transplantation and in cell and organ culture to determine whether the morphologically undifferentiated tumor can be induced to form characteristic epithelial structures resembling renal tubules and whether the enzymes characteristic of renal epithelium will develop as markers of morphologically demonstrable differentiation. In organ culture, both natural (ureteric bud) and heterotopic (fetal spinal cord) inductive tissues will be used as potential inducers as well as chemical agents which are known to affect other in vitro systems in which morphogenetic differentiation occurs (cAMP, IUDR, DMSO). Initially, the goal of this program is to determine whether the lesser tendency of fetal rat kidney (in comparison with that of the mouse) to form differentiated epithelial tumors after exposure to transplacental carcinogens is due to interspecies differences in cellular responsiveness to mediators of morphogenetic differentiation.

A major characteristic of the response of rodents to chemical carcinogens during intrauterine development is that susceptibility to neoplastic transformation generally is not demonstrable prior to completion of definitive organogenesis, which marks the beginning of the fetal period of development. True embryos, in which undifferentiated tissues are only beginning to form identifiable organ rudiments, are subject to teratogenic damage but are not, in general, at risk for subsequent tumor development as a consequence of exposure to carcinogens during this stage of development. The question arises whether this indicates that potential neoplastic transformants are generated, but are prevented from expressing their neoplastic genotype phenotypically by proliferation to generate a tumor. The latter might be accomplished through cell-cell interactions or other mediators of programmed normal differentiation to which cells altered by carcinogens are still responsive.

Mutagenesis in fetal hamster, rat, mouse, and eventually nonhuman primate tissues will be investigated systematically to establish whether genotoxic damage, demonstrable immediately by the mutation assays, correlates with organ-specific and age-dependent transplacental carcinogenesis by various agents in these species. A future project is planned using the fetal hamster in cell cultures from which morphologic transformation of mesenchymal cells is demonstrable. Prenatal hamsters will be subjected transplacentally to a carcinogenic dose of metabolism-independent carcinogen at different stages of development, from implantation of the blastocyst through late fetal life. Cultures prepared from the conceptuses thus exposed will be studied for the presence of transformed cells in an effort to demonstrate the presence of

latent transformed cells in fetal tissues that appear refractory to carcinogenesis during early development. The fact that transformation is readily demonstrated in fetal hamster fibroblasts that originate from the soft connective tissues in which tumors are not seen following transplacental exposure to carcinogens strongly suggests that such an approach will be fruitful.

Publications

Cortesi, E., Saffiotti, U., Donovan, P. J., Rice, J. M. and Kakunaga, T.: Dose-response studies on neoplastic transformation of BALB/3T3 clone A 31-1-1 cells by aflatoxin B_1, benzidine, benzo[a]pyrene, 3-methylcholanthrene, and N-methyl-N'-nitro-N-nitrosoguanidine. Teratogenesis, Carcinogenesis, and Mutagenesis 3: 101-110, 1983

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05157-04 LCC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Mechanisms of Organ Specificity in Chemical Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. M. Rice Laboratory Chief, LCC, NCI

COOPERATING UNITS *(if any)* University of North Carolina, Chapel Hill, N.C.; Microbiological Associates, Inc., Bethesda, MD; and Chemical Industry Institute of Toxicology, Research Triangle Park, N.C.

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.7	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Factors that modify tissue responses to chemical carcinogens in different organ systems are studied to evaluate their contribution to changing susceptibilities to carcinogenesis in certain tissues during prenatal and postnatal development. Tissues and organ systems currently under study include the nervous system, kidneys, and liver. Modifying processes now being studied include excision repair of DNA, DNA repair mediated by alkyl acceptor protein (AAP), the changing cellular susceptibility to carcinogens during different stages of the cell cycle, and the role of the enzyme gamma-glutamyltranspeptidase as a determinant of susceptibility to the carcinogen azaserine.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Beatrice Chen	Research Chemist	LCC, NCI
Alan Perantoni	Microbiologist	LCC, NCI
Lucy Anderson	Special Expert	LCC, NCI
Amos Palmer	Research Veterinarian	LCC, NCI
Jerrold Ward	Chief, TPPS	LCC, NCI

Objectives:

To define the roles of biochemical processes other than carcinogen metabolism that significantly modify the susceptibility of fetal and selected adult organs to chemical carcinogens. Current specific objectives include the following: to investigate the development of capacity for excision repair and alkyl acceptor protein (AAP) mediated repair of DNA in fetal tissues, including brain and liver, during the course of intrauterine development and to evaluate the role of repair capacity or its absence in high prenatal susceptibility to tumorigenesis in these organ systems in different species; to evaluate the role of the enzyme gamma glutamyltranspeptidase (GGT) in the pronounced organ selectivity of the carcinogen, azaserine, and related substances which are selectively toxic and carcinogenic for organs such as the kidney and pancreas which are characteristically high in levels of GGT; to evaluate the extent to which susceptibility of a specific target cell, the hepatocyte, varies in susceptibility to chemical carcinogens during different stages of the cell cycle, a factor of major importance in understanding the intrinsically high susceptibility to chemical carcinogens of fetal tissues which have high rates of cell division.

Methods Employed:

Cell lines high in GGT and derivative, or mutant, lines low in this enzyme are grown in vitro, and uptake, biotransformation, and toxicity of azaserine are studied by liquid scintillation and HPLC techniques. Enzyme activities are demonstrated histochemically and quantitated biochemically through standard procedures involving the generation of colored enzyme reaction products. Nucleic acid repair in organs taken directly from fetal tissues is accomplished by direct measurement of enzyme activities such as apurinic endonuclease which are required for DNA repair. Pregnant animals including both rodents and non-human primates are treated with various doses of carcinogens at precisely defined times during gestation; and at selected intervals after treatment, fetal organs are dissected under a microscope and dissociated for study. The cell cycle kinetics of the regenerating rat liver are determined after subjecting F344 rats to a two-thirds partial hepatectomy by pulse labeling the regenerating liver with thymidine, quantitating the incorporation of this DNA precursor by liquid scintillation counting of isolated DNA, and by evaluating the proportions

of cells undergoing DNA synthesis and in mitosis through high resolution autoradiographic techniques. Rats are injected via the portal vein with selected doses of direct-acting carcinogens at precisely defined times after partial hepatectomy and the carcinogenic response, as well as the evolution of preneoplastic lesions, is determined as a function of time elapsed since partial hepatectomy and as a function of the stage of the cell cycle at which carcinogen treatment took place. Increased synchrony in regenerating liver is achieved by post-hepatectomy injection with hydrocortisone hemisuccinate at 5 hour intervals for a total of 3 injections.

Major Findings:

Previous reports have documented a positive correlation between gamma-glutamyl transpeptidase (GGT) activity and sensitivity to azaserine toxicity. In addition, glutathione, which is catabolized by GGT protects cells from toxicity to azaserine to form what we believe to be a detoxified conjugate of azaserine. Further chemical data support this hypothesis. An HPLC purified reaction product of azaserine-glutathione incubations has been evaluated by amino acid analysis and mass spectroscopy. This product is composed of the amino acids predicted from hydrolysis of an azaserine-glutathione conjugate. Mass spectroscopy of purified material shows an ionic species at m/e 478, which is equivalent to the molecular, weight of a 1 to 1 azaserine-glutathione conjugate. Actual identification, however, will require NMR analysis.

Quantitative excision repair studies continue in order to explore the relationship between DNA repair activity and organotropic differences in fetal susceptibility to chemical carcinogenesis. Cell suspensions from fetal brain, kidney and liver were assayed for the presence of the DNA repair-associated enzyme apurinic endonuclease (APE) by a procedure developed in this laboratory. APE is estimated by its capacity to reduce the capacity of bacterial DNA coding for the enzyme bata-galactosidase to function in an in vitro DNA-dependent system in which bata-galactosidase is synthesized. The DNA λh80d1acp^{S} was made apurinic (AP) by treatment with acid (pH 4.0) and low heat (37^{o}). AP DNA was exposed to fetal tissue preparations of either enriched nuclear fraction or S100 supernatants, in which APE was estimated. APE was detected only in nuclear fractions, and was inversely correlated with susceptibility to carcinogenesis by direct acting alkylating agents in fetal rat liver, kidney, and brain.

Studies on the effects of chemical carcinogens on the regenerating rat liver have shown that in synchronized hepatocyte populations in regenerating livers, susceptibility to neoplastic transformation by the alkylating agent methyl-(acetoxymethyl) nitrosamine (DMN-OAc) is greatest in cells that are actively synthesizing DNA, and thus that the S phase of the cell cycle, especially its earlier portion, is a period during which there is markedly enhanced intrinsic cellular vulnerability to neoplastic transformation. DMN-OAc, an ester of the presumed reactive metabolite of dimethylnitrosamine, which is dependent on esterase for its activation rather than any oxidative enzyme system, resulted in a high incidence of hepatocellular tumors, nodules, and foci, and caused practically no tumors in other organ systems with the single exception of the thyroid when injected into partially hepatectomized rats via the portal vein. Our experiments indicate that this is the ideal agent to use for cell cycle

studies since levels of esterases do not change over the period of hepatectomy and regeneration in liver and labeling of DNA by DMN-OAc-C^{14} is essentially constant, irrespective of the stage of the cell cycle in regenerating liver when the labeled carcinogen was given. Data from this study, previously reported as in progress, are being prepared for publication. An additional finding of great interest is apparent enhancement, by a promoting mechanism, of carcinogenesis in the thyroid by phenobarbital (cf. project Z01CP05303-02 LCC), confirming findings recently reported in Japan of this second tissue in which the barbiturate is capable of systemic tumor promotion in rodents.

A new study in collaboration with Dr. J. Swenberg, CIIT, Research Triangle Park, N.C., to study the interorgan and intercellular distribution and inducibility of AAP in rodents and non-human primates has been initiated. This modality of DNA repair is present at significantly higher levels in patas monkeys (cf. project Z01CP05092 LCC) than in corresponding tissues of rats. This representative non-human primate species closely resembles man with regard to AAP levels in those tissues that have been investigated in both species.

Significance to Biomedical Research and the Program of the Institute:

Organ specificity in carcinogenesis by different classes of agents is of obvious importance to the problem of extrapolating between species and in understanding the spectrum of tumors ascribed to environmental causes in human beings. Most studies in this area have concentrated on the capacity of target cells to metabolize carcinogens to ultimate reactive forms. Other possible mechanisms have received little attention in comparison and deserve exploration. The biochemical basis of the well-established high fetal susceptibility to chemical carcinogens remains poorly understood. The fetus is clearly at greatly elevated risk from exposure to chemical carcinogens, a fact that must be considered in estimations of relative human risk from environmental exposure to such agents. The factors that contribute to this enhanced susceptibility remain to be adequately evaluated. The role of DNA repair processes, best shown by enhanced susceptibility to UV carcinogenesis in individuals deficient in such repair as a result of the hereditary condition xeroderma pigmentosum, indicates that repair is significant in controlling the consequences of damage to cells inflicted by at least some carcinogenic agents, but the development of this capacity during prenatal life has been studied very little. It could easily contribute, in part, to the susceptibility of fetal tissues which may be deficient in activity or fidelity of DNA repair.

It has long been known that dividing cells are more intrinsically susceptible to chemical carcinogens than post-mitotic cells or cells that are not cycling. The exact reasons for this are not clear; a reasonable hypothesis is that DNA may be more vulnerable to damage in certain phases of the cell cycle, and that in cycling cells, there is a greater probability that DNA repair will not be completed before programmed DNA synthesis encounters a defective portion of the genome bearing a carcinogen-induced lesion. Whether, in fact, cells are especially vulnerable to carcinogens at a particular stage of the cell cycle is thus of importance not only for perinatal carcinogenesis, but for carcinogenesis in adult tissues where mitotic activity is high, including various lining epithelia such as intestinal mucosa which constitute major sites of important neoplasms of man.

Proposed Course:

The prenatal development of capacity for excision repair and for alkyl acceptor protein-mediated repair of DNA in liver, brain, and other tissues will continue to be studied in order to allow meaningful comparison of species of experimental animals that differ greatly in prenatal susceptibility to carcinogens in various organ systems.

Studies on the role of the cell cycle in susceptibility to carcinogenesis, and possibly other forms of genotoxic damage including mutation, will be continued. Techniques developed in the course of these studies, including characterization of the biology and morphology of liver cell tumors in rats resulting from a single transient exposure to an alkylating agent, will be applied elsewhere in the program of this Section. We plan to attempt to resolve apparent discrepancies between capacities for DNA repair in fetal liver and brain in rats and mice and the distinct differences in susceptibility to oncogenesis in these species.

Publications

Blasberg, R. G., Kobayashi, T., Patlak, C. S., Shinohara, M., Miyoaka, M., Rice, J. M. and Shapiro, W. R.: Regional blood flow, capillary permeability, and glucose utilization in 2 brain tumor models - preliminary observations and pharmacokinetic implications. Cancer Treat. Rep. 65: 3-12, 1981.

Chen, B. P., Berman, J. J., Ching, W.-M. and Rice, J. M.: DNA breakage by methyl methane sulfonate and its repair in brain and liver cells cultured from fetal rat and mouse. Chem. Biol. Interact. 44: 63-77, 1983.

Rice, J. M. and Frith, C. H.: The nature of organ specificity in chemical carcinogenesis. In Langenbach, R., Nesnow, S. and Rice, J. M. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Publishing Corp., 1983, pp. 1-21.

Rice, J. M. and Perantoni, A.: Organ specificity and interspecies differences in carcinogenesis by metabolism-independent alkylating agents. In Langenbach, R., Nesnow, S. and Rice, J. M. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Publishing Corp., 1983, pp. 77-105.

Rice, J. M. and Ward, J. M.: Age dependence of susceptibility to carcinogenesis in the nervous system. Ann. NY Acad. Sci. 381: 274-289, 1982.

Ward, J. M. and Rice, J. M.: Naturally occurring and chemically induced brain tumors of rats and mice in carcinogenesis bioassays. Ann. NY Acad. Sci. 381: 304-319, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05299-02 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Interspecies Differences in Transplacental Carcinogenesis and Tumor Promotion

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
B. Diwan Expert, LCC, NCI

COOPERATING UNITS *(if any)*
Microbiological Associates, Inc., Bethesda, MD

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.6	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The phenomenon of tumor promotion is systematically explored in various rodent species in conjunction with transplacental carcinogenesis. Tumor-promoting activities of various barbiturates and other pharmacologically defined classes of tumor promoting substances are determined by sequential administration to animals of a transient, low level exposure to a genotoxic carcinogen followed by the promoting agent under study. The appearance of preneoplastic and neoplastic lesions is quantitated in animal tissues; by comparison of the effects of different agents in different target tissues, structure-promoting activity relationships are established. Organ specificities and interspecies correlations in tumor promotion are investigated for clues to mechanism(s) of action of tumor promoters. Premalignant JB-6 mouse epidermal cells are used to investigate mechanisms of tumor promotion by various drugs and environmental pollutants.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Jerry M. Rice	Laboratory Chief	LCC, NCI
Amos E. Palmer	Research Veterinarian	LCC, NCI
Jerrold M. Ward	Chief, TPPS	LCC, NCI

Objectives:

1) To determine the structure-promoting activity relationships of barbiturates and other classes of tumor-promoting compounds; 2) to characterize and define the limits of their organ- and species-specificities; 3) to utilize these agents to demonstrate the occurrence of potentially neoplastic cells (initiated cells) in organs and tissues which appear refractory to tumor development by transient transplacental or systemic exposure to directly acting or pulse carcinogens alone; and 4) to investigate mechanisms of promoting action of these agents employing promotable JB-6 cells.

Methods Employed:

Precisely timed-pregnant mice, rats, and Syrian hamsters are exposed to chemical carcinogens at defined periods during gestation. Offspring derived from these mothers and young animals exposed to carcinogens by conventional routes are subsequently exposed to non-genotoxic agents known or suspected to promote tumorigenesis in one or more organs. Preneoplastic proliferative lesions and neoplasms resulting from such treatment are identified and classified by histological, histochemical and ultrastructural parameters. Premalignant JB-6 mouse epidermal cells are exposed to suspected tumor promoters and the progression of these cells to tumor cell phenotype is measured by colony formation in soft agar at 14 days.

Major Findings:

The results of one year of study on the promoting abilities of different barbiturates in rat liver indicate that both phenobarbital and barbital are strong tumor promoters; amobarbital is a weak promoter while barbituric acid, which lacks substitution at the 5 position of the pyrimidine ring, does not promote liver carcinogenesis. Basophilic proliferative lesions in mice appeared earlier in animals exposed to DEN followed by amobarbital than in those exposed to DEN alone; however, the kinetics of tumor occurrence were similar for both groups.

Preliminary results from studies in mice suggest that phenobarbital markedly inhibits the development of both preneoplastic and neoplastic liver lesions induced by diethylnitrosamine (DEN) treatment to neonatal animals. In these studies, phenobarbital administration was begun before juvenile mice were weaned. The hypothesis that the inhibitory effect on hepatocarcinogenesis is a consequence of feminization, caused by phenobarbital-induced disturbances of steroid hormone metabolism, is being systematically tested.

A preliminary experiment to explore possible 2-stage tumor promotion in the mouse liver was based on the reported failure of amobarbital to promote hepatocarcinogenesis in the rat, suggesting that it might function as an incomplete or second-stage promoter. Amobarbital was hypothesized to compare with phenobarbital in its capacity to promote liver cell tumors as mezerein compares with tetradecanoyl phorbol-13-acetate in capacity to promote skin tumors in mice. No evidence has been found to support this hypothesis, and demonstrated multiple stages of promotion remains exclusively a property of mouse skin.

The cell culture system of Colburn, in which the mouse epidermal-derived JB-6 epithelial cell line can be promoted to anchorage independence by incorporation of phorbol esters into the soft agar medium, has been used to study the mechanism of action of the tumor-promoting plasticizer di-2-ethylhexyl phthalate (DEHP) and related compounds that are promoters of hepatocellular carcinogenesis in the mouse (see project Z01CP05303-02 LCC), but are not complete promoters for mouse skin. JB-6 cells do not respond to other classes of hepatic tumor promoters such as barbiturates. The system provides a means of exploring possible mechanisms of action of DEHP that postulate competition for cell membrane receptors for regulatory small molecules, and for investigating the mechanisms of cellular specificity in tumor promotion.

Significance to Biochemical Research and the Program of the Institute:

The demonstration of tumor-promoting activity of therapeutic drugs at therapeutic dosage levels is of obvious significance to public health if such promoting action is not limited to rodent species only. Rigorous analyses of structure-promoting activity relationships of various barbiturates and determination of their organ specificity may provide a clue as to their mechanism of action as tumor promoters. If organ-specificity and dosage requirements of these drugs prove to be consistent in many non-human species, it may be possible to predict effects of such agents in man.

Proposed Course:

Systematic analyses of structure-promoting activity relationships of different barbiturates should be extended to at least three rodent species --- mouse, rat and Syrian hamster. Organ specificity in tumor promotion of each barbiturate should be established and dose/effect relationship for each tissue in each species should be determined. If organ-specific activity of barbiturates varies with the structure and if it is consistent in all rodent species, these studies should be extended to non-human primates. Studies employing the promotable JB-6 cell model should be extended in the investigations of mechanisms of tumor promotion by suspected drugs and environmental pollutants. These studies will be carried out in consultation and collaboration with other Sections in LCC working on similar projects in tumor promotion: Office of the Chief (Z01CP05092-05 LCC); Tumor Pathology and Pathogenesis Section (Z01CP05303-02 LCC); and Ultrastructural Studies Section (Z01CP04812-15 LCC).

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05301-02 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biology of Natural and Experimentally Induced Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
J. M. Ward Chief, TPPS, LCC, NCI

COOPERATING UNITS *(if any)*
Biological Response Modifiers Program, Division of Cancer Treatment, NCI

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Natural tumors in rats are characteristic for each strain, while induced tumors may be different biologically and morphologically from natural tumors. Natural tumors from approximately 200 F344 rats were transplanted into the mammary fat pad of weanling F344 rats. The latent period, growth rate, invasiveness and metastatic rate of the transplanted tumor were directly related to the histologic appearance of the tumor and tumor cell type. Malignant and benign tumors were readily transplanted although only the former were invasive or metastatic in syngeneic hosts. Characteristics of growth on transplantation can indicate the degree of malignancy of a primary tumor, but cannot establish that it is biologically benign. Alpha fetoprotein (AFP) was found in induced preneoplastic and benign liver lesions of the mouse as well as in liver cancer using the avidin-biotin peroxidase complex (ABC) immunocytochemical technique. AFP was found only in liver carcinomas of rats. AFP is usually a marker for malignancy of liver tumors in animals and humans. The explanation of this finding in mice is under investigation. Large granular lymphocyte (LGL) leukemias of F344 rats were transplanted and characterized by morphologic, immunocytochemical and functional studies. Tumors of different degrees of differentiation were characterized for the first time. Our studies are the first reports of LGL leukemias in any species. Normal large granular lymphocytes were localized in tissues of nude rats by the use of the ABC immunocytochemical technique. LGL were found in lymphoid tissues, epithelium, tumors and inflammatory lesions. Phenobarbital was found to induce eosinophilic hepatocellular foci of hyperplasia and neoplasms in rat liver which were positive for gamma-glutamyl transpeptidase (GGT) while naturally occurring foci and tumors were usually basophilic and GGT negative. Methapyrilene, a nongenotoxic carcinogen, induced hepatocellular hyperplastic foci, adenomas and carcinomas of several types in rats. Carcinomas were readily transplanted and produced alpha-fetoprotein. Thus, methapyrilene induced liver tumors similar to those induced by genotoxic carcinogens.

PROJECT DESCRIPTION

Names, Titles, and Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Masato Ohshima	Visiting Scientist	LCC, NCI
Craig W. Reynolds	Staff Fellow	BIB, NCI

Objectives:

To characterize the biology and pathology of natural and experimentally induced tumors of rats; to identify differences and significance of differences between natural and induced tumors.

Methods Employed:

Induced and natural tumors of rats are routinely characterized by histologic methods and by immunological, immunocytochemical and ultrastructural methods. Selected lesions are transplanted to correlate biology with histological features. Large granular lymphocyte (LGL) leukemia of aging F344 rats is the major natural cause of death of F344 rats. Ten rats with natural LGL leukemia and ten rats with transplantable LGL leukemia were studied. Tumor cells were isolated from leukemic spleen by Percoll gradients. Cells were assayed for NK activity using a ^{51}Cr-release cytotoxicity assay against mouse lymphoma YAC-1 targets. Cell surface antigens on leukemic cells were analyzed with monoclonal antibodies BC-84, M1/70, W3/13, W3/25, OX-1, OX-7, OX-8, Ia and SIg by continuous flow microfluorometry. Tumors were transplanted to weanling recipients by injection of cell suspensions or implantation of tumor fragments into the inguinal mammary fat pad. Transplantable LGL leukemia lines were also characterized by tumor cell dose and tumor latency and pathology. The avidin-biotin-peroxidase complex (ABC) immunocytochemical technique was used to localize cell surface antigens in tumor cells and normal tissues in fixed tissue sections of euthymic and athymic nude rats. Aging F344 rats were exposed to 500 ppm of phenobarbital (PB) in the water and the effect on life span and pathology determined. Methapyrilene was fed at 1000 ppm in the diet to F344 rats and groups of rats were sacrificed after 5, 10, 15, 30, 40 and 73 weeks of feeding. Liver lesions were quantitated using image analysis techniques and Zeiss stereology software.

Major Findings:

Pathologic diagnosis of benign and malignant tumors by histology alone is subjective in nature and is based on one's experience. Some investigators believe that tumor transplantation is prima facie evidence of malignancy and that some nodular lesions in tissues are not neoplastic, but rather hyperplastic. We transplanted 450 naturally occurring tumors of various types and sizes from 200 F344 rats into the inguinal mammary fat pad of F344 rats and observed the tumors for latent period, growth rate, invasion and metastases. Transplantation of naturally occurring tumors of the F344 rat showed that benign and malignant tumors were readily transplantable but tumor latency and invasion were related to tumor malignancy. Some malignant tumors metastasized in transplanted hosts, but many did not. Many benign tumors were transplanted for

several generations but none were invasive or metastasized in tumor recipients. Selected chemically-induced tumors of the liver, induced by the nongenotoxic carcinogen, methapyrilene were transplanted into the mammary fat pad of F344 rats. Hepatocellular carcinomas grew when transplanted while adenomas and non-neoplastic but proliferative lesions of bile ducts did not. From the results of our experiments and a review of the literature, one may conclude that chemically-induced benign tumors may be more difficult to transplant than naturally-occurring benign tumors. It may be that induced benign tumors depend, in part, on the presence of the carcinogen for progression to carcinoma. These experiments provides support to the concept that tumor transplantation, latency, and morphology are related to degree of malignancy but not to whether or not the tumor is malignant.

Alpha fetoprotein was identified by the avidin-biotin-peroxidase complex (ABC) immunocytochemical procedure only in induced liver carcinomas of rats but in induced and spontaneous hyperplastic foci, adenomas and carcinomas of mice. It was previously believed that AFP was a marker for malignancy in liver tumors; our finding of AFP in preneoplastic and benign liver lesions of mice suggests that the phenotypic expression of AFP in mice, at least, is characteristic of neoplasia rather than malignancy.

The large granular lymphocyte leukemia, the most common cause of death of aged F344 rats, was identified and characterized by immunologic procedures in vitro, and by pathology and functional studies. The LGL leukemia and normal cell counterpart offer a model for the study of the natural killer cell in tumor immunity. Transplantable LGL tumors were characterized as to NK activity. Transplantable tumors were heterogeneous in nature and represented a spectrum of tumors from those with morphologic and immunologic characteristics identical to normal LGL-NK cells to poorly differentiated tumors of possible stem cell origin. We now have several lines of transplantable LGL leukemias, with high, intermediate or low NK activity. The cell surface antigen, OX-8 was localized to the cell surface of LGL tumor cells in tissue sections by the ABC immunocyto-chemical technique. LGL leukemia, although a common spontaneous leukemia of F344 rats, may be induced by a few chemicals including ethylene oxide and some drugs. The identification of the type of leukemia 'induced' by chemicals should aid in the differentiation of induced versus potentially promoted leuke-mias. The OX-8 marker for the large granular lymphocyte (LGL) in nude rats was found on lymphocytes in various lymphoid tissues, especially in bronchial-associated lymphoid tissue and the paracortex of lymph nodes in association with interdigitating cell hyperplasia. The majority of intraepithelial lympho-cytes of the intestine were OX-8 positive. The stroma of tumors induced by NMU or acetoxy-DMN contained lymphocytes which were LGL. Inflammatory lesions, including those of papovaviral parotid sialoadenitis in nude rats, frequently had many LGLs. The papovavirus appears to be a new virus of rats and is cur-rently being characterized by other NCI scientists. Transplantable tumors in nude rats were also found to be infiltrated by OX-8 positive lymphocytes.

In F344 rats, phenobarbital (PB) was shown to induce hepatocellular hyperplastic foci and tumors which were composed of eosinophilic hepatocytes with cell membranes positive for gamma glutamyl transpeptidase. PB also may have increased

prior to basophilic foci and adenomas. Carcinomas arose from both types of adenomas and in situ and were readily transplanted. They produced alpha-fetoprotein. Mucous cholangiofibrosis progressed to a cystic lesion and then to neoplasia in some rats. Thus methapyrilene a so-called 'nongenotoxic' carcinogen, induced preneoplastic liver lesions and tumors similar to those induced by so-called 'genotoxic' carcinogens.

Significance to Biomedical Research and the Program of the Institute:

Rodent tumors are used as endpoints in safety assessment studies for drugs, chemicals, etc., and by investigators in cancer research. Knowledge of the nature and characterization of both natural and induced tumors is necessary for careful and accurate decisions regarding safety and risk assessments and conclusions in animal experiments by investigators. Further characterization of these tumors should aid in accurate assessment of carcinogenesis experiments.

Proposed Course:

Continuation of tumor transplantation studies and characterization of induced tumors relevant to investigations on mechanisms of carcinogenesis, in rodent and non-rodent species. Nude rats will be used to investigate the role of natural killer cells in chemical carcinogenesis.

Publications

Reynolds, C. W., Ward, J. M., Denn, A. C.III and Bere, E. W.: Identification and characterization of large granular lymphocyte (LGL) leukemias in F344 rats. In Herberman, R. D. (Ed.): NK Cells and Other Natural Effector Cells. New York, Academic Press, 1982, pp. 1161-1164.

Ward, J. M.: Increased susceptibility of aged F344/NCr rat liver to the effects of phenobarbital on the incidence, morphology and histochemistry of hepatocellular foci and neoplasms. JNCI. (In Press)

Ward, J. M., Argilan, F. and Reynolds, C. W.: Immunoperoxidase localization of large granular lymphocytes in normal tissues and lesions of athymic nude rats. J. Immunol. (In Press)

Ward, J. M. and Reynolds, C. W.: Large granular lymphocyte leukemia, a heterogeneous lymphocytic leukemia in F344 rats. Am. J. Pathol. 111: 1-10, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05303-02 LCC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pathogenesis and Promotion of Natural and Induced Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. M. Ward Chief, TPPS, LCC, NCI

COOPERATING UNITS *(if any)*

Pathology and Histotechnology Laboratory, Program Resources Incorporated, FCRF; Microbiological Associates, Inc., Bethesda, MD

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Iodine deficiency was shown to be a potent promoter for thyroid tumors induced in rats by nitrosomethylurea (NMU). The deficiency caused goiter and hyperplasia of pituitary thyrotrophs which led to thyroid adenomas by 20 weeks after NMU injection and carcinomas by 34 weeks in all exposed rats. Other types of NMU-induced tumors may have not been promoted. Thy 1.1 was found to be a marker for normal thyroid follicular epithelium while thyroid hyperplasias and tumors were found to be deficient in Thy 1.1.

Di(2-ethylhexyl)phthalate (DEHP), a commonly used plasticiser and phenobarbital (PB) were demonstrated to promote liver tumors induced by diethylnitrosamine. DEHP did not have liver tumor initiating activity, however, or promote or initiate skin tumors in mice, but did promote the JB-6 epidermal mouse cell lines to anchorage independence. The DEHP promoted tumors were more malignant than those promoted by PB.

Formaldehyde was shown to be a weak promoter and initiator in a mouse skin painting study, and may have promoted papillomas to carcinomas, but only after 1 year of experimentation. Sencar mice which are more sensitive to skin tumor promotion were shown to be less sensitive to promotion and initiation in other organs as compared with Balb/c mice.

Liver tumors induced in monkeys by diethylnitrosamine or other carcinogens were shown to progress through similar morphologic stages as in rodents. Gamma-glutamyl transpeptidase, however, is normally present in monkey hepatocytes and was usually deficient in hepatocytes in preneoplastic lesions and liver tumors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Amos Palmer	Research Veterinarian	LCC, NCI
Masato Ohshima	Visiting Scientist	LCC, NCI
Bhalchandra Diwan	Expert	LCC, NCI
Jerry M. Rice	Laboratory Chief	LCC, NCI
Nancy Colburn	Expert	LVC, NCI
Henry Hennings	Senior Chemist	LCCTP, NCI

Objectives:

To characterize the sequential morphologic and biologic steps in the development of cancer, and to systematically study cellular and organ specificity and interspecies differences in response to tumor promoters.

Methods Employed:

The role of initiation and/or promotion in the development of neoplasia is under study using select model systems of mouse and monkey liver, mouse skin and rat thyroid. The mouse liver system was developed in a timed sequence - quantitative study using an automated image analysis computer system, the Zeiss Videoplan. Di(2-ethylhexyl)phthalate and phenobarbital were given continuously in the diet for up to 6 months after a single injection of diethylnitrosamine to weanling male B6C3F1 mice. Mice were sacrificed at 2, 4 and 6 months after DEN injection, and the number and area-perimeter of focal hepatocellular proliferative lesions were measured. Using stereology software, the number of lesions per unit volume and volume of lesions were determined. The role of DEHP as an initiator or promoter in mouse skin and JB-6 epidermal cells was also studied.

The role of dietary iodine deficiency and cell proliferation in tumor promotion was studied. Iodine deficiency leads to goiter (diffuse thyroid hyperplasia). Male F344 rats received a single injection of nitrosomethylurea, followed by dietary iodine deficiency. Control groups received the same diet plus added iodine. Groups of rats were sacrificed 20 or 34 weeks after NMU injection and the thyroid weight and incidence and type of thyroid lesions were quantitated. The pituitary gland was also examined, and stained immunocytochemically for thyroid stimulating hormone. Thyroid lesions were transplanted to characterize their biologic behavior. Immunocytochemical localization of Thy 1.1 antigen was determined, using the avidin-biotin-peroxidase complex (ABC) technique.

Formaldehyde was studied in female Sencar mice to characterize its role in skin tumor promotion and initiation. Groups of 30 mice were given DMBA followed by formaldehyde or followed by TPA. Appropriate controls were DMBA, formaldehyde, TPA, or acetone. The numbers of papillomas, carcinomas and other skin lesions were recorded weekly for up to 78 weeks. In another experiment, Sencar mice were compared with BALB/c mice for skin tumor initiation by one intraperitoneal injection of 7,12-dimethylbenz[a]anthrene (DMBA) or urethane and promotion by

continuous skin application of 12-O-tetradecanolyphorbol-13-acetate (TPA), for 52 weeks. Skin papillomas and carcinomas were counted weekly and all mice were necropsied at 52 weeks. The incidence and types of internal tumors were evaluated histologically.

Continuing studies of the effects of several carcinogens (diethylnitrosamine, ethylnitrosourea) on patas monkeys allows us to study the development of liver lesions. Liver lesions were fixed in cold ethanol for determination of gamma-glutamyl transpeptidase (GGT) enzyme activity. Fixed lesions were also stained for glycogen.

Major Findings:

The mouse liver was shown to provide a quick and efficient initiation-promotion system using diethylnitrosamine as the initiator and phenobarbital (PB) or di(2-ethylhexyl)phthalate (DEHP) as the promotor. Mice sacrificed at 2, 4 and 6 months showed dose and time-related incidences and sizes of hepatocellular proliferative lesions. DEHP promoted basophilic foci and tumors which appeared more malignant than the eosinophilic foci promoted by PB. Alpha fetoprotein (AFP) was found in hepatocytes of foci, adenomas and carcinomas, in contrast to the usual localization of AFP only in liver carcinomas of rats and humans. DEHP did not promote or initiate skin tumors in mice, but did promote the JB-6 epidermal cell lines.

Iodine deficiency was a potent promoter of thyroid adenomas at 20 weeks and carcinomas at 34 weeks after nitrosomethylurea injection. The tumors were deficient in Thy 1.1 antigen while the normal follicular epithelium contained abundant antigen. Other tumors induced by NMU appeared not to be promoted by the iodine-deficient diet, but find analysis is not yet complete.

Formaldehyde was not found to have significant initiating or promoting activity in mouse skin by 52 weeks. By 78 weeks, however, weak initiating and promoting activity was seen. There was an increased rate of conversion of papillomas to carcinomas in formaldehyde promoted mice. The late occurrence of these findings will be evaluated when the histopathology is completed.

Sencar mice developed more skin carcinomas and papillomas than did Balb/c mice when exposed to urethane or DMBA and TPA. The incidence and type of induced or promoted internal tumors were different, however, for each strain. The final tumor incidences will be reported after complete histopathologic evaluation is completed.

Liver lesions induced by carcinogens in monkeys were found to develop through a sequence of morphologic and phenotopic events not unlike those seen in rodents. Focal proliferative lesions are frequently enzyme deficient (GGT) and positive for glycogen. While normal monkey hepatocytes contain low levels of GGT, the foci were deficiency in GGT, a finding in contrast to that in rats where GGT positive foci are found among normal hepatocytes which are deficient in GGT. Although limited numbers of liver carcinomas which have been observed have not allowed us to follow the sequence from foci to carcinomas, continuing studies should allow us to do so (cf. project Z01CP0592).

Significance to Biomedical Research and the Program of the Institute:

The exact mechanism of cell transformation is not known. For each chemical carcinogen, a sequence of pathological steps must be characterized, which may or may not be organ or tissue specific. This sequence could give clues to mechanisms of carcinogenesis. Human cancer risk may depend, in part, on the mechanism of tumor formation, organ specificity of carcinogens and promoters and the events that occur after the initial carcinogen-cell interaction. Prevention of cancer may well depend on knowledge and understanding of these steps.

Proposed Course:

Using select model systems, especially mouse liver, rat thyroid and kidney we will characterize the sequence of biologic and pathologic steps leading to cancer progression or tumor regression. We are particularly interested in studying the role in tumor promotion and pathogenesis of epithelial hyperplasia, induced by both chemical and non-chemical stimuli.

Publications

Ward, J. M., Rice, J. M., Creasia, D., Lynch, P. and Riggs, C.: Dissimilar patterns of promotion by di(2-ethylhexyl)phthalate and phenobarbital of hepatocellular neoplasia initiated by diethylnitrosamine in B6C3F1 mice. Carcinogenesis (In Press)

CONTRACT IN SUPPORT OF PROJECT NUMBERS

Z01CP05157-04 LCC
Z01CP05299-02 LCC
Z01CP05303-02 LCC

MICROBIOLOGICAL ASSOCIATES (N01 CP 15744)

Title: Laboratory Rodent and Rabbit Facility as a Resource to the Laboratory of Comparative Carcinogenesis

Current Annual Level: $611,171 (Divided equally between LCC and LCCTP)

Man Years: 5.0

Objectives:

The purpose of this contract is to provide support services for the Laboratory of Comparative Carcinogenesis (LCC) for long-term treatment, holding, observation, and necropsy of rodents in carcinogenesis investigations emphasizing life-time tumor induction studies. The contract is specifically for conducting experiments that require species or strains of rodents not available from the FCRF Animal Production Area, since such animals cannot be introduced into the LCC animal research facilities at FCRF.

The current contract also provides comparable support at an equal level of effort to the Laboratory of Cellular Carcinogenesis and Tumor Promotion. AAALAC-accredited animal housing facilities, general supportive laboratory facilities, qualified personnel, materials and equipment not otherwise provided by the Federal Government are offered equally to both laboratories. This summary describes only work in support of LCC.

Protocols are developed in collaboration with intramural NCI investigators and approved by an NCI Project Officer. Protocols involve the preparation, handling and administration of chemical solutions to animals according to NCI guidelines for the safety of personnel; holding, treatment, and data collection (including gross pathology data) for mice, athymic nude mice, hamsters, rats, guinea pigs, and rabbits; administration of chemical carcinogens to animals by skin painting, gavage, parenteral injection, intratracheal administration or other routes; feeding of commercially pelleted or meal form diets according to protocol or experimental diets as required; storage of labile animal diets, reagents, tissues, or other materials under conditions of temperature regulation (4°C, -20°C, -70°C, or liquid nitrogen); qualitative or quantitative analyses of carcinogen preparations, tissues of carcinogen-treated animals and other biochemical investigations as required; and preservation and supply of tissue samples to be evaluated by light microscopy, electron microscopy, autoradiography, biochemistry, or histochemistry at NCI.

Major Contributions:

This contract, together with its companion contract at Litton-Bionetics, N01CP01039, made possible all research involving administration of chemical

carcinogens to laboratory animals initiated during the first year of LCC operation, when no facilities were available at FCRF. It has provided facilities to carry out studies on comparative effects of different barbiturates as tumor promoters in mice; to begin studies of sequential prenatal initiation/postnatal promotion in rats; to analyze the varying susceptibility of rat hepatocytes in vivo to initiation of carcinogenesis during different stages of the cell cycle; and to study the ability of formaldehyde to initiate or promote tumorigenesis in mouse skin. Most uniquely, it made possible a study on the effects of retinoids on naturally occurring prostatic carcinoma in aging ACI rats, which were available only from a single non-NIH source. It continues to serve the crucial function of providing a means of conducting carcinogenesis research in uniquely suitable but non-SPF rodents, currently including congenic strains of mice varying in expression of murine retroviruses (in collaboration with Dr. W. P. Rowe, NIAID), and Mongolian gerbils, that vary strikingly from other rodents in their responses to chemical carcinogens.

Proposed Course:

To continue to provide a comprehensive carcinogenesis research facility for studies requiring long-term holding of rodent species and strains that cannot be accomodated at FCRF because they are not free of all detectable potential pathogens. This contract terminates on December 29, 1983, and is now being recompeted and divided into two separate resource contracts, one for LCC and one for LCCTP.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05350-01 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Thymic Microenvironment During T-Cell Lymphoma Development

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
U. I. Heine Chief, USS, LCC, NCI

COOPERATING UNITS *(if any)*
Pathology Institute, University of Cologne, Cologne, Germany

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Maturation and proliferation of prethymic T-progenitor cells to mature lymphocytes depend on regulatory mechanisms in the thymus where the T-progenitors must interact with non-lymphoid, epithelial cells to be able to differentiate. The thymic epithelial cells provide a specific microenvironment capable of directing proliferation and maturation. It has been shown previously in mice that during the early phase of M-MuLV induced lymphomagenesis prethymic stem cells of the T-cell lineage derived from blood-forming tissues accumulate in the thymus where they encounter a differentiation block; subsequently, uncontrolled proliferation of the stem cells will lead to generalized lymphoma. The aim of this study is to elucidate the mechanism of the intrathymic differentiation block of prethymic lymphoid stem cells that gives rise to systemic malignant lymphoma of the Thy$^-$ cell type. In vivo experiments, using the Moloney virus-induced lymphoma in the BALB/c mouse as a model, have been performed and the phenotype and distribution of the major thymic cell populations have been characterized at different stages of tumorigenesis by light and electron microscopy. Immunofluorescence studies for the presence of thymopoietin II and serum thymus factor were carried out to determine the functional state of the epithelial cells. The results of our experiments show that the reticular epithelial cells of the thymus, which provide a microenvironment necessary for the differentiation of prethymic stem cells to lymphocytes of the T-lineage, are a prime target for transformation as these cells undergo phenotypic changes and are rendered functionally defective prior to lymphoma development. It is suggested that incompetent epithelial cells cause the progressive accumulation of non-differentiating T-cell precursors producing a dysregulative lymphoma.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

James L. Junker Staff Fellow LCC, NCI

Objectives:

Our principal aim is to identify the role of the thymic microenvironment in T-cell lymphoma development. Special emphasis is directed towards the study of the thymic epithelial cells, as the latter may represent a critical target cell that by dysfunction initiates leukemogenesis.

Methods Employed:

Malignant lymphomas were induced by infecting newborn BALB/c HAN mice with 0.2 ml Moloney murine leukemia virus suspension, 6.8 log FFU/ml. Infectivity studies were carried out at the University of Cologne, whereas the cell biology studies were pursued at the Laboratory of Comparative Carcinogenesis in Frederick MD. Thymic tissue, obtained from control and infected animals at biweekly intervals, was subjected to histological and cytological examinations using standard procedures for light, transmission and scanning electron microscopy. The quantitation of the cell population was performed on semithin sections in weekly intervals until tumor development. The functional state of the thymic epithelial cells was tested by identifying thymic hormone production by immunofluorescence using antisera against thymopoietin II and serum thymus factor.

Major Findings:

Current evidence shows that the thymic reticular epithelial cells have a major role in the maturation of prethymic stem cells of the T-cell lineage to functionally mature T-cells. Characterization of the reticular epithelial cells in the thymic cortex revealed in control and infected animals the presence of two differentiation stages of these cells classifiable as an immature type, resembling the epithelial cell at the periphery of the thymus primordium, and a mature cell type. Quantitation of the different cells during lymphoma development indicates a shift in the cell population as the number of mature reticular epithelial cells declines early during leukemogenesis, coinciding with a progressive degeneration of these cells. These events coincide with elevation in the number of immature epithelial cells and the prevalence of a new epithelial cell type of hyperchromatic appearance. Concomitantly, immunofluorescence studies for thymopoietin II and serum thymus factor show a marked decrease of these hormones only in epithelial cells of infected mice. The accumulation of lymphoblasts in the thymic cortex of infected animals, denoting lymphoma development, was found to be secondary to the shift in the population of the reticular epithelial cells.

Our data, concerning the chronological sequence of increase in as well as depletion of the different cell types in the thymus during leukemogenesis, which coincides with the appearance of deficiencies in the hormonal competence of the reticular epithelial cells, suggest that a prime target for viral infection is the thymic reticular epithelial cell.

Significance to Biomedical Research and the Program of the Institute:

One of the missions of the National Cancer Institute is the elucidation of the mechanisms leading to tumor production and, consequently, the establishment of protocols for treatment. Research of animal models of human lymphomas provides insight into such mechanisms. The aim of this study is to investigate changes in the thymic microenvironment that may be causative for the development of T-cell lymphomas. Results of this study may serve as a basis for further investigations pertaining to mechanisms of cell-cell interactions during tumor promotion in general, which is a research area of major importance in this Laboratory.

Proposed Course:

The work will be continued as a study in comparative carcinogenesis to evaluate the thymic microenvironment in mice expressing varying capability for the induction of lymphoma. We also plan to culture thymic epithelium and raise monospecific antibodies against a variety of epithelial cell proteins that may serve as markers in the characterization of functionally defective thymic epithelium. The availability of such markers will be of importance for the general research community.

Publications

Heine, U. I., Krueger, G. R. F., Karpinski, A., Munoz, E. and Krueger, M. B.: Quantitative light and electron microscopic changes of thymic reticular epithelial cells during Moloney virus induced lymphoma development. J. Cancer Res. Clin. Oncol. (In Press)

Krueger, G. R. F., Karpinski, A., Heine, U. I. and Koch, B.: Differentiation block of prethymic lymphocytes during Moloney virus induced lymphoma development secondary to a thymic epithelial defect. J. Cancer Res. Clin. Oncol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05351-01 LCC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ultrastructural Characterization of Normal and Neoplastic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. L. Junker Staff Fellow, LCC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Ultrastructural Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies involve collaboration in the Laboratory on specific problems in carcinogenesis including the characterization of neoplastic cells and the study of tumor histogenesis. The aim of the present investigation is to define by high resolution and immunoelectronmicroscopy changes in natural killer cells and targeted tumor cells which correlate with natural killer cell activity. Large granular lymphocytes (LGL) have been identified with natural killer cell activity. OX-8, a monoclonal antibody which reacts with a cell surface antigen of LGLs and cytotoxic/suppressor T lymphocytes, has been used in immunoelectron microscopy of lymph nodes from nude rats which lack the T cells. A postembedding staining procedure was used which gave moderate preservation of lymphoid cell structure, but poor preservation of nonlymphoid cells. Cells which stained positively with the antibody displayed characteristics consistent with their being LGLs (kidney shaped nucleus, cytoplasmic granules). Further investigations of the interactions between LGLs and tumor cells in vivo and in vitro will follow establishment of a protocol which gives good ultrastructural preservation and specific antibody staining.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Ursula I. Heine	Chief, USS	LCC, NCI
Jerrold M. Ward	Chief, TPPS	LCC, NCI

Objectives:

To identify at the ultrastructural level those cells which react with OX-8 antibody, a monoclonal antibody which is specific for a cell surface antigen on natural killer cells and cytotoxic/suppressor T lymphocytes. To use this antibody as a marker with which to study natural killer cell activity in nude rats.

Methods Employed:

Immunoelectron microscopy is carried out on lymph nodes from nude rats which are fixed in glutaraldehyde and embedded in Epon-Araldite. Immunoperoxidase antibody staining is performed on 4 μm thick sections after removal of the plastic, using the ABC (avidin-biotin-peroxidase complex) system. After re-embedding the material, ultrathin sections are cut and examined by transmission electron microscopy.

Major Findings:

Specific membrane staining of a subpopulation of lymphocytes has been achieved. Morphology of these cells, showing kidney shaped nuclei and cytoplasmic granules, is consistent with the previous identification of these cells as large granular lymphocytes.

Significance to Biomedical Research and the Program of the Institute:

Development of a marker system for identifying natural killer cells in intact tissue at the ultrastructural level would be useful to the study of interactions between natural killer cells and tumor cells, and would, thereby, help in the understanding of natural defense mechanisms against cancer.

Proposed Course:

Although we have been successful in observing cell surface membrane staining on LGLs in the electron microscope, ultrastructural preservation of the surrounding tissue is not yet satisfactory. Alternative tissue preparation techniques will be investigated and tested. Following establishment of a successful protocol, interactions between natural killer and tumor cells will be examined ultrastructurally to further understanding of the mechanisms by which natural killer cells accomplish their cytocidal activity.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05352-01 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Metabolic and Pharmacological Determinants in Perinatal Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
L. M. Anderson Expert, LCC, NCI

COOPERATING UNITS *(If any)*
Lawrence Hospital, Bronxville, N.Y.; Sloan-Kettering Institute, Rye, N.Y.

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The metabolism and pharmacokinetics of carcinogens in pregnant, fetal, and infant mice are correlated with tumor incidence. Suckling Swiss mice were treated with dimethylnitrosamine (DMN). Liver and lung tumors resulted. Pretreatment of the mothers of these mice with Aroclor 1254, a mixture of polychlorinated biphenyls (PCBs), resulted in transmammary exposure of the sucklings and had several interesting effects on DMN-caused tumorigenesis. The PCBs provided significant protection against initiation of liver and lung tumors. Induction in the sucklings of an enzyme effecting detoxification of DMN was demonstrated and was postulated to be the mechanism of the observed protection. A second effect of the PCBs was a significant increase in the percent mice with livers overgrown by tumor, a phenomenon which was thought to be due to liver tumor promotion by the PCBs. Thus the interaction of maternally-derived PCBs with DMN tumorigenesis was complex, giving protection against tumor initiation but also stimulating tumor growth. The effect of vehicle and diet on rate of absorption of ^{14}C-DMN from the stomachs of adult and suckling mice has been investigated, since this rate determines both length of time of exposure of the gastric mucosa and pharmacokinetics of carcinogen contact with distal target organs. Ethanol or oil retarded DMN loss from the stomachs of both adults and sucklings, and the nature of maternal diet may have influenced rate of gastric DMN departure in fed sucklings. A study of effect of diet and vehicle on oral DMN tumorigenesis in infant mice is planned. In an investigation in progress, the metabolism and pharmacokinetics of methylcholanthrene (MC) in maternal, fetal, and placental tissues are measured and correlated with incidence of tumors induced transplacentally by MC. Genetic backcrosses of C57BL/6 mice, which are highly inducible for the enzyme metabolizing MC, and DBA/2 mice which are noninducible, are used as the pharmacogenetic model, providing both inducible and noninducible fetuses in mothers that are either inducible or noninducible.

PROJECT DESCRIPTION

Names, Titles, and Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Ottavia Barbieri	Guest Researcher	LCC, NCI
Jerry M. Rice	Chief, PCS	LCC, NCI

Objectives:

To discover and characterize cellular and organismal events and phenomena that are critical or modifying determinants in perinatal tumorigenesis. Particular attention has been given to metabolism of carcinogens by maternal and fetal or neonatal tissues and related pharmacokinetics, and correlation of these findings with tumor incidence data. It is expected that this multifaceted approach will indicate the extent to which variation in rates of carcinogen activation and/or metabolic clearance in maternal and fetal tissues and placenta influences the perinatal carcinogenesis process. Effect of diet and vehicle on rate of absorption of carcinogens from the suckling gastrointestinal tract has also been studied, since this rate determines both length of time of exposure of the gastric mucosa and pharmacokinetics of carcinogen contact with distal target organs.

Methods Employed:

In a study of modification of tumorigenesis in suckling Swiss mice, Aroclor 1254 (a mixture of polychlorinated biphenyls and a potent inducer of mixed function oxygenase activity) was administered to pregnant mice on the 19th day of gestation. A previous investigation had shown that such treatment resulted, via transmammary transfer of the PCBs, in a large increase in the enzyme dimethylnitrosamine (DMN) demethylase in the livers of the suckling offspring. Control pregnant mice received oil. Offspring of both groups were given a carcinogenic dose of DMN by i.p. injection on the 4th or 14th day of life. Tumor incidences were then compared at 28 weeks or 18 months of age.

DMN absorption from the stomachs of suckling and adult Swiss mice was studied by intragastric administration of a low dose (about 10 μg/kg) of ^{14}C-DMN. The mice were killed after various intervals and the stomachs homogenized for determination of remaining radioactivity. The effects of full versus empty stomachs, oil, and ethanol on DMN absorption were followed. In other experiments, stomachs were isolated by ligature in anesthetized mice and DMN injected through the stomach wall.

In a systematic investigation of the role of genetically-variable metabolism of methylcholanthrene (MC) in transplacental carcinogenesis, three experimental series are proceeding in parallel, utilizing genetic backcrosses between C57BL/6 mice, which are genetically highly inducible with regard to aryl hydrocarbon metabolism, and DBA/2 mice, which are noninducible. In matings of these strains inducibility is inherited as a Mendelian dominant. The genetic crosses used are DBA mothers x (DBA x C57BL)F_1 males and F_1 mothers x DBA males. Thus, both

inducible and noninducible offspring are present in either an inducible or noninducible mother. In a bioassay experiment, fetuses in both types of genetic cross are subjected to one of several doses of MC administered transplacentally. Basal and induced metabolic phenotype are determined by use of partial hepatectomy before necropsy. In this way changes in carcinogen metabolism resulting from prenatal imprinting can also be observed. Metabolic phenotype will be correlated with tumor incidence. Pharmacokinetic studies of disposition of MC and its metabolites in tissues of mothers and fetuses of the two genetic crosses are proceeding in parallel, as are determinations of metabolism of MC by cell-free preparations of maternal and fetal tissues. A convenient, rapid method for measurement of total metabolism of ^{14}C-MC to primary and secondary products has been developed and validated.

Major Findings:

Treatment of suckling Swiss mice on the 4th or 14th day of life with 5 mg/kg DMN caused liver and lung tumors, as expected. Pretreatment of the mothers of these mice with Aroclor 1254 had several interesting effects on DMN-caused tumorigenesis in the progeny. First, among the sucklings given DMN on postnatal day 14, the PCBs provided significant protection against initiation of liver and lung tumors, compared to mice whose mothers had not received PCBs. This protective action was most evident for lung tumors in males and females at 18 months of age and for liver tumors in males at 28 weeks of age. Induction of enzyme(s) effecting detoxification of DMN was postulated as the mechanism of the observed protection. This interpretation was supported by lack of protective effect in the sucklings treated on postnatal day 4, when the enzyme induction was of lesser magnitude. A second effect of the PCBs was seen in mice at 18 months of age: exposure to this chemical mixture resulted in significant increases in percent mice with livers overgrown by tumor compared to those mice given only DMN. This effect was most pronounced in males and females given DMN on postnatal day 4, but was seen also in males exposed to DMN on day 14; it was hypothesized to be due to liver tumor promotion by the PCBs. Thus, lactationally-delivered PCBs may reduce the numbers of DMN-initiated tumors in target organs of the offspring, but also may have a liver tumor promoting effect not evident until more than 6 months later. Storage of PCBs in body fat with slow release over time may have contributed to this effect.

When ^{14}C-DMN was given intragastrically to mature male Swiss mice, loss of radioactivity from the stomachs was rapid and was completed within 30 min. for fasted mice; fed mice still had 10 per cent of administered dose present in their stomachs after 60 minutes. Only small amounts of DMN were detected in the upper portion of the small intestine during the first 1-5 min. after administration. Experiments involving direct injection of DMN into stomachs and intestines isolated by ligature showed that absorption occurred primarily from the intestines. Loss from the stomachs was slowed by both olive oil and 50 per cent ethanol, each of which resulted in significant retention of DMN in the stomachs of fasted mice 5-30 minutes after administration. DMN was given at about 1/3 the adult dose to suckling mice (15 days old). Disappearance of label from the stomachs was delayed in fed versus 4-hour fasted sucklings and possibly in those whose mothers were fed a high-fat diet. These results indicate that the amount and nature of the stomach contents and/or age has an effect on both

length of time of exposure of the gastric mucosa to a low dose of DMN and on the rate at which it enters the general circulation.

Significance to Biomedical Research and the Program of the Institute:

Our experiments have shown that an environmental contaminant which concentrates in milk, PCBs, can, after administration to pregnant animals, modify tumorigenesis in their suckling offspring by another environmental contaminant, DMN. The effects were complex and included reduction in numbers of tumors initiated, but also probable promotion of liver tumors. The protective effect confirms the general principle of reduction in tumors by inducers of enzymes which metabolize systemic carcinogens, and shows that this protection may occur during the perinatal period. The fact that liver tumor promotion may have occurred, with effects seen up to 18 months of age, after a brief exposure of only 3 weeks during the suckling period draws attention to the vulnerability of the young animal. In the human context, both protection and tumor promotion by agents encountered during childhood, with consequences during adult life, are matters that might be subjects for fruitful epidemiological studies.

Modification of release of DMN from the stomachs of adult and suckling mice by factors such as meal size, oil, and ethanol could have implications in evaluations of human risk after ingestion of nitrosamines from various sources. Both length of time of exposure of the gastric mucosa and the pharmacokinetic fate of the chemical in the body would be affected. Such studies could be extended to humans by use of noncarcinogenic nitrosamines.

Proposed Course:

The effect of genetically-variable induction of carcinogen metabolism on the transplacental tumorigenesis process is being studied in biochemical, pharmacokinetic, and bioassay experiments as described above, with methylcholanthrene as the carcinogen and C57BL/DBA genetic backcrosses as the animal model. This work will be continued and completed. In a second phase, the pregnant mice will be treated with the noncarcinogenic inducer beta-naphthoflavone prior to transplacental methylcholanthrene. Such experiments will reveal the relative contributions of maternal and fetal pharmacogenetics to potentiation of or protection against transplacental carcinogens. If clear results are obtained, the experimental approach will be extended to other mouse strains and other classes of carcinogens, in order to establish the generality of the findings.

The effect of maternal diet and of vehicle on rate of loss of dimethylnitrosamine from the stomachs of suckling mice will be systematically investigated. Particular attention will be paid to the amounts and kinds of lipid in the maternal diet and to the effects of lipid, ethanol, cow's milk, and infant formula as vehicles for DMN administered intragastrically to fasted sucklings. These results will be used in the design of experiments to assess the effects of maternal diet and carcinogen vehicle on tumor incidence in infant mice given DMN per os.

Publications

Anderson, L. M., Van Havere, K. and Budinger, J. M.: Effects of polychlorinated biphenyls on lung and liver tumors initiated in suckling mice by N-nitrosodimethylamine. JNCI (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05353-01 LCC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Sensitivity Factors in Special Carcinogenesis Models

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
L. Anderson Expert, LCC, NCI

COOPERATING UNITS *(if any)*
Lawrence Hospital, Bronxville, N.Y.; Memorial Sloan-Kettering Cancer Center, New York, N.Y.; Litton-Bionetics, Inc., Rockville, MD

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Animal tumorigenesis models with unique qualitative or quantitative sensitivity to chemical carcinogens are utilized to study mechanisms and modifying factors in carcinogenesis. In a project employing athymic nude mice, transplacental or intraperitoneal exposure of nudes to the direct-acting carcinogen ethylnitrosourea (ENU) caused the appearance of skin tumors (sebaceous adenomas and papillomas), with an incidence ten times greater than that of the nu/+ littermates. An investigation of the reason for this difference is in progress and includes topical treatment of nu/nu and nu/+ mice with carcinogens and uv light, thymectomy of nu/+ newborns and thymus transplants to nu/nu animals. Strain A mice have high quantitative sensitivity to the induction of lung tumors by carcinogens and have been used as a model for assessing the effects of very low doses of the environmental carcinogen, dimethylnitrosamine (DMN). After 16 weeks of exposure to 500 ppb DMN, 44 per cent of the males had a lung tumor, compared with 8 per cent of controls. Even at 10 ppb DMN, 20 per cent of the males were tumor-bearing. This model is now being improved by systematic investigation of effect of diet and of exposure time, and will then be used for a large dose-response study. Determination of the effects of concurrent administration of ethanol, which inhibits liver metabolism of DMN, is also planned. Different mouse strains vary in their responsiveness to inducers of aryl hydrocarbon metabolism. Benzo[a]pyrene was administered intrarectally to ICR/Ha mice, which are moderately inducible, and C57BL/6, whose induction response is twofold that of the ICR mice. The noncarcinogenic inducer beta-naphthoflavone or oil was given 24 hours prior to the BP. Although no colon tumors resulted, neoplasms of the lung, forestomach, mammary gland, and lymphoid tissue were seen, and all were reduced significantly in incidence by prior treatment with beta-NF. The protective effect was greater by a factor of 2 in the C57BL/6 mice, compared to the ICRs. A further investigation is planned involving 6 mouse strains of varying inducibility and per os administration of the carcinogen, to confirm the generality of this finding.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Jerry M. Rice Chief, PCS LCC, NCI

Objectives:

To discover, develop, and utilize animal tumorigenesis models with unique qualitative or quantitative sensitivity to chemical carcinogens, in order to open new avenues of experimental approach in study of mechanisms and modifying factors in carcinogenesis. In particular, attention has been focused on three mouse models: (1) The nude (nu/nu) mouse is being used to study the effects of lack of thymus gland and/or of abnormal skin morphology on carcinogenesis; (2) Low-dose effects of an environmental carcinogen have been demonstrated by exploitation of the high sensitivity of strain A mice to lung tumorigenesis; and (3) Genetic differences among mouse strains with regard to responsiveness to inducers of mixed function oxygenase enzymes are utilized to confirm the potential usefulness of inducers as protective agents in carcinogenesis.

Methods Employed:

In the project employing the nude mouse, BALB/c nu/nu and nu/+ littermates were exposed transplacentally or by intraperitoneal injection to the direct-acting carcinogen ethylnitrosourea (ENU). Mice were killed when moribund or at 18 months of age and all lesions examined histologically.

In the experiments with strain A mice, mice were fed semi-purified diets and exposed to 10-1000 ppb dimethylnitrosamine (DMN) in their drinking water for varying intervals of their lives. Lungs were fixed in Bouin's solution and examined carefully for tumors. Controls were maintained in parallel.

The effect of genetic differences in enzyme induction on tumorigenesis was studied in C57BL/6 mice, in which aryl hydrocarbon metabolism is highly inducible, and in ICR/HA mice, in which this activity is only moderately inducible. The effective, non-carcinogenic inducer, beta-naphthoflavone (beta-NF), was administered by i.p. injection once per week. Controls received vehicle (olive oil). After 24 hours the mice received 1 mg benzo[a]pyrene (BP), an environmental polycyclic aromatic hydrocarbon, by intrarectal instillation. The mice were killed when moribund or at 18 months of age and all tumors and other lesions examined histologically.

Major Findings:

Among the nu/nu and nu/+ mice exposed transplacentally to ENU, skin tumors, including both papillomas and sebaceous adenomas, developed on the nude mice

with almost tenfold greater incidence than on their haired littermates. Dose dependency was demonstrated: after an ENU dose of 50 mg/kg maternal body weight, tumors were found of 45 per cent of nu/nu and 5 per cent of nu/+ mice, whereas these percentages were 23 per cent and 3 per cent after 10 mg/kg. Skin tumors were also induced on nude mice but not on haired controls by direct intraperitoneal treatment with ENU. These results constitute the first demonstration that nude mice may have higher than normal susceptibility to carcinogenesis under some circumstances.

Strain A mice were exposed to 10, 50, 100, 500, and 1000 ppb DMN in their drinking water for 16 weeks, starting at 3-4 weeks of age. Unequivocal tumorigenic effects were seen with the two highest dose groups; the greatest tumor incidence was seen with the males given 500 ppb, 44 per cent of whom had at least 1 tumor. Even at 10 ppb DMN, the incidence of male tumor-bearing mice was 2.5 times that of controls (20 per cent versus 8 per cent), although the number of mice was too small for statistical confirmation of a real effect at this dose.

Intrarectal treatment of female C57BL/6 and ICR/Ha mice with BP did not result in any colonic tumors, even though metabolism of BP to proximate carcinogenic derivatives by colonic mucosal homogenates was demonstrated, and BP was found to be cytotoxic to the colon mucosa. However, the BP did cause primary lung tumors, forestomach papillomas, lymphomas, mammary carcinomas, and sarcomas in one or both strains, presumably as a result of systemic absorption of the carcinogen. Pretreatment with beta-NF resulted in a significant reduction in the numbers of all forms of BP-caused neoplasms except for the sarcomas, whose numbers were unaffected by the beta-NF. Overall, inducer pretreatment reduced total incidence of neoplasms by about 30 per cent in the ICR/Ha mice and by about 60 per cent in the C57BL/6 mice. This difference is of particular interest in light of the fact that C57BL/6 mice respond to inducers with an increase in aryl hydrocarbon metabolism that is twice that seen in Swiss mice.

Significance to Biomedical Research and the Program of the Institute:

The demonstration of special sensitivity of nude mice to skin carcinogenesis provides a new model system for study of determining and modifying factors in chemical carcinogenesis. Further experiments are required to reveal whether the selective effect of ENU on the nude skin resulted from absence of thymus-dependent immune surveillance, or from unique properties of nude skin, or some other characteristic associated with the nu/nu genotype. In any case, the model can be utilized to shed light on physiological and/or cellular situations which predispose to high responsiveness to a carcinogen.

Much attention has been given to the question of whether the ppb level of contamination of food, air, etc. by carcinogens poses a cancer risk to the human population. The sensitive strain A mouse lung tumorigenesis model provides a direct means for measuring the carcinogenic effects of these low doses, and for gathering information about dose-response relationships, effects of diets and co-carcinogens, sensitivity at different times of life, etc. Such data may be useful in assessments of the impact of chronic low-dose exposure of human populations to carcinogens.

Intervention in the carcinogenesis process is one possible means of reducing the impact of exogenous carcinogens on humans. Reduction in the effect of

systemic carcinogens by administration of enzyme inducers is a well-documented phenomenon, with few exceptions known; our results showed that, at least for 2 mouse strains, the protective effect was proportional to the genetically-determined responsiveness to the inducer. These findings are of particular interest in light of the fact that humans are also probably genetically variable with regard to responsiveness to inducers, and they commonly encounter inducers in food, cigarettes, smoke, etc. It is possible that the phenomenon of protection through induction could be exploited to identify human risk groups and to reduce cancer risk by manipulaton of diet.

Proposed Course:

The first step in investigaton of the mechanism of selective carcinogenesis in nude mouse skin will be an attempt to discern the relative contributions of absence of thymus-dependent immune system function versus special properties of nude skin. Thymectomies will be performed on newborn nu/+ mice exposed transplacentally to ENU, and thymuses will be implanted into nu/nu mice as soon as congenic animals are available. Attempts will be made to induce tumors of the skin directly by application of various chemical initiators and tumor promoters and by ultraviolet light. Success with this effort will make it possible to induce tumors on skin transplanted between nu/nu and nu/+ mice and thereby to dissect the contributions of skin and immune surveillance. These studies will be coordinated and integrated with projects being carried out by other investigations in this laboratory on cellular specificity in two-stage epidermal carcinogenesis.

At present efforts are underway to determine optimum exposure time for demonstration of a difference between low-dose DMN-induced and spontaneous strain A lung tumors and to examine the effect of diet on the low-dose tumorigenesis phenomenon. Once diet and exposure time have been systematically established, a dose-response study of the effects of 1-1000 ppb DMN will be undertaken with a sufficiently large number of mice for statistical demonstration of effect. The effects of concurrent administration of ethanol will be examined and are expected to be of particular interest, since ethanol is an effective inhibitor of liver metabolism of DMN and results in maintenance of high blood levels of the carcinogen. Blood levels of DMN in these experiments are being determined in collaboration with Drs. Peter Magee and George Harrington at Temple University.

The apparent correlation between the genetics of inducibility and the protective effect of inducers will be confirmed by administration of a moderate oral dose of BP to six strains of mice, with and without prior treatment with the inducer beta-NF. These strains will include 2 highly inducible, 2 non-inducible, and 2 of intermediate inducibility. If the correlation of protection with inducibility is upheld, further experiments will include chronic administration of both inducer and carcinogen in the food, in closer approximation to a human exposure situation.

Publications

Anderson, L. M., Deschner, E. E., Angel, M. and Herrmann, S. L.: Murine colonic mucosal metabolism and cytotoxicity of benzo[a]pyrene. Oncology 39: 369-377, 1982.

Anderson, L. M., Last-Barney, K. and Budinger, J. M.: Sensitivity to carcinogenesis in nude mice: Skin tumors caused by transplacental exposure to ethylnitrosourea. Science 218: 682-684, 1982.

Anderson, L. M., Priest, L. J., Deschner, E. E. and Budinger, J. M.: Carcinogenic effects of intracolonic benzo[a]pyrene in beta-naphthoflavone induced mice. Cancer Lett. (In Press)

Anderson, L. M., Van Havere, K. and Budinger, J. M.: Lung tumorigenesis in strain A mice by low doses of dimethylnitrosamine. In Magee, P. N. (Ed.): Nitrosamines and Human Cancer. New York, Cold Spring Harbor, 1982, pp. 538-542.

ANNUAL REPORT OF

THE LABORATORY OF EXPERIMENTAL PATHOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

The Laboratory of Experimental Pathology plans, develops and implements research on the experimental pathology of carcinogenesis, especially concerned with the induction of neoplasia by chemical and physical factors in epithelial tissues, including: (1) development, characterization and evaluation of experimental pathology models of human cancer, such as cancers of the respiratory tract, by in vivo and in vitro carcinogenesis methods; (2) development and characterization of tissue culture systems for quantitative study of the effects of carcinogens alone or in combination; (3) research on mechanisms of carcinogenesis correlating different levels of biological organization, from whole organisms (human and animal), organs and tissues, to the cellular, subcellular and molecular levels.

General research objectives:

The main program of investigations in the Laboratory of Experimental Pathology (LEP) continues to be focused on two correlated problems: (a) the comparative pathogenesis of chemically induced neoplastic disease, particularly in lining epithelia, which are the tissues of origin of most human cancers, studied at all levels of biological organization, ranging from human tissues and animal models to organ and cell cultures and to molecular interactions; and (b) the interactions resulting from concurrent effects of different carcinogens and cofactors in multifactorial carcinogenesis mechanisms.

Sequential series of biological models linking molecular, cellular and organ levels:

There is a fundamental need to relate the process of carcinogenesis to the specific characteristics of the tissues and cells from which the induced tumors originate. Experimental chemical carcinogenesis is the result of chemical biological interactions characterized by pathologic responses that are typical of the different tissues and cells of origin. Human cancer is characterized by a similarly wide variety of pathologic response patterns.

In order to correlate mechanisms of carcinogenesis, investigated at the cellular and molecular level, with the corresponding events in animal and human tissues and organs, it is important to connect the different levels of observation. The approach developed to pursue this goal consists of the study of interactions of carcinogens in a series of biological systems of increasing complexity, but closely related to each other in a step-by-step sequence. Such systems include molecular targets in defined microenvironments, organized cell systems, target tissues in culture and in vivo, and finally organs and whole organisms, including not only models of animal pathology but also human pathology. Such an approach requires the development of a range of biological models related to each other and ultimately to human cancer pathology. A great deal of progress has occurred in this direction in the past two decades through major advances in experimental pathology, cell biology and biochemistry, to which previous work in the LEP has substantially contributed. The current LEP program represents a logical sequence to these advances.

A systematic investigative approach is pursued for studies on the mechanisms of carcinogenesis in a sequential series of biological target systems, both in vivo and in vitro. The series of interrelated systems, which was partly developed in the previous program of LEP and is further extended in its current program, includes the following components: (a) human pathology studies of histopathogenesis and cell differentiation in epithelial carcinogenesis; (b) in vivo animal systems for short-term and long-term studies on target epithelial tissues and organs, including animal models for carcinogenesis closely comparable to their human pathology counterparts (e.g., the hamster model for respiratory carcinogenesis); (c) organ explant culture systems for target epithelia and outgrowth cultures of epithelial cells from animal and human sources (e.g. respiratory, urothelial and epidermal epithelia); (d) mammalian epithelial cell systems for neoplastic transformation; (e) mammalian cell systems for neoplastic transformation in embryo or newborn cells or cell lines; (f) mammalian cell mutagenesis systems; (g) mammalian cell systems for the analysis of DNA damage and repair and other cytotoxic effects; (h) bacterial mutagenesis systems; (i) systems for the analysis of specific molecular targets, such as specific membrane receptors, protein kinases, and keratin; and (j) systems for the identification of genes and gene products involved in the transformation process, particularly DNA transfection systems for mammalian cell transformation, as well as gene cloning and structural identification.

Emphasis on epithelial systems:

In the past decade, major emphasis was given in LEP to the development of culture systems for human epithelial tissues and cells (in the Human Tissues Section) and for mouse epidermal culture systems (in the In Vitro Pathogenesis Section). LEP is now engaged in further methodological cell culture developments, not only for some human cell systems (e.g. urothelium), but also for those epithelial tissues and cells that are derived from experimental animal models closely related to human cancer pathology and are well known for their response to carcinogens, such as the hamster respiratory tract model and the mouse skin model.

Three main epithelial systems are currently studied in LEP:

(A) The hamster respiratory carcinogenesis model (Saffiotti 1964, 1968) has become well established as closely resembling the pathogenesis of the human bronchogenic carcinoma. Organ culture systems were established from the target tissues of the hamster respiratory system. Subsequent work in other laboratories has recently led to the preliminary development of respiratory epithelial cell culture and transformation systems. New chemically defined culture conditions are now being developed in LEP for the growth and transformation of hamster respiratory epithelia, in order to investigate specific cellular mechanisms controlling cell differentiation and transformation. Concurrently, in vivo studies on the segmental response of the respiratory tract to various carcinogens and cofactors are under way.

(B) The mouse epidermal carcinogenesis model has been widely studied for decades in vivo for its response to full carcinogens and/or promoting agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA). Previous work in LEP led to the development of primary culture methods for mouse keratinocytes and their transformation (Yuspa et al, 1978). Mouse epidermal cell lines transformable by promoting agents (JB-6 and clonal sublines) were also established (Colburn et al, 1980). New chemically defined media are being developed for primary and secondary cultures

of mouse keratinocytes and for their transformation by carcinogens. Studies are under way in collaboration with the Cell Biology Section, Laboratory of Viral Carcinogenesis, on the JB-6 clonal subline that are either sensitive or resistant to promotor-induced transformation. It was found that promoter-sensitivity can be transmitted by DNA transfection from the sensitive to the resistant clones. The identity of the gene or genes responsible for the susceptibility to promoter-induced transformation in this epithelial system is under investigation.

(C) Human urothelium (bladder and ureter epithelium) is cultured using new chemically defined media, and the conditions for optimal response to growth and transformation are under investigation. The corresponding animal models for chemically induced bladder carcinogenesis were previously studied in the hamster and rat and may provide useful culture counterparts for in vivo/in vitro studies; the carcinogenic activity of several chemical carcinogens on the human bladder has been well established in occupational and environmental studies.

Development of chemically defined culture conditions for studies on mechanisms of differentiation and transformation in epithelial systems:

Methods for chemically induced neoplastic transformation of epithelial cells in culture started to develop in the last decade, but these methods need to be further extended, and more rigorously defined from a quantitative point of view. As new and better defined culture conditions are established for target epithelial cell systems, their response to carcinogens needs to be correlated with the mechanisms of neoplastic transformation investigated at the molecular level. All three of the epithelial systems described above have comparable patterns of response to specific treatments and culture conditions that can lead, on one hand, to terminal differentiation, senescence and cell death and, on the other hand, to progressive cell growth, anchorage independence and neoplastic transformation. An important condition for studies on these mechanisms is the ability to grow the target epithelia in chemically defined culture media, replacing serum with selected additions of hormones and growth factors. Factors that control either continuous cell growth or the induction of senescence and terminal differentiation -- and the escape from senescence of transformed cell populations -- are under investigation.

In addition, an established mouse embryo fibroblastic cell line, BALB/3T3 clone A31-1-1, was characterized for its mutagenic and transformation responses in relation to several parameters. This cell line was also selected for the development of culture media in which serum addition is lowered or eventually replaced by addition of chemically defined factors.

Elimination of serum additions from the culture media in all these systems will not only dispose of a source of uncontrolled biological variables from batch to batch, but also make it possible to identify the specific role of individual growth factors in the control of cell growth and transformation.

Mechanisms of concurrent effects of different carcinogens and cofactors in multifactorial carcinogenesis:

Mechanisms of chemical carcinogenesis resulting from concurrent exposure to different carcinogens have so far received relatively little attention in the field of carcinogenesis studies and yet multiple exposures to different carcinogens represent the common realistic condition of human contact with carcinogens.

Studies of combined effects of carcinogens in the past were mostly based on in vivo animal experiments, usually limited to two carcinogens at a time. Several examples of marked synergism were reported.

The development of the above described biological models has made it possible to study combined effects of carcinogens and/or cofactors in relevant in vitro and short-term systems for mutagenesis and cell transformation. Methods in molecular biology and biochemistry are used to extend these studies to investigate the molecular mechanisms involved in the induction of neoplastic transformation by chemical carcinogens singly or in combination.

Combinations of carcinogenic factors or co-factors active in multifactorial carcinogenesis include: (a) carcinogens of the same chemical class; (b) carcinogens of different chemical classes; (c) complete and incomplete carcinogens (initiators, promoters and co-carcinogens); (d) carcinogens and modifiers acting on their tissue distribution, retention and response (e.g., particulate materials in respiratory tract), and on their metabolic activation and detoxication; (e) carcinogens and factors acting on the cellular expression of neoplastic transformation; and (f) carcinogens and altered genes, activated or derepressed to produce permissive conditions for neoplastic transformation.

Studies on concurrent effects were addressed to defining quantitative responses to the following parameters in the BALB/3T3 cl A31-1-1 system: a) single and split dose treatments with carcinogens; b) treatments of variable duration; c) exposure to carcinogens during different phases of the cell cycle; and d) concurrent exposure to different carcinogens. Additional studies on combined exposures were conducted in mouse epidermal cells and in the hamster respiratory tract model in vivo and in vitro.

Molecular studies of genes involved in the control of neoplastic transformation are currently addressed to testing the hypothesis that different genes may be activated by different carcinogens and/or cofactors, e.g. promoting agents, and that transformation may result from the combined effects of these different genes. Transforming DNA's have been obtained from several chemically transformed cell lines. The identification of a gene (or genes) responsible for susceptibility to promoting agents and transfectable to promoter-resistant epithelial cells is presently under investigation.

In summary, the focus of LEP research is addressed to the further elucidation of combined and possibly complementary mechanisms that would induce neoplastic transformation when concurrently or sequentially activated to their respective permissive conditions, in cellular systems biologically correlated with the main epithelial tissues of origin of human cancers in vivo and in corresponding cell culture models, maintained by optimally defined culture conditions.

OFFICE OF THE CHIEF

(1) Provides overall scientific direction and administrative coordination to the Laboratory's intramural research program and its supporting resources; (2) participates in research projects in all components of the Laboratory and provides collaborative research coordination of staff activities and resources; (3) conducts bibliographic research and data analysis; (4) conducts research on carcino-

genesis mechanisms and quantitative studies on the interactive effects of combined exposures to different carcinogens and cofactors, using in vivo and in vitro systems established in the Laboratory.

The continuously developing state of knowledge on mechanisms of chemical carcinogenesis was examined and interpreted for the purpose of contributing criteria for the identification and evaluation of carcinogenic effects of chemical and physical agents; methods for qualitative and quantitative carcinogenesis risk evaluation were also further analyzed. Specific review and analyses of data from the literature as well as from original research were conducted on carcinogenesis studies on air pollutants, particularly motor exhausts and combustion products; on mineral oils; and on the evaluation of mixed exposures to carcinogens and correlations of data from in vivo and in vitro systems.

The laboratory activities of investigators assigned to this Office included research projects on molecular mechanisms of carcinogenesis as well as collaboration on projects in the Respiratory Carcinogenesis Section and in the Tissue Culture Section.

Four types of studies were developed in the following research areas.

(A) - Quantitative studies on the concurrent induction of cytotoxicity, mutation and transformation were continued, using the mouse embryo cell line BALB/3T3 clone A31-1-1. This cell line, obtained from T. Kakunaga (Laboratory of Molecular Carcinogenesis, NCI), was characterized in a series of experiments which demonstrated its susceptibility to the induction of neoplastic transformation by a number of carcinogens with different metabolic pathways, including polycyclic aromatic hydrocarbons, aflatoxin, aromatic amines, nitrosamides and arsenic. A mutation assay for ouabain resistance was established for this versatile cell line, making it available for studies on concurrent effects of carcinogens on cytotoxicity, mutation and transformation.

A procedure for cell synchronization was developed for cells in exponential growth phase by inducing mitotic block with serum-free medium for 3 days and then releasing the cells from mitotic block by addition of serum. Treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 30 min at various points during the cell cycle showed a dissociation in cell cycle dependence between mutation and transformation frequencies in this cell line, since high susceptibility for mutation induction was found throughout the S phase and low susceptibility in the G1 phase, while no significant differences were observed during the different phases of the cell cycle for the induced frequencies of neoplastic transformation.

Split-dose protocols, with varying time intervals between equal split doses of MNNG, were investigated in comparison with single dose treatments: no evidence was found for recovery from sublethal damage in the split-dose tests regardless of whether the second dose was administered during or after the period of DNA damage, as determined by alkaline sucrose sedimentation and by alkaline elution methods.

A new phenomenon was observed in the BALB/3T3 cl A31-1-1 cells with MNNG exposures maintained for different periods of time after different initial exposure concentrations. The exposure periods required for maximal induction differed for ouabain resistant mutations (30-60 min), for cytotoxicity (about 120 min) and for neoplastic transformation frequencies (120-240 min). The half-

life of MNNG under the test conditions was determined spectrophotometrically to be about 65 min. The observed temporal dissociation in duration of exposures required for maximal effect suggests different targets in the mechanisms of mutation and transformation in this system. Current studies are investigating the basis for the events responsible for continued induction of transformation after the inducibility of mutations has been saturated.

(B) - DNA mediated gene transfer techniques and molecular cloning methods were used to investigate the identity and multiplicity of transforming genes activated by different carcinogens in various target cells. Different fibroblastic embryo cells lines are studied as recipients in DNA transfection assays for the induction of transformation by DNA from cell lines that were chemically transformed by different carcinogens. Preliminary positive results were obtained with transformation induced in NIH/3T3 cells by transfected DNA from 3 out of 4 benzo(a)pyrene transformed BALB/3T3 cell lines and by DNA from 4-nitroquinoline-1-oxide transformed hamster embryo cells. DNA from the transformed NIH/3T3 cells was extracted and found to be transforming after a second round of transfection. Molecular characterization of the transforming DNA's is under way.

In the mouse epidermal cell lines JB-6 and clonal sublines, selected as sensitive or resistant to transformation induced by promoting agents and expressed as anchorage independence, it was found that DNA from promotion-sensitive cells when transfected to resistant cells would render them sensitive to promoter-induced transformation. This remarkable finding was obtained in collaboration with the Cell Biology Section, LVC. This collaboration continues with studies on molecular cloning of prospective transforming genes. A jointly operated P-3 laboratory unit was established. Methods used include: DNA and RNA isolation, purification, enzyme restriction analysis, sucrose gradient and gel electrophoresis, Southern and Northern blotting and the construction and screening of genomic libraries in selected plasmid and phage vectors. These methods are now ready to be used in studies with the different cell systems described above, that have been characterized in this laboratory.

(C) - Molecular mechanisms in multistage carcinogenesis are investigated in the mouse epidermal JB-6 cell line with its clonal variants with a positive or negative mitogenic response (M^+ or M^-) to promoting agents and/or with a positive or negative promotion response (P^+ or P^-). Studies on the 2-deoxyglucose (2-DG) uptake response in these cell lines showed that uptake is required for the mitogenic response to TPA. Epidermal growth factor (EGF) and serum both markedly enhance hexose uptake and mitogenesis, while insulin affects neither process. EGF receptors are present on cell lines that respond to mitogenic stimulation but are undetectable in the unresponsive cell lines. In EGF-receptorless cells, about 25% of the normal complement of EGF receptors could be reconstituted with normal mouse liver plasma membranes, thus restoring a similar degree of mitogenic responsiveness to TPA and EGF.

A phosphoprotein species (pp80) which changes in response to TPA was identified in JB-6 clonal sublines. This phosphoprotein was characterized and found to represent approximately 2% of total phosphoprotein in whole JB-6 cell lysates. Transformed JB-6 cell lines were found to lack pp80 and they no longer produce it in response to TPA. The role of this phosphoprotein is being investigated.

(D) - Studies on the retention, distribution, metabolism and binding of carcinogens in target tissues and cells have been recently initiated using both in vivo and in vitro model systems. Carcinogens that have been studied so far include MNNG (in relation to cellular models of transformation) and benzo[a]pyrene (in relation to its metabolism in respiratory carcinogenesis and in the presence of cofactors).

RESPIRATORY CARCINOGENESIS SECTION

(1) Conducts research on the pathogenesis of cancers in the respiratory tract and on their induction by carcinogens, alone or in combination, using animal models closely related to human pathology and corresponding in vitro systems; (2) investigates the carcinogenic effects of chemical and physical agents on the respiratory tract, their quantitative aspects and their pathogenetic mechanisms; (3) studies mechanisms of cell differentiation and carcinogenesis in respiratory and related epithelia; (4) provides pathology expertise, resources and collaboration to other components of the Laboratory in the study of epithelial carcinogenesis.

The research activities developed in this Section are devoted to the characterization of respiratory carcinogenesis model systems in vivo and in vitro and to the elucidation of mechanisms of epithelial carcinogenesis by chemical and physical factors, alone or in combination. This Section also provides expertise on pathology research for in vivo animal carcinogenesis studies as well as on inorganic substances in carcinogenesis. The programs are closely correlated with those of the other LEP components.

The hamster respiratory carcinogenesis model (Saffiotti, 1964; 1968) was selected for further studies on combined effects of different factors and for the development of the corresponding bronchial epithelial organ culture and cell culture models in collaboration with the Tissue Culture Section. This hamster model was already extensively studied and shown to be similar to its human counterpart in its differentiation and pathogenesis and therefore represents the model of choice for studies on mechanisms of induction of bronchogenic carcinoma.

For studies requiring syngeneic animals, a Syrian golden hamster inbred strain (15:16/Bio.EHS) was obtained from NIEHS, tested and found free of viral and parasitic contamination, and established as a breeding colony at the Animal Production Area, FCRF. A clean breeding colony of a non-inbred strain (Syrian/CG.FOD) was also established.

Studies of cellular characterization in the different segments of the respiratory tract following intratracheal instillations with particular carriers were undertaken. Studies on different inorganic particulates and different carcinogens, alone or in combination, were developed. Histologic, histochemical, ultrastructural, immunochemical and autoradiographic techniques are used for the characterization of the segmental response of the respiratory epithelium to different carcinogens and their combinations. Light and electron miscroscopic characterization of the types of cellular differentiation and on their distribution in the hamster respiratory tract at different ages showed preliminary evidence that a primitive cell type is found in the respiratory tract at very young ages but no longer in adults. Two long-term in vivo studies on respiratory carcinogenesis by N-methyl-N-nitrosourea (NMU) and 2,6-dimethylnitrosomorpholine were completed and their pathology evaluated. An unusual finding was the induction by NMU applied topically to hamster tracheas in buffered solution at pH 4.5, of spindle cell carcinomas, which were characterized by electron microscopy: this is a rare

tumor type, previously unreported in respiratory carcinogenesis. Combined effects of topically applied insults to the respiratory epithelium, e.g., scarification and application of particulate materials, are being studied in combination with NMU treatments.

Chemicals, that are capable of inducing selective toxic or proliferative effects in different segments of the respiratory tract, are investigated singly and in combination. The compounds 3-methylindole and 2-methylnaphthalene, present in tobacco smoke, and selectively toxic for the non-ciliated bronchiolar lining cells (Clara cells) and alveolar type I cells in several species, are being investigated for their possible role in respiratory carcinogenesis in the hamster model, in which their toxic effects were characterized.

Arsenic was selected for studies on the interaction of organic and inorganic carcinogens as a unique example of discrepancy between positive human findings and negative animal tests. Metabolic species differences and possible requirements for a cofactor role in carcinogenesis are under investigation. Preliminary results indicate that inorganic arsenic is methylated in vivo by the mouse much more effectively than by the rat; arsenic was found to bind to human keratins in vitro; the role of arsenic binding in the induction of hyperkeratosis and keratinizing cell tumors is under investigation. Trivalent arsenic was found more cytotoxic than pentavalent arsenic for cultures of primary BALB/c mouse epidermal cells and of the BALB 3T3 clone A31-1-1 cell line; in the latter system, trivalent arsenic was found to induce neoplastic transformation, while pentavalent arsenic appeared negative. The role of arsenic in epithelial carcinogenesis is under investigation in studies of combined exposures with other carcinogens.

Organ culture and explant outgrowth cell culture methods for the hamster respiratory epithelium are being developed in collaboration with the Tissue Culture Section (see below).

TISSUE CULTURE SECTION

(1) Conducts research on cell culture systems for the characterization and quantitative study of neoplastic transformation induced by chemical and physical carcinogens; (2) develops and characterizes organ and cell culture systems for carcinogenesis studies, especially those derived from epithelia known for their susceptibility to carcinogens in vivo, such as the respiratory tract epithelium; (3) conducts research on mutagenesis, neoplastic transformation, differentiation, and on their expression mechanisms and relationships; and (4) provides expertise, resources and collaboration on tissue culture methods for the entire Laboratory.

The research program of the Tissue Culture Section is directed towards an understanding of changes in the control of growth and differentiation associated with chemical and physical carcinogenesis at the molecular and cellular levels. Emphasis is placed on carcinogenesis of epithelial cells derived from important organ sites of human cancer, e.g., the respiratory tract, the epidermis, the urothelial system (including the bladder and ureter) and the prostate. To accomplish these goals, existing cell culture systems will be further refined and characterized and new systems developed from both rodent and human tissues.

During the first full year of operation of the Section, necessary equipment has been installed and made operational and procedural details established for the operation of the tissue culture laboratory. Several research projects have been established, in collaboration with other components of LEP.

A common research theme was developed, relating to replicative culture of several epithelial cell types (bladder, epidermis, trachea), all of which undergo squamous terminal differentiation in conventional media containing serum. In separate studies for each of these 3 systems, it was found that replacement of serum by hormones and growth factors, coupled with a reduction in Ca^{++} levels, effectively prevented terminal differentiation and made it possible to obtain significant growth of the cell populations.

Serum-free media have been developed for human urothelium, mouse epidermis and hamster trachea. The work with human bladder and ureter, initiated by M.E. Kaighn in Pasadena, was continued in collaboration with his former colleagues at the Huntington Medical Research Institutes, Pasadena, CA. A serum-free medium has been developed using as a base Ham's MCDB 152 medium (developed for human epidermal keratinocytes) supplemented with 7 to 8 hormones and growth factors. Addition of serum or increase in Ca^{++} concentration was found to induce terminal differentiation in this system. These cells have been shown to be epithelial by ultrastructural studies with transmission and scanning electron microscopy and have the normal human karyotype.

Mouse keratinocytes have been grown for several passages in serum-free medium (MEM with low Ca^{++} and with growth factors). As with human urothelium, both serum and normal Ca^{++} levels induce terminal differentiation. This system is being characterized by electron microscopy, keratin profile analysis and growth studies.

Development of a replicative culture system from Syrian hamster tracheal epithelium is under way. Extensive squamous terminal differentiation was induced in explants cultured with serum or with ordinary Ca^{++} levels in this system, as had been observed in the two other systems mentioned above (human bladder and mouse epidermis). The serum-free medium MEM with low Ca^{++} and with growth factors, developed for mouse keratinocytes, also gave the best results thus far for the hamster tracheal epithelium. Characterization of the cells is under way.

The next phase of these studies will be to characterize the cellular response in the above epithelial systems after exposure to carcinogenic agents. Treated cells will be studied for changes in culture longevity, response to growth factors, anchorage-independent growth, morphology and karyotype. Selected cell lines will be tested for tumorigenicity in athymic nude mice.

Other studies were conducted in this Section, in collaboration with the Respiratory Carcinogenesis Section, to investigate the toxicity and transforming activity of arsenic salts in epithelial cells; work was focused on the effects induced in mouse epidermal keratinocyte cultures, for which optimal culture conditions were studied as reported above; the effects of arsenic in inducing escape from terminal differentiation and anchorage independence were investigated in preliminary experiments.

The Tissue Culture Section also collaborated with investigators in the Office of the Chief in studies on the induction by chemical carcinogens of cytoxicity,

ouabain resistant mutations and neoplastic transformation in the mouse embryo fibroblast cell line BALB/3T3 clone A31-1-1 and on their relation to phases of the cell cycle and to duration of exposure. Culture conditions and protocols were developed for the toxicity, mutation and transformation assays. In order to avoid the variations brought into this system by the use of different serum batches, the development of serum-free culture conditions was undertaken for transformation assays in this cell line. Successful chemical induction of transformation was obtained with the serum level reduced from 10% to 3%. These studies are continuing, to determine if serum levels can be further lowered or totally replaced with appropriate chemically defined factors. These methods are expected to contribute to a better definition and quantitation of studies on mechanisms of transformation.

Other studies in collaboration with investigators in the Office of the Chief were devoted to cell systems used for cell tranformation by transfection of DNA transforming sequences. NIH/3T3 cells were used for transfection with DNA's obtained from chemically transformed cells from the mouse BALB/3T3 clone A31-1-1 system or from hamster embryo cells. Comparative studies with NIH/3T3, BALB/3T3 and C3H 10T 1/2 cell lines are under way.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04490-07 LEP

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Evaluation and Prevention of Carcinogenic Effects

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Umberto Saffiotti Chief, LEP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.1	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Primary objectives of this work are to examine current knowledge in the field of chemical carcinogenesis and related fields and to identify criteria for the evaluation of carcinogenic effects of chemical and physical agents. Risk assessment methods are reviewed with consideration of qualitative and quantitative criteria. Laboratory methods and biological models for the detection and quantification of the carcinogenic activity of chemicals are examined and evaluated, with particular emphasis on animal models and in vitro models for carcinogenesis studies.

Data on the experimental design and results of carcinogenicity bioassays of mineral oils (petroleum lubricating base oils and derived products) were analyzed and reviewed for inclusion in the Monograph series of the International Agency for Research on Cancer on the Evaluation of the Carcinogenic Risk of Chemicals to Humans.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

None.

Objectives:

To examine current knowledge and experimental data in the field of chemical carcinogenesis and related fields, to examine criteria for the evaluation of carcinogenic hazards in the human population and for assessment of their risk, and to plan research approaches, relevant to the study of occupational and environmental cancer prevention, for development in laboratory work.

Methods Employed:

Examination and evaluation of biological models, methods and findings used for the detection of carcinogenic activity and the identification of mechanisms of carcinogenesis. Analysis of results of carcinogenesis studies, including experimental design, metabolism, biochemical changes, pathology and statistical evaluations. Analysis of occupational and environmental exposure data and of interactions of carcinogens with human tissues. Review and documentation of environmental carcinogenesis data and their evaluation.

Major Findings:

A thorough bibliographic review was conducted on experimental designs and results of carcinogenicity bioassays of mineral oils (petroleum lubricating base oils and derived products) for the Monograph on this subject of the International Agency for Research in Cancer (IARC). The chemical characterization of the test materials reported in the literature was generally poor, with some notable exceptions in which details of chemical and physical properites were given. Carcinogenic activity was generally associated with higher boiling point fractions and with higher contents in polycyclic aromatic hydrocarbons.

Significance to Biomedical Research and the Program of the Institute:

Criteria for environmental health and cancer prevention need to be based on sound scientific grounds. Analysis of different methodological approaches to the study of the carcinogenic process--at the human, animal, cellular and molecular levels--provides a strong basis for identifying specific criteria for the evaluation and risk assessment of carcinogens and for further research.

Proposed Course:

Continuation of these activities, with emphasis on research approaches and data analysis.

Publications:

Saffiotti, U.: Evaluation of mixed exposures to carcinogens and correlations of in vivo and in vitro systems. Environ. Health Perspect. 47: 319-324, 1983.

Saffiotti, U.: Occupational carcinogens in relation to the multifactorial orgin of cancer: Experimental pathology approaches. In Prevention of Occupational Cancer-International Symposium, Occupational Safety and Health Series No. 46, Geneva, International Labour Office, 1982, pp. 17-25.

Saffiotti, U.: The search for biological models to investigate human carcinogenic risks: Human pathology and experimental carcinogenesis correlations at the organ, tissue and cellular level, in vivo and in vitro. In Castellani, A. (Ed.): The Use of Human Cells for the Evaluation of Risk from Physical and Chemical Agents. New York, Plenum Press, 1983, pp. 571-586.

Saffiotti, U.: The search for criteria to assess the risks resulting from carcinogenic agents. In Castellani, A. (Ed.): The Use of Human Cells for the Evaluation of Risk from Physical and Chemical Agents. New York, Plenum Press, 1983, pp. 13-22.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04491-07 LEP

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Quantitative Studies on Concurrent Factors in Neoplastic Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Umberto Saffiotti Chief, LEP, NCI

COOPERATING UNITS *(if any)*

Laboratory of Toxicology, Istituto Superiore di Sanita', Rome, Italy

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.6	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mouse embryo cell line BALB/3T3 clone A31-1-1 was used for quantitative studies on cytotoxicity, mutagenicity and neoplastic transformation induced by different carcinogens. A ouabain resistance (oua-r) mutational assay was established for this cell line with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Optimal expression time for oua-r mutations was found to be independent from dose of carcinogen; a linear dose response relationship was obtained for induction of oua-r mutations by MNNG. Split-dose treatments with various intervals between doses were negative for recovery from sublethal damage with MNNG but positive with X-rays; mutation and transformation frequencies were not significantly different after single or split doses of MNNG regardless of time interval between doses. Although MNNG-induced mutation frequencies were found to vary with phases of the cell cycle (maximum in S phase), transformation frequencies did not; this dissociation suggests different underlying mechanisms.

A new phenomenon was observed in studies with MNNG exposures maintained for different periods of time after different initial concentrations. Half-life of MNNG in the cultures was about 65 min. The exposure periods required for maximal induction were: 30-60 min for oua-r mutations, about 120 min for cytotoxicity and 120-240 min for transformation; the ratio of transformation to mutation frequencies was within the same order of magnitude at short exposure times and increased to a more than 20-fold ratio at times of 240 min or longer. This temporal dissociation in exposure time required for maximal induction of mutation and transformation supports the hypothesis that transformation is dependent on factors other than a single gene mutation and offers a useful model for investigating the molecular events occurring during this differential time of exposure.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Margherita Bignami	Guest Researcher	LEP, NCI
M. Edward Kaighn	Expert	LEP, NCI
Corrado Ficorella	Visiting Fellow	LEP, NCI
Richard L. Norman	Staff Fellow	LEP, NCI

Objectives:

To determine conditions under which carcinogenic effects can result from concurrent exposure to different carcinogens and/or cofactors, and to analyze the mechanisms of such interactive effects in multifactorial carcinogenesis. To develop in vitro model systems for concurrent induction of cytotoxicity, mutagenicity and neoplastic transformation and to define the interrelationships of these endpoints in response to multiple factors.

Methods Employed:

The BALB/3T3 clone A31-1-1 mouse embryo cell line was used under the test conditions previously standardized in this laboratory for transformation assays with different carcinogens. Optimal conditions were determined for a mutational assay for ouabain resistance in this cell line using N-methyl-N-'nitro-N-nitrosoguanidine (MNNG). Split dose protocols, with varying time intervals between equal doses, were used to investigate recovery from sublethal damage induced by MNNG in comparison with X-rays. DNA damage and repair were determined by alkaline sucrose sedimentation analysis and subsequently by alkaline elution. Cell proliferation was determined autoradiographically after pulse-labelling with tritiated thymidine (^{3}H-TdR). Cell synchronization was obtained by plating cells in logarithmic growth phase in serum-free medium (MCDB 402 with 3 added factors) for 3 days (which induces mitotic block), and then feeding them with complete medium; ^{3}H-TdR incorporation was measured after pulse-labelling at different times following release from the mitotic block; the labelling index was determined autoradiographically. The half-life of MNNG was calculated from spectrophotometric determinations made under the conditions of test, as well as in serum-free and cell-free medium.

Major Findings:

Methods were established for concurrent induction of ouabain resistant (oua^{r}) mutations and neoplastic transformation, as well as cytotoxicity, in the BALB/3T3 clone A31-1-1. Using the direct acting alkylating agent MNNG, the optimal expression time (48 hours) for ouabain resistance was found to be independent from the dose of carcinogen; a linear dose-response relationship was obtained for oua^{r} mutations with increasing doses of MNNG.

Comparison of cytotoxicity induced by single versus split dose treatment with MNNG showed no recovery from sublethal damage, while this effect was confirmed with X-rays. No significant differences were detected in the frequencies of oua^{r} mutation or neoplastic transformation with single or split doses of MNNG,

regardless of whether the second dose was given during or after the period of DNA repair, again showing differences in the response of this cell line to chemical or physical agents.

Treatment of this cell line with MNNG for 30 min at various points during the cell cycle showed maximal induction of oua^r mutations throughout the S phase and low induction in the G_1 phase, but a constant level of transformation frequencies was found in G_1, early S and late S phases; these results show a dissociation of mutation from transformation in their cell cycle dependence in this cell line, which differs in this respect from the C3H 10T1/2 line.

Exposure times to MNNG were extended for periods ranging from 30 min to 72 hrs, after starting concentrations of 0.5 and 2 ug/ml in complete medium; the results showed that the plateau for maximal induction of oua^r mutations was reached early (30-60 min), the plateau for cytotoxicity was reached later (about 120 min) and the plateau for neoplastic transformation was reached still later (120-240 min). The half-life for MNNG in the cultures was about 65 min, and appeared to be independent of the amount of serum and the number of cells present, under the conditions of test. The marked dissociation observed in the exposure times required for MNNG to induce maximal levels of mutation and transformation supports the hypothesis that neoplastic transformation is dependent on factors other than a single gene mutational event. The ratios of transformation to mutation frequencies were within the same order of magnitude for short treatment times (<60 min), while they increased to more than a 20-fold difference for exposure times of 240 min or longer. Similar studies are underway with another alkylating agent, methylnitrosourea (MNU). (Additional studies with this cell system are reported in other LEP Projects, #Z01 CP 0527502 and Z01 CP 05278-02. The Salmonella mutation assay, previously used in this project, was not utilized in the current year).

Significance to Biomedical Research and the Program of the Institute:

These studies are part of a long-term project designed to investigate the quantitative response of cellular systems to the induction of mutation and neoplastic transformation, as a basis for quantitative studies on combined effects of different carcinogens and cofactors. The present findings support the hypothesis that different molecular mechanisms are involved in the induction of mutation and of transformation; they underline the fact that different cell systems currently used for mutation and transformation studies have different characteristics in their response to chemical and physical agents in relation to certain biologic parameters, (e.g. cell synchronization is required to demonstrate transformation by MNNG in C_3H 10T1/2, but not in BALB/3T3-A31-1-1). The temporal dissociation of exposure times required for maximal induction by MNNG of mutation and transformation provides a new biological phenomenon for investigating the differences between mutation and transformation mechanisms, especially in relation to toxicity mechanisms.

Proposed Course:

Confirmation of the observed phenomena using another alkylating agent, MNU; analysis of the factors involved in the continuing induction of transformation during exposure periods when the mutagenic response is already saturated.

Comparison of split-dose treatment by a single agent with sequential treatments with different agents.

Publications:

Bignami, M. and Saffiotti, U.: Mutagenesis and morphological transformation by N-methyl-N'-nitro-N-nitrosoguanidine in the BALB/3T3 clone A31-1-1 cell line. Carcinogenesis 4: 419-425, 1983.

Cortesi, E., Saffiotti, U., Donovan, P. J., Rice, J. M. and Kakunaga, T.: Dose-response studies on neoplastic transformation of BALB 3T3 clone A31-1-1 cells by aflatoxin B_1, benzo[a]pyrene, 3-methylcholanthrene and N-methyl-N'-Nitro-N-nitrosoguanidine. Teratogenesis, Carcinogenesis and Mutagenesis 3: 101-110, 1983.

CONTRACT IN SUPPORT OF THIS PROJECT

MICROBIOLOGICAL ASSOCIATES (N01-CP95637-58)

Title: Biochemistry and Cell Culture Resource
Project C: BALB/3T3 Transformation

Current Annual Level: $0 (terminated on 2/23/83)

Man Years: .2 for LEP

Objectives: Propagation, maintenance and storage of BALB 3T3 cells; screening of bovine serum lots; selection of target subclones; cytotoxicity and transformation assays with BALB/3T3 cells.

Major Contributions: Seven lots of newborn calf serum were tested and found inadequate for support of cell growth and transformation; four lots of fetal bovine serum were tested and all found adequate (one lot however resulted in a higher than expected transformation rate for untreated cells). Clones A31-1 and A31-1-1 (subclones A, C and K) were compared for response to graded doses of BP and MNNG: subclone A31-1-1C was the cell line of choice and was used for all subsequent work. Toxicity and transformation tests were carried out with different compounds at different doses and for different durations of exposure. Compounds tested included: polycyclic aromatic hydrocarbons (benzo[a]pyrene, benz[a]-anthrancene, 7,12-dimethybenz[a]anthracene and 3-methylcholanthrene), aromatic amines (1-naphthylamine, 2-naphthylamine, 4-aminobiphenyl, benzidine, 3,3'-dichlorobenzidine, o-tolidine and aniline) and other compounds (aflatoxin B_1, ethylenethiourea and safrole). Tests for synergistic effects were conducted with certain combinations of exposures. Tumorigenicity assays in nude mice showed sarcoma development with inoculation of transformed cells. This project was terminated and the final results are currently being evaluated.

Proposed Course: Contract expired on 2/28/83.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05265-02 LEP

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Chemical Carcinogens on Transforming DNA Sequences and Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Umberto Saffiotti Chief, LEP, NCI

COOPERATING UNITS *(if any)*
Laboratory of Tumor Cell Biology, NCI; Laboratory of Viral Carcinogenesis, NCI; Laboratory of Molecular Oncology, NCI; Biological Carcinogenesis Program, FCRF

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using recently developed DNA mediated gene transfer techniques and molecular cloning methods, this project investigates the identity and multiplicity of transforming genes activated by different carcinogens in various target cells, including epithelial cells.

DNA transfection procedures are used to study transformation of different recipient cell lines (NIH/3T3, BALB/3T3 cl A31-1-1 and $C_3H10T1/2$ cl 8) by high molecular weight DNA extracted from cell lines transformed by several different carcinogens. Preliminary positive results in NIH/3T3 cells were obtained with DNA from 3 out of 4 lines of BALB/3T3 cells transformed by benzo[a]pyrene, and with DNA from hamster cells transformed by 4-nitroquinoline-1-oxide. Restriction enzyme analysis is used to characterize the transforming DNA's.

In collaboration with the Cell Biology Section, LVC, NCI, a P-3 laboratory unit was established. In order to study effects of carcinogens on cellular genes, methods are being set up for cloning prospective genes in plasmid and phage vectors. The transforming DNA was isolated from a mouse epidermal cell line (JB-6) transformed by the promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA) and this DNA was shown to be expressed in the transformed cells since it was DNAse I hypersensitive. Sensitivity to transformation induction by tumor promoters, characteristic of promoter-sensitive subclones of the JB-6 mouse epidermal cell line, was shown to be transferable to promoter-resistant subclones by transfection of DNA from the sensitive cells. The identity of the gene or genes responsible for this property of epithelial cells is being investigated. In addition, a human genome library from sperm DNA was constructed in the Cheron 4A phage vector.

PROJECT DESCRIPTION

This Project now consists of two parts which were developed at different times and are best described separately; they will be soon integrated into a unified program.

Part A: Characterization of transforming effects of genes activated by chemical carcinogens.

Part B: Identification of transforming genes involved in chemical carcinogenesis, including second-stage events. (Started: February, 1983)

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Part A	Margherita Bignami	Guest Researcher	LEP, NCI
	M. Edward Kaighn	Expert	LEP, NCI
	Eric H. Westin	Expert	LTCB, NCI
	Donald Blair	Expert	LMO, NCI
Part B	Michael I. Lerman	Visiting Scientist	LEP, NCI
	Thomas D. Gindhart	Expert	LEP, NCI
	Nancy H. Colburn	Expert	LVC, NCI

Objectives:

Part A: An important issue in chemical carcinogenesis concerns the multiplicity of possible targets whose alteration by carcinogens leads to malignant transformation. Recently developed DNA mediated gene transfer (DMGT) techniques, e.g. transfection, have allowed the identification of transforming genes in a wide variety of human and rodent tumors lines. Transforming genes of human and rodent origin can be characterized according to their restriction endonuclease pattern (REP) of sensitivity to inactivation in transfection assays. Little evidence has been obtained so far as to whether different carcinogenic agents activate identical or different transforming genes in the same cells. While the published results suggest that different agents may regularly activate the same transforming genes in the same cells, such results are still quite limited and no systematic study has actually been made of the transforming genes activated by chemical carcinogens of different classes in one cell type. Published data indicate that the aneuploid mouse embryo cell line, NIH/3T3, can be made to express transformation by transfection with many different transforming genes, suggesting that a multiplicity of genes could induce the final steps required for transformation in a susceptible cell type.

Specific objectives are: 1) to determine whether carcinogens known to damage DNA in different fashions activate the same or different transforming genes in the same target cell as detected by DMGT; 2) to determine whether carcinogens which act synergistically activate the same or different transforming genes when administered singly and in combinations; and 3) to identify specific alterations in DNA resulting in the expression of transforming genes.

Part B: The long-range objectives of this study are to identify human transforming genes involved in the mechanisms of chemical carcinogenesis, including second-stage or promotion-like events.

As a first approach to the problem of identifying and cloning the genes activated by chemical carcinogens, an animal cellular model was chosen, i.e. the mouse epidermal cell line JB-6, with its variant subclones sensitive to the transforming effects of promoting agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA). Promoter sensitivity, expressed by the induction of anchorage-independent growth, is a property of some JB-6 subclones, while others are promoter-resistant. Collaborative studies of the Cell Biology Section, LVC and of this Laboratory have shown that DNA-mediated transfection can be used to transfer promoter-sensitivity from the sensitive to the resistant clones (Colburn et al., 1983a and 1983b). This system was therefore selected to investigate the identity of the gene(s) responsible for TPA-dependent transformation; recombinant DNA methods are used for the molecular cloning of this gene or genes. These techniques will then be used to select and identify other genes that may be activated by chemical carcinogens and to isolate the corresponding genes from human cells.

Methods Employed:

Part A: BALB 3T3 clone A31-1-1 cells have been transformed in this laboratory by several different carcinogens. Multiple independently derived transformed cell lines are either available or being developed. High molecular weight DNA's from each transformed line are extracted and transfected into NIH/3T3 cells by the calcium phosphate DNA precipitation technique, and are evaluated for the ability to induce transformed foci in NIH/3T3 cells. DNA's which reproducibly transform the indicator cells are typed according to their pattern of inactivation by restriction endonucleases.

Part B: In collaboration with the Cell Biology Section, LVC, a P-3 laboratory unit was established and equipped for growing and purifying plasmids and phage preparations. Methods are being developed for cloning prospective genes in plasmid and phage vectors. Techniques being used for molecular cloning include DNA and RNA isolation, purification, enzyme restriction, separation on sucrose gradients and gel electrophoresis, Southern and Northern blotting techniques, as well as construction and screening of partial and complete genomic libraries. DNA-mediated transfection in the JB-6 system is used to select for the cloned genes.

Major Findings:

Part A: The transfection procedures were reproduced in two different clones of NIH/3T3 cells and calibrated with the cloned v-mos gene (in collaboration with Drs. D. Blair and E.H. Westin). High molecular weight DNA was extracted from several transformed cell lines derived from the mouse cell line BALB/3T3 cl A31-1-1 treated with the following carcinogens: benzo[a]pyrene (BP); 7, 12-dimethylbenz[a]anthracene (DMBA); N-methyl-N'-nitro-N-nitrosoguanidine (MNNG); tryptophane pyrolysate; and ultraviolet light (UV). Replicate experiments were conducted with different transformed foci induced by each agent. Preliminary results obtained by transfection of these DNA preparations indicate that DNA's from 3 out of 4 BP-transformed lines induced transformation in NIH/3T3 cells. DNA extracted from

Syrian golden hamster embryo cells transformed by 4-nitroquinoline-1-oxide (4-NQO) and transfected in NIH/3T3 cells produced transformed foci with a transfection efficiency of 0.05 - 0.15 foci/ug DNA, determined in 4 replicate experiments. DNA's extracted from these transformed foci were found to induce transformation in a second round of transfection. Restriction enzyme analysis of the 4-NQO induced transforming DNA showed that the transforming activity was inactivated by Hind III and EcoR1 but not by BamH1 and Xba.

Part B: This project started in February 1983; the initial effort was devoted to establishing new facilities and methodologies.

In order to study the effects of carcinogens on cellular proto-oncogenes (c-onc), a battery of viral oncogenes (v-onc) has been compiled; 12 v-onc plasmids were grown so far, their DNA was purified and they are now ready to use as probes.

Experiments are in progress to identify genes responsible for the susceptibility to TPA-induced transformation in promoter sensitive (P^+) and resistant (P^-) subclones of the mouse epidermal JB-6 cell line. Transforming DNA was isolated from nuclei of a TPA-transformed JB-6 cell line (T^3) and it was found to be expressed in the transformed cells since it was DNAse I hypersensitive. The identity of the active gene or genes is investigated by using v-onc probes and RNA from the transformed T^3 cells. The gene(s) activated by TPA in the P^+ cell lines is now cloned in a shuttle type plasmid vector and is being purified by the sib-selection technique.

A human genome library from sperm DNA was constructed in the Cheron 4A phage vector and will be used to study the structure of human proto-oncognes in the genome of germ cells.

Significance to Biomedical Research and the Program of the Institute:

Part A: If all carcinogenic agents activate the same transforming gene in the same cell, then the pre-existing phenotype of the target cell would critically determine the transformation response to carcinogens in general and the identity of the agent itself would be relatively unimportant; only one transforming gene would exist for a cell type, but different carcinogens might activate it by different molecular mechanisms; combinations of transforming agents would all be expected to activate only one critical DNA sequence constituting a "final common pathway" for the cell type under study; and nonadditive interactions between carcinogens would occur only at the level of procarcinogen metabolism rather than at the DNA level. If different carcinogenic agents activate different transforming genes in the same cell, then clearly different target transforming DNA sequences could exist for a single cell type and combinations of transforming agents may exert their synergistic action by activation of genes capable of synergistic functions. The answers to these questions are of critical importance to understand cancer pathogenesis and to evaluate the interactions of environmental and genetic factors in the induction of cancer.

Part B: The methods of molecular cloning, applied to the identification of transforming genes activated by chemical carcinogens and tumor promoters in epithelial cells, offer new insight into the genetic events implicated in the malignant transformation of epithelial tissues, from which most human cancers arise.

Proposed Course:

Part A: Continuation of these studies will include: comparative evaluation of the susceptibility to transfection of different established cell lines (e.g. NIH/3T3, BALB/3T3 and C3H10T 1/2) and further characterization of the transforming activity of chemically induced DNA alterations.

Part B: The current studies, initiated in February 1983, will be continued using both animal epithelial cell systems and human tissues. Attempts will be made to transfer the active genes to normal human cells.

Publications:

Colburn, N. H., Talmadge, C. B. and Gindhart, T. D.: Transfer of phorbol ester promotability by transfection of DNA from promotable into nonpromotable cells. Prog. Nucleic Acid Res. Mol. Biol. 29: 107-110, 1983.

Colburn, N. H., Talmadge, C. B. and Gindhart, T. D.: Transfer of sensitivity to tumor promoters by transfection of DNA from sensitive into insensitive mouse JB6 epidermal cells. Mol. Cell. Biol. 3: 5-13, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05273-02 LEP

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Mechanisms in Multistage Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Thomas D. Gindhart Expert, LEP, NCI

COOPERATING UNITS *(if any)*

Laboratory of Viral Carcinogenesis, NCI

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.6	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Objectives of these studies are to analyze the molecular mechanisms underlying specific stage transitions in multistage carcinogenesis, comparing the mechanisms of promoting agents with those of carcinogens, singly or in combinations.

Enhancement of hexose transport and the presence of receptors for epidermal growth factor (EGF) have been studied for their role in the mitogenic effect of phorbol esters on post-initiated mouse epidermal cell lines. Clonal variants which undergo a mitogenic response to 12-O-tetradecanoylphorbol-13-acetate (TPA) enhance their hexose uptake rate in response to phorbol esters and possess EGF receptors, while mitogenically unresponsive variants lack both the hexose uptake response and EGF receptors. Reconstitution of EGF-receptorless cells with receptors from mouse liver membranes partially restores the mitogenic response to TPA. Mitogenic effects of tumor promotors appear to depend on enhancement of hexose uptake and of EGF receptors. Glucose appears to be specifically required for this effect: L-glutamine will support normal growth but not promoter-driven mitogenesis.

A major cell membrane associated phosphoprotein has been found in JB-6 cells which transiently increases in response to TPA but is uninducible in transformants. Its regulation appears linked to mechanisms underlying transformation of JB-6 cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Marion P. Copley	Staff Fellow/ Guest Researcher	LEP, NCI
Umberto Saffiotti	Chief	LEP, NCI
Nancy H. Colburn	Expert	LVC, NCI

Objectives:

The objectives of these studies are to analyze the molecular mechanisms underlying specific stage transitions in multistage epithelial carcinogenesis. Malignant transformation of mammalian cells by chemical carcinogens proceeds by progression through stages which can be phenotypically recognized in vivo and in parallel cell culture systems. Biochemical differences between populations of cells at successive stages can initially be utilized as stage-specific molecular markers and then exploited to analyze the underlying mechanisms responsible for progression of cells from one stage to the next. The biological model currently utilized is the mouse epidermal cell line JB-6 with its subclones selected for sensitivity to promoting agents. Other epithelial models, being characterized in other projects of this laboratory, will be used for further studies; emphasis will be placed upon comparison of the mechanisms of tumor promoting agents with those of carcinogens administered singly and in combinations.

This project includes two specific parts. Part A: Determination of the roles of altered hexose transport and EGF receptors in the mitogenic and transformation responses to tumor promoters in mouse epidermal cell lines; Part B: Evaluation of changes in protein kinase (PK) activities as epithelial cells pass through defined stages of preneoplastic progression.

Methods Employed:

Part A: Clonal sublines of the JB-6 mouse epidermal cell line are used to study the roles of hexose transport and EGF receptors in preneoplastic progression. The effects on the rate of ^{3}H-deoxy-D-glucose (2-DG) uptake of TPA, EGF, insulin and glucose deprivation were determined for eight clonal sublines among which the ability to proliferate at plateau density in response to TPA clearly diverges from the ability to respond to TPA with promotion of transformation. EGF receptors were measured on intact cells with ^{125}I-EGF. EGF receptorless cells were reconstituted with mouse liver plasma membranes containing EGF receptors.

Part B: The same battery of clonal variants of mouse epidermal cell plus their transformed counterparts was labeled with ^{32}P-orthophosphate and the phosphoprotein patterns of whole cell lysates and subcellular fractions were analyzed by one and two dimensional gel electrophoresis. Possible differences between promotion sensitive and resistant JB-6 cell lines in regulation of the calcium and phospholipid dependent protein kinase-C (PK-C) are being sought by varying the conditions of the PK-C assay. Possible differences between these cell lines in substrates for this enzyme are being studied by gel electrophoresis of in vitro reaction products.

By analyzing pp changes in just a single step of a multistage sequence, the total number of changes will be minimized and the essential/non-essential ratio maximized. Tumor promoters are known to directly activate PK-C, a recently described protein kinase present in all cells. By analyzing changes induced by a single, well studied promoting agent in matched pairs of cell lines, which differ in their transformation response to that agent, relevant pp changes can be identified (those which occur only in promotion sensitive cell lines).

Major Findings:

Part A: Variants with both mitogenic and promotion response to TPA (M^+P^+) show twofold enhancement of 2-DG uptake in log phase growth and at plateau. Several independent lines of evidence indicate that the hexose uptake response is absolutely required for mitogenesis in response to TPA. All variants defective in the mitogenic response to TPA (M^-P^+, M^-P^-) fail to increase 2-DG uptake rates in response to TPA during all phases of growth. EGF and serum strongly enhance 2-DG uptake and mitogenesis, while insulin affects neither process. A non-toxic means of blocking TPA's mitogenic response which also eliminated the hexose uptake response was devised. Cell growth is normal in medium containing 10% and 1% of the normal concentrations of glucose, but the mitogenic response to TPA is selectively abolished. Glutamine will not substitute for glucose. An M^+ spontaneous revertant which arose from an M^- cell line regained the hexose uptake response to TPA in association with the mitogenic response. EGF receptors are present on mitogenically responsive lines and undetectable on lines not showing these responses. Reconstitution of EGF receptorless cells with mouse liver plasma membranes restored about 25% of the normal complement of EGF receptors and a similar degree of mitogenic responsiveness to TPA and EGF.

Lines resistant to mitogenic stimulation by TPA (M^-) tend to transform spontaneously with passage in culture while variants defective in the promotion response but still showing the mitogenic response to TPA (M^+P^-) have not transformed over dozens of passages. Variants which show both responses to TPA also spontaneously transform with passages but at a low rate. These (M^-) lines are being evaluated as representing a new intermediate stage in preneoplastic progression.

Part B: A phosphoprotein species (pp80) which changes in response to TPA has been found in clonal sublines of JB-6 mouse epidermal cells. It has the following properties: molecular weight 80,000 Daltons, isoelectric point 4.5, microheterogeneity in terms of both molecular weight and isoelectric point characteristic of glycoproteins, localization to the membrane fraction of JB-6 cells from which it is readily solubilized by 1% Triton X-100. It was found that pp80 is a major cellular phosphoprotein in JB-6 cells representing approximately 2% of total cellular phosphoprotein in whole cell lysates. TPA treatment (10 ng/ml) increases the amount of ^{32}P labelled pp80 by 2-3 times in non-transformed JB-6 cells. This enhancement is first detectable within 30 minutes, peaks at 1 hr and returns to baseline levels within 24 hrs, with a steady gradual decline when followed for as long as 72 hrs. Variant cell lines resistant to promotion to anchorage independence by TPA show the same pattern of response as susceptible lines. pp80 is increased by TPA but not by EGF. Dibutyryl c'AMP at 10^{-3} molar inhibits the effect of TPA on pp80 when administered concomitantly.

Transformed JB-6 cell lines lack pp80 and fail to produce it in response to TPA. Loss of the pp80 response to TPA is likely to be closely linked to mechanisms maintaining the transformed phenotype in JB-6 cells since four independently derived transformants lacked the response while five different non-transformed cell lines possessed the response.

Significance to Biomedical Research and the Program of the Institute:

Identification of specific molecular mechanisms by which epithelial cells progress from one stage to the next in multistage carcinogenesis should contribute to a more complete understanding of the pathogenesis of epithelial cancers.

Identification of the phorbol ester receptor's protein kinase substrates should lead to characterization of the defects in the physiologic system which is the cellular target of tumor promoting agents in post-initiated cells. This physiologic system is perturbed by many agents capable of triggering the latter steps of preneoplastic progression, including the chemical carcinogen dioctylphthalate and DNA-damaging oxidants such as H_2O_2. However only phorbol esters bind to a specific receptor common to all mammalian cells except erythrocytes.

Stimulation of cell division by TPA is thought to play a role in earlier stages of preneoplastic progression in the mouse skin model of multistage carcinogenesis. The finding that the mitogenic response of mouse epidermal cells to TPA in vitro requires both stimulation of hexose uptake and receptors for EGF suggests that agents which counteract promoter enhancement of hexose uptake or EGF receptor function may inhibit early preneoplastic progression.

The M^- lines have elevated rates of 2-DG uptake characteristic of transformed cells but do not grow in soft agar. These results show that a high rate of hexose uptake alone is insufficient for expression of the transformed phenotype and suggest that the M^- lines represent a new substage of preneoplastic progression intermediate between the M^+P^+ cell lines and transformants.

Identification of a change in substrate for the protein kinase-c activated by TPA, associated either with susceptibility to TPA induction of the transformed phenotype in epithelial cells or with its subsequent TPA-independent maintenance, should lead to a more detailed description of the biochemical reactions underlying these events.

Proposed Course:

Determine the tumorigenicity in vivo of the M^- cells and compare them with transformed JB-6 cells.

Since total cellular pp's may not reflect changes in key regulatory subpopulations, specific PK activities and phosphoproteins will be studied in subcellular fractions of pre-labeled, treated cells and in in vitro labeling reaction mixtures made by reconstituting subcellular fractions.

Publications:

Colburn, N. H., Gindhart, T. D., Dalal, B. and Hegamyer, G. A.: The role of phorbol ester receptor binding in responses to promoters by mouse and human cells. In Langenbach, R., Nesnow, S. and Rice, J. M. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Press, 1983, pp. 189-200.

Colburn, N. H., Gindhart, T. D., Hegamyer, G. A., Blumberg, P. M., Delclos, B., Magun, B. and Lockyer, J.: The role of phorbol diester and EGF receptors in determining sensitivity to TPA. Cancer Res. 42: 3093-3097, 1982.

Copley, M. P., Gindhart, T. D. and Colburn, N. H.: Hexose uptake as an indicator of mouse epidermal cell resistance to the mitogenic activity of TPA. J. Cell Physiol. 114: 173-178, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05274-02 LEP

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Respiratory Carcinogenesis by Chemical and Physical Factors in the Hamster Model

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Umberto Saffiotti Chief, LEP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION
Respiratory Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.4	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies on the mechanisms of bronchogenic carcinoma induction by chemical and physical factors, alone or in various combinations, are pursued in the hamster respiratory carcinogenesis model (Saffiotti, 1968). In vivo studies use two newly established Syrian golden hamster colonies: inbred 15:16/Bio.EHS and outbred Syrian/CG.FOD. Treatments include: intratracheal instillation of carcinogens absorbed on particulate carriers, alone or in combinations; systemic vs/ topical treatments; and combined treatments with carcinogens and co-factors. Respiratory epithelial tissue responses are characterized by histological, ultrastructural, histochemical and biochemical methods, and by their study in organ and cell culture conditions.

Light and electron microscopy studies have given preliminary evidence that the respiratory epithelia of very young hamsters contain a large cell population with primitive differentiation that is absent in older animals.

The tumor response induced by different carcinogens in different segments of the respiratory tract is being characterized. Among other tumor types, the previously unreported induction of spindle cell carcinomas, a rare tumor type, was obtained with topical administration of N-methyl-N-nitrosourea. Regionally selective toxic agents are investigated; the toxic effects of 3-methylindole and 2-methylnaphthalene (two components of cigarette smoke with selective toxicity for Clara cells and alveolar type I cells in certain species) have been studied in the hamster model.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Sherman F. Stinson	Biologist	LEP, NCI
Richard L. Norman	Staff Fellow	LEP, NCI
Hildegard M. Reznik-Schüller	Visiting Scientist	LETM, NCI

Objectives:

The main objective of this project is the elucidation of the mechanisms by which brochogenic carcinoma, a major form of human cancer, is induced by chemical and physical factors, alone or in various combinations.

The main biological model selected for these studies is the hamster respiratory carcinogenesis model, originally developed by intratracheal administration of saline suspensions of carcinogens carried by fine inorganic particles (Saffiotti, et al., 1964, 1968). This model was subsequently extensively studied both in intramural and in collaborative research and differentiation markers were characterized for basal cells, intermediate cells, mucous granule cells and keratin containing cells. The cellular responses and the types of tumors induced by carcinogens in the hamster model are closely similar to their human counterparts. The role of different carcinogens acting synergistically was identified in this system, especially for polycyclic hydrocarbons in combinations with N-nitroso compounds. The special role of particulate carrier materials was identified in a series of studies with particulates having different physical characteristics; their pathogenetic mechanisms remain to be further investigated. An adequate induction model remains to be defined for the pathogenesis of small cell undifferentiated carcinomas.

Pathogenetic studies of this model received new emphasis, in this and other laboratories, with the development of organ culture methods for respiratory epithelia and with their use in investigating the interaction of carcinogens with target-cells. (See Project # Z01 CP 05277-02 LEP).

Specific objectives of this project include the following studies:

A) In vivo studies: Establishment and pathological characterization of specific pathogen free colonies of inbred and outbred hamsters. Establishment of optimal treatment procedures including intratracheal instillations. Study of the response to particulates alone or as carriers of carcinogens absorbed on their surface, singly or in combination; study of the role of fibrogenic dusts; and study of mechanisms involved in the effects of particulates on retention, distribution and metabolism of carcinogens on epithelial cell proliferation and on the induction of neoplastic transformation. Study of the effect of age of the hamsters on the populations of susceptible cells in the respiratory tract and the type of respiratory neoplasms induced. Study of the conditions and mechanisms of synergistic effects of carcinogens in the respiratory tract. Study of the effects of chemicals with selective target effects in different regions of the respiratory tract (such as polycyclic aromatic hydrocarbons with various carriers, certain N-nitroso compounds, 3-methylindole and 2-methylnaphthalene).

B) In vitro studies: Determination of the optimal conditions for the explant of tracheobronchial hamster epithelium and the establishment of organ cultures and primary epithelial cell cultures, from adult and/or newborn hamsters. Determination of the conditions for correlating in vivo and in vitro studies on the effects of carcinogens. These in vitro studies are pursued jointly with project No. Z01 CP 05277-02 LEP "Hamster bronchial epithelial culture carcinogenesis model."

Methods Employed:

Controlled breeding at the FCRF Animal Production Area, under specific pathogen free conditions, of two colonies of Syrian golden hamsters, inbred 15:16/Bio.EHS and outbred Syrian/CG.FOD. Establishment of lifetime and serially sacrificed colony control and treatment groups with general histopathological study and special investigation of respiratory tract cell differentiation and carcinogenesis. Intratracheal instillations of solutions and of particulate suspensions. Segmental treatments of the respiratory tract using a special cannula (Schreiber, et al., 1975), designed to expose only a localized region of the trachea to specific soluble carcinogens. Systemic treatments. Study of respiratory epithelia by histologic, histochemical, autoradiographic and immunochemical methods and by scanning and transmission electronmicroscopy. Epithelial tissue isolation, fractionation and use for biochemical analysis of carcinogen localization, metabolism, and binding.

Carcinogens to be studied include polycyclic aromatic hydrocarbons, N-nitroso compounds, aflatoxin and arsenic (see Project No. Z01 CP 05275-01 "Arsenic metabolism and carcinogenesis"). Particulate materials under consideration include the oxides of iron, magnesium, titanium and aluminum, as well as carbon, talc and silica (quartz, cristobalite, tridymite) and others to be selected.

Two components of cigarette smoke, 3-methylindole (3MI) and 2-methylnaphthalene (2MN), shown by others to induce selective damage to the nonciliated bronchiolar lining cells (Clara cells) and alveolar type I cells of the lungs of several animal species, are studied for their effects on tumor incidence and carcinogen metabolism. The LD_{50} for i.p. injections of 3MI or 2MN in hamsters and the highest acceptable dose for repeated administrations are determined. The time course, affected cell types and extent of damage following single or multiple injections are studied by light and electron microscopy.

Concurrent exposures to 3MI or 2MN and polycyclic aromatic hydrocarbons, such as would occur during smoking, are being investigated in the hamster respiratory carcinogenesis model. Concurrent administrations of BaP absorbed on Fe_2O_3 particles intratracheally and of 3MI or 2MN systemically are used to evaluate the effects of 3MI and 2MN on BP metabolism (EtAc soluble, H_2O soluble), on levels of (^{3}H)BaP binding to respiratory tract epithelium in vivo and on tumor incidence.

Major Findings:

The following results have been obtained so far.

1 - Baseline studies: The two hamster colonies were established and reached a satisfactory production level. Experimental studies were conducted to define the normal histology and physiology of the hamster strains being used in this project.

Light and electron microscopy were used to characterize the cellular composition of the respiratory tract epithelium at different ages; preliminary results indicate that the airways of very young hamsters contain large numbers of a primitive type of cell which is not found in older animals; further characterization of this and other types of cells is under way. Various physiologic parameters such as food and water intake and urine and fecal output were recorded, for hamsters held in metabolism cages for periods up to 30 days. These data will be used as baseline values for designing future metabolic studies.

2 - Differential susceptibility to respiratory carcinogens: Three major long-term studies were started in collaboration with Dr. H.M. Reznik-Schüller to examine any differences in type and incidence of respiratory neoplasms induced by 12 weekly subcutaneous injections of DEN in hamsters which were 1 day, 4 weeks and 8 weeks old at initiation. Animals have been killed at 4 week intervals, and a large group is being held for lifetime observations. Proliferative changes are characterized by light and electron microscopy.

Long-term in vivo studies were completed to evaluate the respiratory carcinogenic effects of two N-nitroso compounds: a) N-methyl-N-nitrosourea (MNU), applied topically to the tracheas of hamsters in buffered solution at pH 4.5, induced not only mixed epidermoid-adenocarcinomas, similar to the most common types of respiratory neoplasm in humans, but also spindle cell carcinomas, a rare variant of squamous carcinoma which has not previously been reported in experimental respiratory carcinogenesis studies; b) 2,6-dimethylnitrosomorpholine given intragastrically or subcutaneously induced adenomas, papillomas and carcinomas of the upper respiratory tract (nasal cavities, larynx and trachea); oral administration of retinoids decreased the incidence of induced pulmonary tumors.

3 - Combined effects of N-methyl-N-nitrosourea (MNU) and topical factors: A series of pilot studies was started to test whether various topically applied insults to the respiratory tract can enhance the respiratory carcinogenic effect of MNU given systemically; MNU alone was not found to induce respiratory tumors when injected systemically in hamsters, but it is a potent respiratory carcinogen by topical administration. Ferric oxide (Fe_2O_3) instilled intratracheally is the first insult being studied. Results are so far only preliminary.

4 - Effects of regionally selective toxic agents on respiratory carcinogenesis: Pilot studies were conducted to determine acute toxicity of single systemic or intratracheal applications in hamsters of varying doses of 3-methylindole and 2-methylnaphthalene. 3MI was shown to be toxic to hamsters and the LD_{50} for a single i.p. injection was determined. Intratracheal or systemic exposure to single doses of 3MI results in acute toxic changes of the terminal bronchiolar epithelium which are completely resolved in survivors after one week. Studies of the effects of chronic administration are under way; the highest acceptable dose for repeated administrations is below 25 mg/kg.

5 - Data reviews: Literature data and original results were analyzed and critically reviewed to establish a background for the design of new experimental plans; reviews were completed on the effects of polycyclic aromatic hydrocarbons on experimental respiratory carcinogenesis, on factors involved in laryngeal carcinogenesis and on the pathology and carcinogenesis of nasal cavity, nasopharynx and upper respiratory tract tumors.

Significance to Biomedical Research and the Program of the Institute:

This project is addressed to the elucidation of pathogenetic mechanisms of one of the major forms of human cancer; it is expected to contribute new knowledge on the conditions of concurrent or synergistic effects of different agents in respiratory cancer induction, a topic highly relevant to the understanding of human susceptibility to the multiple exposures that concur in lung cancer causation. This project is also expected to contribute basic knowledge in the poorly explored field of mechanisms of epithelial carcinogenesis and to provide an experimental pathology basis for the selection of cellular epithelial models of neoplastic transformation.

Proposed Course:

This project is designed to include long-term studies of several years' duration as well as shorter-term studies. It is closely interrelated to other projects in the laboratory on carcinogen metabolism and synergism and on epithelial cell culture and transformation.

Publications:

Reznik, G. and Stinson, S. F. (Eds.): Comparative Nasal and Nasopharyngeal Carcinogenesis: Boca Raton, CRC Press (In press)

Stinson, S.F.: Conclusions on comparative nasal carcinogenesis. In Reznik, G. and Stinson, S. F. (Eds.): Comparative Nasal and Nasopharyngeal Carcinogenesis. Boca Raton, CRC Press (In press)

Stinson, S. F.: Nasal cavity cancer in laboratory animal bioassays of environmental compounds. In Reznik, G. and Stinson, S. F. (Eds.): Comparative Nasal and Nasopharyngeal Carcinogenesis. Boca Raton, CRC Press (In press)

Stinson, S. F. and Reznik, G.: Adenocarcinoma of the upper respiratory epithelium in the rat. In Jones, T. C., Mohr, U. and Hunt R. D. (Eds.): Pathology of Laboratory Animals. Washington, D.C., I.L.S.I. (In press)

Stinson, S. F. and Reznik-Schüller, H. M.: Neoplasms of the mucosa of the ethmoid turbinates in the rat. In Jones, T. C., Mohr, U. and Hunt R. D. (Eds.): Pathology of Laboratory Animals. Washington, D.C., I.L.S.I. (In press)

Stinson, S. F., Reznik-Schüller, H. M., Reznik, G. and Donahoe, R.: Spindle cell carcinoma of the hamster trachea induced by N-methyl-N-nitrosourea. Am. J. Pathol. 111: 21-26, 1983.

Stinson, S. F. and Saffiotti, U.: Experimental laryngeal carcinogenesis. In Ferlito, A. (Ed.): Cancer of the Larynx. Boca Raton, CRC Press (In press)

Stinson, S.F. and Saffiotti, U.: Experimental respiratory carcinogenesis with polycyclic aromatic hydrocarbons. In Reznik-Schüller, H. M. (Ed.): Comparative Respiratory Carcinogenesis, Vol. II, Experimental Respiratory Tract Carcinogenesis, Boca Raton, CRC Press, 1983, pp. 75-93.

CONTRACT IN SUPPORT OF THIS PROJECT

UNIVERSITY OF MARYLAND (NO1-CP-25605)

Title: Hamster Respiratory Carcinogenesis Resource for In Vivo/In Vitro Correlation Studies

Current Annual Level: $196,496

Man Years: 6.9

Objectives: To conduct in vivo respiratory carcinogenesis studies in the Syrian golden hamster and to provide in vitro techniques for the culture of respiratory epithelium to complement the in vivo studies.

Major Contribution: During this first year of contract, in vivo and in vitro techniques and methodology were developed, standardized and optimized. Hamsters from various sources were evaluated for microbiological and parasitological profiles, to select the optimal source of animals. Pilot studies were conducted to analyze the effect of mechanical and chemical trauma associated with intratracheal instillations on cell turnover rates throughout the respiratory tract. Serial sacrifice experiments were undertaken to quantify the cell proliferation induced by topical epithelial injury.

Proposed Course: With the completion of preliminary studies, experiments are currently being initiated to elucidate the mechanisms of the regional specificity of the respiratory tract epithelium to tumor induction with different carcinogenic and promoting agents. Various combinations of carcinogens and cofactors, including chemical and mechanical agents, will be utilized in both in vivo and in vitro systems and their effects on morphology, cell kinetics and metabolism will be studied.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05275-02 LEP

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Arsenic Metabolism and Carcinogenesis Studies

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Umberto Saffiotti Chief, LEP, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Respiratory Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.8	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Arsenic (As) carcinogenesis is a unique example of discrepancy between positive human findings and negative animal tests. Two hypotheses are investigated in an attempt to clear this discrepancy: (a) species-specific differences of As metabolism between humans and laboratory rodents used in carcinogenesis tests; (b) requirements for combined exposures with other agents in co-carcinogenic interactions. It was found that the mouse is able to methylate inorganic arsenic in vivo; this biotransformation is similar to that in humans and considerably more effective than that occurring in the rat.

In vitro culture models were selected in which to investigate arsenic metabolism and its interaction with other compounds. Transformation and toxicity studies were conducted using the mouse embryo cell line BALB/3T3 clone A31-1-1. In this system, trivalent As was found to have higher toxicity than pentavalent As; transformed foci were induced by trivalent As while pentavalent As was negative.

Primary mouse epidermal cell cultures are also used. The conditions needed for arsenic methylation and the effect of arsenic on epidermal cell differentiation and keratin synthesis in culture are studied. Growth conditions were successfully established for these cultures by replacing serum with four defined factors in low calcium basal medium. Primary hamster epidermal cell and bronchial epithelial cell culture systems are considered for future use.

In vivo carcinogenesis protocols are developed according to the information obtained from in vivo and in vitro metabolic studies and from studies of target cells in culture.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Federico Bertolero	Visiting Fellow	LEP, NCI
M. E. Kaighn	Expert	LEP, NCI

Objectives:

These studies are aimed at the development of in vitro and in vivo models for investigating the role of arsenic and other metals in carcinogenesis, particularly in epithelial tissues.

The study of metal carcinogenesis is important because of the ubiquity of the exposure and the persistence of metals in the environment. Because environmental exposure to metals generally occurs concomitantly with other complex exposures to xenobiotics, particular attention will be given to studies on the potential interactions of metals in combination with other carcinogens or cocarcinogens.

Conclusive epidemiological evidence exists for an increased risk for lung cancer and for non-melanotic skin cancer following exposure to inorganic arsenic. Animal experiments, however, have so far mainly resulted in negative findings. Two main hypotheses are investigated to explain this discrepancy: (a) species-specific metabolic differences between human subjects and laboratory rodents used so far in carcinogenesis tests (mostly rats and mice); and (b) requirements for combined exposures with other agents in a cocarcinogenic interaction. Recent studies have shown a significantly different metabolism of inorganic arsenic in rats, as compared with human and other mammals (Marafante, E., Bertolero, F., Edel, J., Pietra, R. and Sabbioni, E.: Sci. Total Environ. 24: 27-29, 1982). Once absorbed, inorganic arsenic is methylated in vivo through pathways which have not yet been identified. Arsenic methylation is commonly regarded as a detoxification process although its implications for other pathways of cellular metabolism are unknown. The increase in cancer risk observed in epidemiological occupational studies has been attributed mainly to the presence of inorganic trivalent arsenic, whereas the less toxic pentavalent compound was generally regarded as less harmful: however, inorganic pentavalent arsenic can be reduced in vivo to the more toxic trivalent form. New HPLC methods, available for the speciation of arsenic compounds, are used to elucidate this important problem.

Arsenic might act as a cocarcinogen in combination with other agents. Very limited experimental data, mostly negative, are available so far from animal studies; recent unpublished results from a study in hamsters by Pershagen et al (personal communication) suggest a cocarcinogenic effect on the induction of adenomatous tumors in the hamster lung. Further in vivo carcinogenesis studies are designed on the basis of metabolism and cellular studies.

Defined cell culture models were selected to study arsenic compounds for their metabolism, carcinogenic activity and possible interaction with other xenobiotics. Initial studies were conducted using the mouse cell line BALB/3T3 clone A31-1-1 and primary cultures of mouse epidermal cells. These mouse cell systems were selected because As metabolism in this species was reported to be

qualitatively similar to that observed in humans. The BALB/3T3 Cl A31-1-1 cell line was characterized for transformation studies in this Laboratory (see Project Z01-CP-04491-07 LEP). The mouse epidermal primary cell culture model was selected for the following reasons: a) primary keratinocytes can be grown in culture and can be chemically transformed under controlled conditions, as shown by S. H. Yuspa and coworkers in previous projects from this Laboratory; b) arsenic and several other metals are accumulated in vivo in keratinizing tissues, and arsenic is known to induce hyperkeratosis; and c) the induction of tumors by arsenic, so far demonstrated only in human observations, occurs in tissues that are capable of undergoing keratinization, i.e. epidermis and respiratory epithelium.

Methods Employed:

1- Analytical methods. (a) Separation of As binding sites and metabolites obtained in vivo and from tissue culture systems, by conventional preparative biochemistry techniques, i.e.,: tissue homogenization, differential ultracentrifugation, gel chromatography, gel electrophoresis and membrane filtration; (b) speciation of the diffusible metabolites, performed by two methods: Ion-exchange chromatography on AG50 resin that allows the separation of trivalent and pentavalent inorganic arsenic from the monomethylated and dimethylated metabolites; and HPLC separation on a C18 reversed-phase column that improves speciation by permitting the separation of all four arsenic metabolites with a single elution (Brinckman, F.E., et al.: J. Chromatogr. 191:31-46, 1980); and (c) detection and quantification of As metabolites by gammacounting, employing As-74 and As-73 labelled compounds in all metabolic studies.

2- In vivo metabolic studies. Preliminary studies were performed on As metabolism in BALB/c mice to confirm the reported qualitative similarities with the metabolism in humans.

3- Cell culture studies. (a) The BALB/3T3 clone A31-1-1 mouse embryo cell line is used to study toxicity and neoplastic transformation according to methods currently used in this laboratory (See project Z01-CP-04491-07 LEP). (b) The methods for cultivating primary epidermal basal cell cultures from newborn BALB/c mice selectively in low calcium medium were experimentally further developed by combining them with the methods for cell growth in serum-free chemically defined medium (see project Z01-CP-05278-02 LEP).

Major Findings:

Results so far obtained indicate that: a) Inorganic arsenic is methylated in vivo by the mouse more effectively than by other rodent species (rat and rabbit). b) Induction of neoplastic transformation by As was obtained in BALB/3T3 clone A31-1-1 cultures; As^{+3} was found to have higher toxicity than As^{+5}; As^{+3} induced transformed foci at concentrations of 10^{-5} and $10^{-5.5}$M, while As^{+5} was negative; the transformed foci were isolated and found positive in soft agar assays. c) Studies on primary cultures of mouse epidermal cells, to determine optimal culture conditions, showed that effective growth was successfully established for these cultures by replacing serum with four defined factors in low calcium basal medium; epithelial cell identity was verified morphologically and histochemically (See project Z01-CP-05276-02 LEP). Culture conditions are in the process of being standardized for studies on arsenic effects.

Significance to Biomedical Research and the Program of the Institute:

Arsenic carcinogenesis has long been considered a unique example of discrepancy between positive human findings and negative animal tests. The reasons for this apparent divergence need to be found. The elucidation of arsenic mechanisms in carcinogenesis can provide a basis for the understanding of metal carcinogenesis in general and for risk evaluation and prevention. Positive results obtained in transformation studies on cells in culture are expected to provide a lead for investigating the mechanisms of As carcinogenesis.

Proposed Course:

1) Defining the effects of As salts on cell growth and differentiation; 2) Defining the conditions for a qualitative and quantitative transformation assay in serum-free medium for primary mouse epidermal cell cultures; 3) Investigating the direct and/or cocarcinogenic mechansims responsible for the transforming activity of arsenic salts, and their possible genetic changes associated with transformation; 4) Determining the conditions responsible for the effect of As compounds on respiratory tract carcinogenesis in vivo and in vitro in the hamster model.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05276-01 LEP

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Growth Control in Epithelial Cells and its Alteration in Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

M. Edward Kaighn Acting Chief, Tissue Culture Section, LEP, NCI

COOPERATING UNITS *(if any)*

Huntington Medical Research Institutes, Pasadena, CA

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Culture Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human bladder and ureter cells and mouse epidermal cells are being used for studying the mechanism of chemical carcinogenesis in epithelial cells. Serum-free media have been developed for both systems: low levels of calcium and substitution of serum by hormones and growth factors were found to be essential. Increase in calcium or addition of serum induced terminal squamous differentiation in both systems. Both systems are now being extensively characterized by electron microscopy, karyology, growth response to various factors and by their complement of structural proteins and enzymatic activities. Chemical transformation experiments will be conducted when these systems are adequately characterized.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Umberto Saffiotti	Chief	LEP, NCI
Federico Bertolero	Visiting Fellow	LEP, NCI
Raymond T. Jones	Senior Scientist (IPA)	LEP, NCI

Objectives:

The goal of this project is to understand the sequence of changes in the control of growth and differentiation of epithelial cells that are produced by chemical carcinogens. Several epithelial culture systems are now being developed for this purpose. Serum-free media have been developed for normal human bladder and ureter epithelium and for mouse epidermal keratinocytes (see Project Z01 CP 05278-02 LEP). Both cell types are capable of a significant number of divisions under present conditions. A normal human prostatic epithelial cell line (NP-2s) will also be used. Cultured normal human and rodent epithelial cells will be treated with carcinogens singly and in combination. The treated cells will be studied for changes in culture longevity, response to growth factors, anchorage-independent growth, morphology, karyotype, and alterations in enzymatic activites or structural proteins. Selected cell lines will be tested for tumorigenicity in athymic nude mice. An important goal is to identify markers of the transformed phenotype of epithelial cells.

Methods Employed:

Human urothelial cells are obtained as primary outgrowths from bladder or ureter explants established in serum-free defined medium. A normal human prostatic epithelial line isolated in Pasadena is available as frozen stock. Mouse epidermal keratinocytes are isolated by the cold trypsinization and stripping technique. All three cell types have been successfully cryopreserved. Epithelial cells will be exposed to graded, sublethal concentrations of chemical carcinogens. Direct-acting carcinogens will be used during the initial phase of these studies. The toxicity of each compound will be determined by clonal survival experiments, or by its effect on incorporation of tritiated thymidine into DNA. The selection technique of choice will be escape from senescence. In addition, media which selectively favor growth of transformed cells, e.g., switch from low to high calcium or addition of serum, will be used to induce terminal differentiation in normal but not in transformed cells. Appearance of altered growth properties, ultrastructure (both SEM and TEM) and karyological changes will be studied as a function of time after application and of dose level. Growth in soft agar and in nude mice will be used to demonstrate tumorigenicity.

Major Findings:

This project has been focused so far on developing defined media for each epithelial cell system. Human bladder and ureter have been cultured directly in medium HMRI-1, which consists of medium MCDB 152 with the Ca++ concentration increased to 0.1 mM and with the addition of 7 or 8 growth factors. The relative importance of each factor is under investigation. Either the increase of Ca++ concentration

to 1mM or the addition of serum result in terminal squamous differentiation. Primary monolayers have been subcultured several times in HMRI-1. Their growth is population-dependent. Mouse epidermal keratinocytes have also been established and grown in serum-free medium with four growth factors. As with the bladder, the importance of each factor and its optimal concentration are being determined. Keratinocytes have been cloned with and without a feeder layer of BALB/3T3 cells.

Significance to Biomedical Research and the Program of the Institute:

Epithelial cells give rise to the vast majority of human cancers; mechanisms of carcinogenesis in epithelial cells are therefore of critical importance. Both bladder and prostatic cancers are important forms of human cancer. The normal human bladder and prostatic epithelial cultures systems, that have been developed, offer an opportunity to investigate etiologic factors and the mechanism of carcinogenesis in human epithelial cells. The mouse keratinocyte system offers the opportunity of comparing the action of carcinogens in epithelial cells that exhibit a similar type of terminal differentiation and that correspond to a well known animal model of carcinogenesis.

Proposed Course:

The initial phase of this project will continue to provide careful characterization of both the human urothelial cells and the mouse keratinocytes as they grow in serum-free media. Since the human prostate line (NP-2s) still requires 1 to 2% serum for growth, experiments will be conducted to eliminate this requirement. Once culture conditions are adequately defined, the effect of carcinogens will be studied in these cells, initially with direct-acting carcinogens. When each system is more firmly established, work will progress to investigate the effect of metabolism-dependent carcinogens and the combined activity of different carcinogens and promoters.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05277-01 LEP

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Hamster Bronchial Epithelial Culture Carcinogenesis Model

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

M. Edward Kaighn Acting Chief, Tissue Culture Section, LEP, NCI

COOPERATING UNITS *(if any)*

Department of Pathology, University of Maryland Medical School, Baltimore, MD

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Culture Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Syrian golden hamster is the classic animal model for the vivo study of respiratory carcinogenesis. Hamster tracheal explant cultures have been used to study in vitro carcinogenesis. Because of the advantages of using serum-free media in carcinogenesis studies in vitro, various formulations of media were tested for the the explant and subsequent cell culture of hamster respiratory epithelia. Media CMRL-1066, 199, MCDB 152, and modified MEM were tested. All media had growth factors replacing fetal bovine serum. Optimal outgrowths of epithelial cells from tracheal rings were obtained in CMRL-1066 and modified MEM. However, more cells underwent terminal differentiation in medium CMRL-1066 due to its high calcium concentration than in the modified (low calcium) MEM containing insulin, hydrocortisone, bovine pituitary extract and epidermal growth factor. Additional tests are under way on media which allow the replicative culture of cells from the outgrowths. It was found that tracheal rings can be cryopreserved at -70°C for extended periods; this procedure has greatly facilitated the use of tracheal rings in these studies. Similar techniques were developed for the explant culture of hamster larynx and bronchi in serum-free medium. Studies comparing response of the various segments of the hamster respiratory tract are ongoing, using treatments with carcinogens having different specificity in different respiratory tract segments. Tracheal rings removed from hamsters pretreated with diethylnitrosamine were grown in explant culture in serum-free CMRL-1066 and were compared to non-treated controls; preliminary results suggest that carcinogen treatment in vivo appears to facilitate the outgrowth of cells from the tracheal rings. A qualitative response to the in vitro treatment of tracheal rings with 3-methylindole and 2-methylnaphthalene has been observed by comparing the extent of outgrowth of cells from the treated rings to untreated controls.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Raymond T. Jones	Senior Scientist (IPA)	LEP, NCI
Umberto Saffiotti	Chief	LEP, NCI
Sherman F. Stinson	Biologist	LEP, NCI

Objectives:

(1) Development of replicative cell cultures from bronchial epithelia of inbred Syrian hamsters; (2) development of explant cultures from the same source; (3) determination of the growth potential of normal bronchial epithelial cultures and their response to growth factors; (4) investigation of the effect of pretreatment with various carcinogens in vivo on both organ and cell cultures; and (5) comparison of growth behavior and response to carcinogens in epithelial cells from different segments of the laryngeo-tracheo-bronchial tract which show different responses to certain carcinogens.

Methods Employed:

Segments of the laryngeo-tracheo-bronchial tract were removed from anethesized inbred Syrian golden hamsters and were grown in 60 mm Petri dishes as organ explant cultures according to current methodologies. Various formulations of media, all containing growth factors replacing fetal bovine serum, were tested for the maintenance of the explants as well as for cellular outgrowth. They included CMRL-1066, 199, MCDB 152 and modified MEM. Tracheal rings were obtained and cryopreserved in culture medium containing 7.5% DMSO at -70°C. Tracheal rings were also treated in vitro with serial dilutions of 3-methylindole and 2-methylnaphthalene and the outgrowths and explants studied by morphological techniques. Tracheal rings from hamsters treated in vivo with diethylnitrosamine (DEN) were explant-cultured and studied by morphological techniques. The testing of serum-free media for the replicative culture of hamster respiratory epithelial cells are continuing using current methodologies. Respiratory explant and cell cultures are treated with selected chemical carcinogens to study morphological and karyotypic changes, alterations in growth behavior and loss of anchorage dependence. Attention will be paid to the order in which phenotypic changes occur as measured in cultured cells following exposure to carcinogens or respiratory toxins. Such changes will be compared with changes seen in the hamster in vivo, in order to identify sequential steps in the multistage process leading to neoplasia.

Major Findings

Preliminary experiments were conducted to determine whether the laryngeo-tracheo-bronchial tract epithelia from Syrian golden hamsters can be grown in serum-free medium as explants for extended periods. Of the various formulations of media that were tested for the explant and subsequent cell culture of hamster tracheal epithelia, all containing growth factors replacing fetal bovine serum, optimal outgrowths of epithelial cells from tracheal rings were obtained in CMRL-1066 and

modified MEM. However, more cells underwent terminal differentiation in medium CMRL-1066 due to its high calcium concentration than in the modified (low calcium) MEM containing insulin, hydrocortisone, bovine pituitary extract and epidermal growth factor. It was found that tracheal rings can be cryopreserved at -70°C for extended periods; this procedure has greatly facilitated the use of tracheal rings in these studies. Similar techniques were developed for the explant culture of hamster larynx and bronchi in serum-free medium. Studies comparing the susceptibility to carcinogenic responses in various segments of the hamster respiratory tract are ongoing, using treatments with carcinogens having different specificity, in different respiratory tract segments. Tracheal rings removed from hamsters pretreated with diethylnitrosamine were grown in explant culture in serum-free CMRL-1066 and were compared to non-treated controls; preliminary results suggest that carcinogen treatment in vivo appears to facilitate the outgrowth of cells from the tracheal rings. A response to the quantitative in vitro treatment of tracheal rings with 3-methylindole and 2-methylnaphthalene has been determined by comparing the extent of outgrowth of cells from the treated rings to be untreated controls.

Significance to Biomedical Research and the Program of the Institute:

Lung cancer, a leading cause of death, continues to increase in incidence. The hamster respiratory carcinogenesis model, which reflects the histopathology of human lung cancer development, is used as a source of tissues for the cultures. The establishment of an organ culture system, which preserves tissue relationships and the development of replicative epithelial cell cultures, completes the battery of correlated biological models, so that experimental questions can be comparatively investigated at all levels of increasing biological organization. This approach should provide a more precise evaluation of carcinogenic mechanisms in the respiratory tract and contribute to their evaluation in relation to the corresponding human tissue models in vitro and to human pathology in vivo.

Proposed Course:

Studies will be continued to determine optimal conditions for the isolation and culture of hamster laryngeo-tracheo-bronchial epithelial cells in serum-free medium. These studies will focus on the isolation of cells obtained either from explant outgrowths or by enzyme digestions of portions of the respiratory tract. Since preliminary studies using minimal essential medium with low calcium and growth factors have given promising results, this course of study will be continued and extended in order to characterize the cultured cells by morphological and immunocytochemical techniques.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05278-02 LEP

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Serum-free Media and Optimal Conditions for In Vitro Carcinogenesis Studies

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

M. Edward Kaighn Acting Chief, Tissue Culture Section, LEP, NCI

COOPERATING UNITS *(if any)*

Huntington Medical Research Institutes, Pasadena, CA

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Culture Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A major goal of this laboratory is to gain insight into the sequence of molecular and cellular changes which disturb the balance between growth and differentiation during chemical carcinogenesis (See also LEP projects Z01 CP-05276-02 and Z01 CP-05277-02). Several culture systems for normal cells of rodent and human derivation are being used to approach this goal. Human urothelium, mouse keratinocytes and hamster tracheal epithelium in conventional media all undergo squamous terminal differentiation and fail to multiply to a useful extent. To overcome this problem, defined media in which serum is replaced by growth factors have been used successfully in all these systems. In serum-free media substantial cell replication occurs; when switched to media containing serum or increased calcium levels, these cell systems undergo terminal differentiation.

The efficacy of nutrient medium MCDB 402 is also being assessed in the mouse cell line BALB/3T3 cl A31-1-1, not only to obtain appropriate growth control of target cells for transformation studies, but also to provide a baseline for comparison of the growth response of normal and transformed cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Umberto Saffiotti	Chief	LEP, NCI
Corrado Ficorella	Visiting Fellow	LEP, NCI
Federico Bertolero	Visiting Fellow	LEP, NCI
Margherita Bignami	Guest Research	LEP, NCI
Raymond T. Jones	Senior Scientist (IPA)	LEP, NCI

Objectives:

The primary goal is to understand the sequence of molecular and cellular changes leading to alteration of growth and differentiation during chemical carcinogenesis. To approach this goal, several culture systems of both rodent and human origin were chosen. Several of these systems (human urothelium, mouse epidermal keratinocytes and Syrian golden hamster tracheal epithelium) undergo terminal squamous differentiation in conventional cultures with serum. To overcome this problem, serum-free defined media are being developed for all systems. The mouse embryo cell line BALB/3T3 cl A31-1-1, well established for chemically induced transformation, is also used in these studies; because of difficulties associated with the use of different serum lots in this assay, the development of a suitable serum-free culture medium should facilitate quantification and reproducibility of the transformation assay and of related mechanism studies.

Methods Employed:

Keratinocytes from newborn BALB/c mouse epidermis are prepared by cold trypsinization and selective removal of the epidermal layer (according to Yuspa et al.); primary monolayers are then either used immediately or cryopreserved. Primary explant cultures of human urothelial tissues (bladder, ureter) are established in serum-free medium; further outgrowth is obtained by transfer of the tissue to new dishes; the outgrowth is dissociated and either used immediately or cryopreserved. Explant outgrowths of hamster tracheal epithelium are obtained similarly. Phase and electron microscopy are used to monitor cell morphology. To compare the efficacy of serum-free media, growth is measured by increase in cell number or by incorporation of tritiated thymidine into DNA. Keratin is identified histochemically (Rhodamine B) and by immunoperoxidase or immunofluorescence techniques. Gel electrophoresis is employed to identify the profile of keratins synthesized by the various cell types. The BALB/3T3 cell line is used for transformation assays by a standard procedure (see Project Z01 CP 04491-07 LEP); known carcinogens are used in dose response tests in reduced serum and in serum-free media.

Major Findings:

Progress has been made in developing serum-free media for the following four culture systems.

(1) Human urothelium. Work has continued in collaboration with Dr. David Kirk and colleagues (Huntington Medical Research Institutes, Pasadena, CA). A defined serum-free medium has been developed (HMRI-1) that supports primary outgrowth as well as subculture of human bladder and ureter epithelia. HMRI-1 consists of medium MCDB 152 with the addition of 0.1 mM calcium, six growth factors and an extract of the bovine pituitary. The cultured cells were characterized to have epithelial ultrastructural features, normal human chromosomes, HBO surface antigens and keratin. Serum addition or increase in the calcium level were both found to trigger terminal squamous differentiation in this system.

(2) Mouse epidermal keratinocytes. Keratinocytes are isolated from newborn BALB/c mouse skin by use of the trypsin floating technique and differential filtration. These primary suspensions are cultured directly into a serum-free medium supplemented with growth factors. Under these conditions the keratinocytes form monolayers within a week. Primary monolayers are either passed for expansion or cryopreserved. The basal medium was prepared without calcium since this ion is known to induce terminal differentiation in basal epidermal cells. Addition of either calcium or serum was found to induce terminal differentiation. Cellular identity has been verified morphologically and the presence of keratin demonstrated histochemically. Culture conditions are being standardized.

(3) Syrian golden hamster tracheal epithelium. To develop serum-free medium for this target tissue used in carcinogenesis studies (see Project #Z01 CP 05277-02 LEP), the effectiveness of several media formulations for explant outgrowth was tested. CMRL 1066, M-199, MCDB 152 and low calcium MEM were supplemented with growth factors in place of serum. Because of its high calcium concentration, medium CMRL 1066 rapidly induced terminal differentiation in the cell outgrowth, while fewer cells underwent terminal differentation in the epithelial outgrowths of tracheal rings grown in the low calcium MEM containing insulin, hydrocortisone, bovine pituitary extract and epidermal growth factor.

(4) BALB/3T3 cl A31-1-1 mouse embryo cell line. Experiments were designed to eliminate or reduce dependence on serum in the transformation assay with this cell line (see Project #Z01 CP 04491-07 LEP). The results already show the effectiveness of reducing the serum level from 10% to 3%. This was made possible by substitution of MCDB 402 for MEM as basal nutrient. Sustained growth of this BALB/3T3 cell line has been achieved in MCDB 402 with added growth factors. Further experiments are under way using this medium in transformation assays.

Significance to Biomedical Research and the Program of the Institute:

Two major problems are associated with mammalian transformation systems in current use for evaluation of carcinogens and their mechanisms of action: (1) quantification is made difficult by lot-to-lot variations in serum quality; and (2) extrapolations of results between different species as well as different cell-types are of questionable validity. Since most human cancers are epithelial in origin, there is a critical need to develop replicative cultures of human and animal epithelial cells. Experience has shown that epithelial cell types capable of sustained multiplication can be isolated from many tissues if serum supplementation is avoided. This development of defined media will make it possible to isolate appropriate target cells for carcinogenesis studies as well as to eliminate the variability imposed by the use of serum.

Three epithelial cell systems in use in this laboratory (human urothelium, mouse keratinocytes and hamster trachea) undergo a similar terminal differentiation in serum-supplemented media. This effect can be reduced by substituting growth factors for serum and by reducing the concentration of Ca^{++} as shown by others. In normal cells, the relation between terminal differentiation and replication is under delicate control; this balance is shifted in favor of replication by early events in carcinogenesis. Availability of serum-free epithelial systems will make it possible to investigate the molecular changes induced by carcinogens independently of terminal differentiation and of other uncontrolled variables associated with serum use.

Proposed course:

Defined media for serum-free epithelial cultures from human urothelium, mouse epidermis and hamster trachea will be further optimized. Each system will be appropriately characterized by electron microscopy and histochemistry and by gel electrophoretic analysis of the keratin profiles. Since all three systems require bovine pituitary extract, identification of the active fraction of this extract will be initiated. Work will continue to reduce the serum level required for the BALB/3T3 transformation assay.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF HUMAN CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1982 to September 30, 1983

The Laboratory of Human Carcinogenesis conducts investigations to assess (1) mechanisms of carcinogenesis in epithelial cells from humans and experimental animals; (2) experimental approaches in biological systems for the extrapolation of carcinogenesis data and mechanisms from experimental animals to the human situation; and (3) host factors that determine differences in carcinogenic susceptibility among individuals.

The scientific and managerial strategy of the Laboratory is reflected in its organization into three sections, i.e., In Vitro Carcinogenesis Section (IVCS), Carcinogen Macromolecular Interaction Section (CMIS), and Biochemical Epidemiology Section (BES). Scientifically, the emphasis is on the role of inherited or acquired host factors as important determinants in an individual's susceptibility to environmental or endogenous carcinogens and cocarcinogens. Our investigations of host factors involve interspecies studies among experimental animals and humans, cover the spectrum of biological organization ranging from molecules to the intact human organism, and are multidisciplinary, including molecular and cellular biology, pathology, epidemiology, and clinical investigations. Two sections (IVCS and CMIS) devote their major efforts to more fundamental and mechanistic studies. The scientific findings, techniques, and concepts developed by these two sections and, of course, the scientific community at large, are utilized by the BES in selected and more applied studies of carcinogenesis and cancer prevention. The laboratory-epidemiology studies in this section require the expertise found in the IVCS and CMIS and in the NCI Epidemiology Program. Resources needed by the Laboratory are unique and complex. For example, collection of viable normal as well as neoplastic epithelial tissues and cells - well characterized by morphological and biochemical methods from donors with an epidemiological profile - requires the continued cooperation among donors and their families, primary care physicians (internists, surgeons, house staff), surgical pathologists, nurses, epidemiologists, and laboratory scientists.

Remarkable progress has been made during the last few years by this and other laboratories in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. We have developed chemically defined media for long-term culture of human bronchus, colon, esophagus, and pancreatic duct. Primary cell cultures of human epithelial outgrowths have been obtained from many different types of human tissues. Isolated epithelial cells from human bronchus and esophagus can be transferred three or more times and can undergo more than 30 cell divisions. Human bronchial epithelial cells can also be grown in serum-free culture medium. Morphological, biochemical, and immunological cell markers have been used to identify these cells as unequivocally of epithelial origin.

The availability of nontumorous epithelial tissues and cells that can be maintained in a controlled experimental setting offers an opportunity for the study of many important problems in biomedical research, including carcinogenesis. For example, the response of human bronchial epithelial cells (enhanced growth or differentiation) after either exposure to carcinogens and/or tumor promoters or DNA transfection by oncogenes is being actively investigated. Parallel investigations using epithelial tissues and cells from experimental animals allow investigators to study interspecies differences in response to carcinogens, cocarcinogens, and anticarcinogens. Clonal growth of normal human pleural mesothelial cells in serum-free culture medium has also been achieved so that the in vitro transformation by asbestos of these cells can be studied.

Metabolism of Chemical Carcinogens and Formation of Carcinogen-DNA Adducts. One important use of cultured human tissues has been in the investigation of the metabolism of chemical carcinogens because (1) many environmental carcinogens require metabolic activation to exert their oncogenic effects; (2) the metabolic balance between carcinogen activation and deactivation may, in part, determine a person's oncogenic susceptibility; and (3) knowledge of the comparative metabolism of chemical carcinogens among animal species will aid efforts to extrapolate data on carcinogenesis from experimental animals to humans. We and our coworkers have systematically examined the metabolism of procarcinogens of several chemical classes which are considered to be important in the etiology of human cancer. Procarcinogens of several chemical classes can be activated enzymatically to electrophilic reactants that bind covalently to DNA in cultured human tissues. The studies of activation and deactivation of representative procarcinogens have revealed that the metabolic pathways and the predominant adducts formed with DNA are generally similar between humans and experimental animals. Wide quantitative interindividual differences (50- to 150-fold) are found in humans and other outbred animal species. When the metabolic capabilities of specimens from different levels of biological organization are compared, the profile of benzo[a]pyrene metabolites is similar in cultured tissues and cells, but subcellular fractions, e.g., microsomes, produce a qualitative and quantitative aberrant pattern.

The metabolism of benzo[a]pyrene was studied in both epithelial and fibroblastic cells initiated from the same bronchus specimens. The total metabolism of benzo[a]pyrene and binding of its ultimate carcinogenic metabolite was 3-fold higher in the epithelial than in the fibroblast cells. No qualitative differences in the metabolic profile of benzo[a]pyrene between the explant culture and the epithelial cell cultures were observed.

To test the interactive effects of cell types in the metabolic activation of carcinogens and to further assess interindividual differences among people, human tissue- and cell-mediated mutagenesis assays have been developed. The fact that terminally differentiated cells, such as pulmonary alveolar macrophages, can activate benzo[a]pyrene and mediate an increase in frequencies of mutations and sister chromatid exchanges in cocultivated "detector" cell populations, i.e., Chinese hamster V79 cells, suggests that nontarget cells of chemical carcinogens may play an important role in the activation of environmental carcinogens.

Because the specific carcinogenic agents responsible for the etiology of colon cancer are not known, we are systematically studying the metabolic activation of several chemical classes of carcinogens. The following carcinogens are converted by cultured human colon to metabolites that bind to DNA: benzo[a]-pyrene; 6-nitrobenzo[a]pyrene; aflatoxin B_1; N-nitrosodimethylamine; 1,2-di-methylhydrazine; and 3-amino-1,4-dimethyl-5H-pyride(4,3-b) indole. The latter compound is formed by pyrolysis of tryptophan. During the next fiscal year, we plan to study several other amino acid pyrolysis products because of their high potential as colonic carcinogens in humans.

In order to understand the interaction of complex chemical mixtures, such as tobacco smoke and diesel exhaust, with biological systems, the p32 postlabeling method of Randerath has been modified. Several different carcinogen-DNA adducts were formed when tobacco smoke condensate was incubated with DNA in the presence of liver microsomes. The identification of these adducts are presently being attempted.

The extrapolation of data from studies of N-nitrosamine carcinogenesis between experimental animals and humans is a pressing problem. Abundant evidence of N-nitrosamine carcinogenesis from both in vitro and in vivo studies using experimental animals has accumulated. Although N-nitrosamines are widespread pollutants, the carcinogenicity of these chemicals in humans has been difficult to prove by epidemiological studies. In vitro studies comparing pathobiological responses of N-nitrosamines in humans and experimental animals offer an approach to solve this problem at least at the cellular and tissue level of biological organization. N-Nitrosamines can be metabolized by cultured human epithelial tissues and cells. Quantitative differences in metabolism and alkylation of DNA are found among humans, among various organs within an individual, and among adult versus fetal tissues. Whether these differences are sufficient to influence an individual's cancer risk and organ site is as yet unknown.

DNA Repair. Although DNA repair has been extensively studied in human fibroblasts, lymphoid cells, and neoplastic cells, little information is available concerning DNA repair in normal human epithelial cells. Using the methodology to culture human bronchial epithelial and fibroblastic cells developed in our laboratory, we have initiated studies to investigate DNA damage and repair caused by chemical and physical carcinogens as examined by alkaline elution methodology, BND cellulose chromatography, unscheduled DNA synthesis, and high pressure liquid chromatographic analysis of the formation and removal of carcinogen-DNA adducts. As we reported last year, human bronchial epithelial cells repair single-strand breaks in DNA damaged by X-radiation, UV-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines at rates similar to bronchial fibroblasts.

During metabolic activation, N-nitrosodimethylamine yields equal molar quantities of methyl carbonium ions and formaldehyde. Both of these metabolites can react with nucleophilic sites in cellular macromolecules, carbonium ions by alkylation, and aldehydes via formation of unstable alkyl-ol derivatives preferably with amine groups ($R\text{-}HN\text{-}CHOH\text{-}R_1$). The monomethylol derivatives of formaldehyde can form intermediary labile products that by secondary reaction can yield stable methylene bridges between macromolecules. Although the alkylating metabolites of N-nitrosamines and their cytotoxic, mutagenic,

and carcinogenic effects have been extensively studied, the possible contribution of other metabolites, especially aldehydes, has not received much attention. We have investigated the effect of formaldehyde on the repair of X-ray-induced single-strand breaks. Human bronchial cells were exposed to X-rays and then incubated with or without the presence of formaldehyde, and the repair of DNA single-strand breaks was measured. The presence of formaldehyde significantly inhibited the repair of the X-ray-induced single-strand breaks correlating with the potentiation of cytotoxicity in human cells and mutation frequency in Chinese hamster V79 cells by combinations of the agents. Formaldehyde, a common environmental pollutant and metabolite of carcinogenic N-nitrosamines, also inhibits repair of O^6-methylguanine, decreases O^6-alkylguanine transalkylase activity, is mutagenic at high concentrations (> 100 μM) and potentiates the cytotoxicity and mutagenicity of the methylating agent, N-methyl-N-nitrosourea in normal human cells, Exposure to formaldehyde may lead to the dual genotoxic mechanism of both directly damaging DNA, i.e., formation of DNA-protein crosslinks and single-strand DNA breaks, and inhibiting repair of mutagenic and carcinogenic DNA lesions caused by alkylating agents and physical carcinogens.

The molecular events and their fidelity during DNA repair in human cells are unknown. New techniques in molecular and cellular biology provide new opportunities to investigate the mechanisms of DNA repair. For example, human DNA fragments are being transfected into xeroderma pigmentosum cells by a procedure developed by LHC staff and DNA repair-sufficient cells selected by ultraviolet irradiation. Using the unique cotransfected microbial sequences as molecular tags, the transfected human DNA repair gene(s) are being isolated and identified.

Interactive Effects between Chemical Carcinogens and Hepatitis B Virus in Liver Cancer Carcinogenesis. Liver cancer incidence is high in areas with both food contamination by carcinogens, such as aflatoxin B_1, a liver carcinogen in experimental animals, and a high incidence of chronic active viral hepatitis. Due to the insensitivity of epidemiological methods, the role of single agents or combinations of agents is uncertain. Our long-term goal is to study the interactive effects of hepatitis B virus and chemical carcinogens, such as aflatoxin B_1, in the malignant transformation of cultured human hepatocytes. During this fiscal year, and in collaboration with coworkers at the Cancer Institute, Beijing, People's Republic of China, methods to culture tissue explants and epithelial cells from fetal human liver have been developed. Metabolism of chemical carcinogens has been investigated, and the role of core antigen gene in the cytopathology of hepatitis B virus has been studied.

In order to access the role of chemical carcinogens, e.g., aflatoxin B_1, in human liver carcinogenesis, we have initiated several projects with coworkers at the Cancer Institute, Beijing, People's Republic of China. For example, the metabolic activation of aflatoxin B_1 and other carcinogens, such as N-nitrosodimethylamine and benzo[a]pyrene, have been investigated using cultured human fetal liver explants. One major aflatoxin B_1-DNA adduct was formed by addition of aflatoxin B_1-2,3oxide to the 7-position of guanine. This reaction product is unstable, and the imidazole ring of the guanine will open to stabilize the molecule. The major aflatoxin B_1-DNA adduct was similar to the one formed in fetal human liver explants and in rat liver in vivo, an organ susceptible to the carcinogenic action of aflatoxin B_1.

In a second related project, we collected urine samples in Murang'a district, Kenya, for analysis of aflatoxin B_1-guanine, a "nucleic acid repair product." It has previously been shown that food samples collected in this district are known to be contaminated with aflatoxin B_1, and a positive correlation exists between the dietary intake of aflatoxin B_1 and the incidence of liver cancer. The urine samples collected at the out-patient clinic of Murang'a district hospital were concentrated on C_{18}Sep-Pak columns, and aflatoxin B_1-guanine was isolated by high pressure liquid chromatography in two different systems. Eight of 106 samples had a detectable level of a compound whose synchronous fluorescence spectrum was identical to chemically synthesized aflatoxin B_1-guanine. The spectrum did not show any bathochromic shift when pH was made alkaline. These results are an indication of interactions between the ultimate carcinogenic form of aflatoxin B_1 and cellular nucleic acids in vivo and further support the hypothesis that aflatoxin B_1 may play an important role in the etiology of human liver cancer.

We are also investigating the cytopathological and oncogenic effects of hepatitis B virus. Using protoplast fusion method to transfect human cells with pSV2-derived plasmids at frequencies greater than 10^{-3}, it is possible to test the biological effect of hepatitis B virus genes independent of the viral structures required for infection. A pSV2gpt$^+$ plasmid constructed to carry a subgenomic fragment of hepatitis B virus that contained the core antigen gene (HBc gene) was transfected into human cells. The cytopathologic effects of HBc gene expression were observed immediately after the transfection. A human epithelial cell line was stably transfected with the HBc$^+$ gene by selecting recipient cells for gpt$^+$ expression. The gpt$^+$/HBc$^+$ cell line was used to determine that growth in serum-free medium or 5'-azacytidine treatment stimulates the production of the HBc gene product (HBcAg). These factors were used to test a hepatocellular carcinoma that has carried the entire hepatitis B virus genome since its isolation from a chronically infected patient. This cell line was stimulated to produce the HBc gene product in response to the same factors that stimulated HBcAg production in the gpt$^+$/HBc$^+$ cell line constructed by transfection. The temporal relationship of the cytopathologic response to HBc gene expression was similar for both cell types, indicating a primary role for HBc gene expression in the cytopathology of hepatitis B virus-infected human liver. Such information may be useful for development of therapeutic regimens for chronically infected patients and may provide insight into the biological nature of the virus at the genetic and molecular levels. Methylation of cytosine in 5' flanking DNA sequences of the hepatitis B virus core antigen gene appears to control its expression. Since the core antigen may be responsible for the cytopathologic effects of hepatitis B virus, controlling core antigen gene expression by methylation could give preneoplastic and neoplastic liver cells a selective growth advantage during carcinogenesis. Consistent with this hypothesis is our recent result showing methylation of the promoter-enhancer DNA sequences of the core antigen gene which inhibits its expression in a human hepatocellular carcinoma.

Cellular Differentiation and Transformation. Our operational definitions of normal, premalignant, and malignant cells are biological, e.g., differentiated state, growth, altered cellular affinities and architecture, and tumorigenicity when injected into the appropriate host. Methods for the culture of human epithelial tissues and cells provide an opportunity to investigate the biology and molecular mechanisms of carcinogenesis directly in human target cells and

to conduct studies comparing carcinogenesis in cells from experimental animals and humans.

We have focused our primary attention on two sites of human cancer, i.e., bronchus and esophagus. As noted above, our initial effort was devoted to developing methods to culture and unequivocally identify human epithelial cells. We are now studying the factors controlling growth and differentiation of these normal cells and their malignant counterparts; the ability to culture these normal and malignant cells in chemically defined media is essential for such studies. For example, blood-derived serum, platelet lysates, or calcium ions (> 1 mM) and small amounts of serum induce terminal squamous differentiation in the normal bronchial epithelial cells, but not in carcinoma cells which continue to grow and, in some cases, grow at a faster rate. This information is being used in the design of in vitro carcinogenesis experiments in which these inducers of terminal differentiation are being used in a strategy to provide selective advantage of preneoplastic and neoplastic cells. Comparative studies of normal and malignant cells also reveal a striking difference in their pattern of cytoskeleton proteins, including keratins, and their production of polypeptide hormones. In addition, remarkable differences in keratin patterns were noted among adenocarcinomas, squamous cell carcinomas, and small cell carcinomas of the lung.

We have recently initiated studies of the effects of tumor promoters and cocarcinogens on cultured human epithelial cells. For example, data have been obtained for phorbol, 12-O-tetradecanoylphorbol13-acetate (TPA), teleocidin B, 2,7-dichlorgdibenzo-p-dioxin (DCDD), and 2,3,7,8-tetrachlorodibenoz-p-dioxin (TCDD) (10^{-7} M, 10^{-8} M, and 10^{-9} M) and for cigarette smoke condensate (0.1, 1, 10 µg/ml), catachol, benzo[a]pyrene, benzo[e]pyrene, and pyrene (1.5×10^{-6}, 10^{-7}, 10^{-8} M). The effects of teleocidin B, TPA, phorbol, TCDD, and DCDD were each assessed by cellular morphology, clonal growth rate, and enzyme assays, i.e., plasminogen activator (PA), ornithine decarboxylase (ODC), and aryl hydrocarbon hydroxylase (AHH). Bronchial epithelial cells were inoculated into a maintenance medium (LHC-0) or a growth medium (LHC-4). Teleocidin B and TPA had similar effects on growth, morphology, and enzyme activities. At > 1 nM, squamous differentiation ensued; division ceased, cells became larger with dense nuclei, cell boundaries became indistinct, and cross-linked envelopes formed. Teleocidin B or TPA caused an elevation of ODC activity in LHC-0 and a decrease of ODC in LHC-4. Both compounds caused an increase in PA and a decrease of AHH activity in both media. TCDD caused a 15% decrease in cell growth at 100 nM, and increased ODC and AHH in both media, but increased PA only in LHC-4. DCDD did not alter growth, but its biochemical effects were similar to, although less marked than, those of TCDD. In summary, TPA and teleocidin B cause similar effects in bronchial epithelial cells, TCDD induced both ODC and AHH in these cells, and the cellular response to these agents is altered by composition of the medium. Cigarette smoke condensate and catachol caused an increase in AHH (138% and 126%, respectively) and had no effect on formation of cross-linked envelopes. Cigarette smoke condensate had no effect on PA, while catachol caused a slight decrease (79%). Benzo[a]pyrene or pyrene caused a decrease in AHH (87% and 78%, respectively), an increase in PA (133% and 124%, respectively), and had no effect on crosslinked envelopes. Benzo-[e]pyrene had little effect on any of these tests.

A group of compounds which enhance intracellular cyclic AMP levels increase ODC activity and some also increase clonal growth. Many of these compounds were found to increase ODC activity in LHC-O without increasing clonal growth. Such compounds include cholera toxin (10^{-8} M), isobutylmethylxanthine (10^{-6} M), forscolin (10^{-6} M), L-epinephrine (1.5×10^{-6} M), and isoproterenol (1.5×10^{-6} M). The β-adenergic hormones were investigated more extensively. A synergistic interaction among undefined factors in pituitary extract, epidermal growth factor, and adrenergic hormones to stimulate clonal growth of the normal human bronchial epithelial cells is supported by our findings. L-Epinephrine or isoproterenol do not stimulate clonal growth in the absence of pituitary extract or epidermal growth factor, but in the presence of these compounds, clonal growth increases from 0.95 population doublings per day (PD/D) to 1.2 population PD/D. These findings also indicate that since ODC is increased by the β-adenergic hormones in LHC-O without an increase in clonal growth, the increase in ODC activity is necessary but not sufficient for cell division. Of the 25 compounds tested thus far for effects on clonal growth, one has been found to stimulate the growth of cells in the absence of epidermal growth factor or pituitary extract. Bombesin at 0.15 μg/ml stimulates an increase in the clonal growth of normal human bronchial epithelial cells from 0.4 PD/D to 0.7 PD/D. In contrast to epidermal growth factor, this increase in growth is not accompanied by an increase in cell migration. Bombesin also increases the colony forming efficiency. When cells are grown in the presence of epinephrine, it has been found that the synergistic properties of pituitary extract can be partially replaced by bombesin at 0.15 μg/ml and endothelial cell growth supplement at 20 μg/ml.

The pattern of 5-methylcytosine residues in mammalian DNA has recently been found to be crucial to the control of genetic expression. Decreases in DNA 5-methylcytosine content are known to alter the level of differentiation of cells in culture. Thus, changes in DNA 5-methylcytosine patterns may be critical to the process of carcinogenesis. Human tumor DNA is being probed for DNA methylation pattern alterations in selective DNA sequences and genes. Since chemical carcinogens have been shown to decrease genomic 5-methylcytosine levels in BALB/3T3 cells, DNA from carcinogen-treated human epithelial cells is also being probed for changes in 5-methylcytosine patterns. New methods to assess 5-methylcytosine content in non-dividing differentiated human cells are being developed.

Epidemiological studies have established that exposure to asbestos fibers is the primary cause of mesothelioma in the industrialized world. Because the latency period for this disease averages 40 years and because there has been a marked increase in the use of asbestos during and since World War II, an epidemic of mesothelioma has been predicted for the latter part of this century. Carcinogenesis studies with animals have shown that mesothelioma can be caused by intrapleural or intraperitoneal injections of asbestos. However, the long-term effects of asbestos fibers on human mesothelial cells in culture have not been reported previously. To study this important problem, methods to culture replicative normal mesothelial cells from adult human donors have been developed. The cells contained keratin and hyaluronic acid-mucin, exhibited long, branched microvilli, and retained the normal human karyotype until senescence. The mesothelial cells were 10 and 100 times more sensitive to the cytotoxicity of asbestos fibers than were bronchial epithelial or fibroblastic cells, respectively, from normal adult humans. Exposure of the mesothelial

cells to amosite asbestos caused chromosomal rearrangements, including dicentrics. These aneuploid mesothelial cells have an extended population doubling potential of more than 35 divisions beyond the culture life span (30 doublings) of the control cells. Mesothelial cells have distinct keratin proteins and have a remarkable ability to regulate their cytoskeletal composition; the content of keratin or vimentin in the cytoskeleton reflects the growth conditions. We speculate that this uniquely fluid cytoskeleton may be more easily perturbed by penetrating asbestos fibers than are the cytoskeletons of other cell types and that this leads to an increased risk for chromosomal instability and transformation.

Another important problem in human carcinogenesis concerns the mechanism responsible for the cocarcinogenic effect of asbestos in enhancing the tumorigenicity of tobacco smoke in the bronchial epithelium. We and our coworkers have initiated investigations to define the effects of asbestos on cultured bronchial epithelial tissues and cell. The differential cytotoxic activity of various asbestos and glass fibers was estimated by measuring the inhibition of epithelial cell growth as a function of fiber concentration. The data show that various fiber types have different effects on human bronchial epithelial cells. Chrysotile was extremely toxic; amosite and crocidolite were less toxic; glass fibers were only mildly toxic. For comparison, human bronchial fibroblastic cells were also exposed to fibers and were found to be markedly more resistant (more than 10-fold) than the epithelial cells to all the types of asbestos tested.

Monolayers of bronchial epithelial cells were used to investigate the effect of amosite asbestos at the cellular level. With scanning electron microscopy and high voltage electron microscopy, amosite fibers were ingested by human bronchial epithelial cells. In contrast to macrophages, the fibers penetrated the surface of epithelial cells without the development of filopodia. While macrophages seem to phagocytose fibers along both the long and short axes, epithelial cells seem to take up fibers along the short axis with only a membrane sleeve surrounding each fiber. Examination by scanning high voltage and transmission electron microscopy with associated energy dispersive X-ray spectra clearly revealed that short fibers ($< 12\ \mu m$) were taken up quickly by the cells. Asbestos was present within the cells by 2 hours after exposure, and by 28 hours, many fibers were found in the cytoplasm and occasionally in the nucleus.

Asbestos fibers induce abnormal cell growth. Addition of amosite asbestos (10, 100, or 1,000 $\mu g/ml$) to human respiratory mucosa in explant culture caused numerous focal lesions including squamous metaplasia and dysplasia. When examined by scanning electron microscopy, the epithelial lesions appear as focal elevations of nonciliated cells. Cytopathological aberrations of the bronchial epithelial cells were manifested by cellular polymorphism and variation in nuclear size. Since it is important to know whether there are asbestos fibers in the cytoplasm of cells involved in the lesions, studies are now being done using X-ray microanalysis in combination with transmission electron microscopy including high voltage electron microscopy. These studies are being extended to determine the progression of these lesions and eventually their malignant potential.

Activation and/or modification of cellular oncogenes is likely to be important in carcinogenesis, especially in the latter phases of this multistage process. Because the vast majority of the studies utilized an interspecies assay, i.e., transfection of human tumor DNA into mouse NIH 3T3 cells, we are using human epithelial cells as recipients of oncogene DNA transferred into the cells by a modified protoplast fusion method or carried as part of an oncogenic viral vector. Transfection of primary human bronchial cultures with plasmids carrying either the vKras or vHras oncogenic complementary DNA results in alteration of cell growth properties. These experiments have demonstrated that epithelial cells become "serum resistant" in LHC-4 growth medium at a frequency of 10^{-4} to 10^{-5} units. Experiments with complementary sets of oncogenes are currently in progress. We have isolated "potentially transformed" cells after transfection with vHras but not vKras. The origin of these cells (i.e., epithelial, fibroblastic), the presence of vHras gene, and production of p21 transforming factor in these cells is being assessed. The protoplast fusion method described for high-frequency transfection of human cells also provides a method for application to general problems in human somatic cell genetics. The ability to transfect genes at frequencies greater than 10^{-3} is sufficient to attempt isolation of single copy genes from genomic libraries linked to selectable markers.

Using the second approach, human bronchial epithelial cells have been infected by Kirsten sarcoma virus with an amphotropic murine leukemia virus helper. This viral infection results in epithelial cell colonies which do not differentiate in response to serum as do normal epithelial cells. These transformed cells also have extended growth, but no cell lines have yet been isolated which grow indefinitely. The presently achieved efficiency of transformation in this system is about 0.2% that of transformation of mouse NIH 3T3 cells by the same virus.

Biochemical Epidemiology. The primary goal of biochemical and molecular epidemiology is to identify individuals at high cancer risk by obtaining pathobiological evidence of (1) high exposure of target cells to carcinogens and/or (2) increased host susceptibility due to inherited or acquired factors. Laboratory methods have been recently developed to be used in combination with analytical epidemiology to identify individuals at high cancer risk. These methods include: (1) techniques to assess specific host susceptibility factors; (2) assays that detect carcinogens in human tissues, cells, and fluids; (3) cellular assays to measure pathobiological evidence of exposure to carcinogens; and (4) methods to measure early biochemical and molecular responses to carcinogens.

Currently available techniques exist that would allow the utilization of biochemical and molecular measures to better characterize exposure to carcinogens, to serve as intermediate end points on the path to malignancy, to identify measures which halt or reverse this process, and to investigate the mechanisms of human carcinogenesis. Included in these investigations would be the following: (1) efforts to evaluate body burden of chemical carcinogens in studies of occupational and general environmental cancer risk factors; (2) sophisticated analyses of air, water, and biological specimens for carcinogenic and mutagenic substances in conjunction with specific analytical studies; (3) search for evidence of viral infection including viral segments or oncogenes in the DNA of individuals at high risk of cancers that may be associated with infectious agents or heritable states; (4) evaluation

of disturbances in immune function as they may relate to malignancies, particularly those of the hematopoietic system; (5) investigation of the relationship between micronutrients and a variety of epithelial cancers; and (6) determination of the relationship between macronutrients, including dietary fat and subsequent hormonal changes, to subsequent risk of breast, endometrial, and perhaps colon cancer.

Examples of these multidisciplinary studies in cancer etiology are described. First, the finding of wide differences in carcinogen metabolism and amount of carcinogen-DNA adducts among individuals suggested the hypothesis that the metabolic balance between activation and deactivation of chemical procarcinogens may determine in part an individual's oncogenic susceptibility. We are comparing the metabolism of benzo(a)pyrene including the formation of DNA adducts in nontumorous bronchial mucosa from donors with or without lung cancer. The binding values of benzo(a)pyrene to DNA are higher in nontumorous tissues from patients with squamous cell carcinoma when compared to those with adenocarcinomas or no cancer. Binding values were also higher in individuals who have a family history of cancer. Additional cases are being collected in this laboratory-epidemiology study.

Our investigations of carcinogen metabolism in cultured human cells revealed that the major carcinogen-DNA adducts formed are identical to those found in experimental animals in which the chemical is carcinogenic. This important finding has encouraged us and others to search for such adducts in people exposed to environmental carcinogens. Using hybridoma and rabbit produced antibodies specific for selected carcinogen-DNA adducts and highly sensitive enzyme radioimmunoassays and complementary biophysical approaches, e.g., photon counting synchronous fluorimetry, we are currently examining specimens from populations known to be exposed to carcinogens, e.g., benzo[a]pyrene, and aflatoxin B_1. For example, roofers who work with hot asphalt are exposed to high levels of polynuclear aromatic hydrocarbons including benzo[a]pyrene. Antibodies detecting benzo[a]pyrene-DNA antigens have been used to demonstrate this adduct in peripheral blood cells from many, but not all, workers. The significance of this interindividual difference is as yet unknown. In addition, the formation of carcinogen-DNA adducts may represent only the initial stages of carcinogenesis and measures of later stages, i.e., tumor promotion and progression, are also needed to predict an individual's cancer risk.

Classic genetic linkage studies have been used to predict the risk of developing diseases. Until recently, disease-associated polymorphisms could be assessed only by analyzing gene products, e.g., cell surface histocompatibility antigens or blood group substances commonly referred to as A,B,H. Advances in molecular biology have now made it possible to measure genetic polymorphisms at the DNA level. This approach utilized restriction enzyme catalyzed endonucleolytic cleavage and DNA hybridization with gene-specific probes to detect base-pair substitution and fragment length polymorphisms. The potential of this technology is exemplified by the recent identification of individual DNA polymorphisms associated with diabetes mellitus and hemoglobinopathies. A similar molecular approach using specific DNA probes, e.g., oncogenes or ectopic hormones, is being used to potentially detect and characterize DNA polymorphisms in individuals who are oncogenetically predisposed.

A human C-type retrovirus has been found to be associated with certain adult T-cell malignancies. Lymphocytes from these patients were established in long-term tissue culture using the human T-cell growth factor. Studies were carried out to examine the cell surface markers of cells infected and with producing the human T-cell lymphoma virus (HTLV). HLA typing was performed on these cells. The virus was transferred to human umbilical cord blood lymphocytes by coculture. Cytotoxic lymphocytes were developed against an autologous cell line established from one of the patients. Cell lines established from patients with the adult T-cell leukemias and lymphomas expressed more than the expected two alloantigens controlled by the HLA-A or HLA-B locus. Alloantisera detecting these altered determinants were confined to the HLA-AW19 cross-reactive group and the HLA-B5 cross-reactive group. A monoclonal antibody detecting a polymorphic epitope on HLA alloantigens of these two cross-reactive groups was developed by Dr. Bart Haynes. This monoclonal antibody was found to react with all cells infected with HTLV and producing products of this virus. Human umbilical cord lymphocytes infected with the virus by coculture also expressed altered HLA alloantigenic determinants. These alterations mirrored those seen with tumor cell lines established from patients. Thus, the appearance of neoantigens suggests an association of HTLV provirus replication and HLA alloantigenic expression. Cell surface markers were examined on cell lines established from patients and cell lines established by coculturing with the human T-cell lymphoma virus. These cells were predominantly OKT4+ (helper phenotype). These cells also expressed increased HLA-DR and an antigen detected by the Tac antibody which detects the receptor for T-cell growth factor. A cytotoxic T-cell line, established from a patient with the HTLV-associated disease, killed autologous cells and another cell line, but was not cytotoxic for other cell lines established from patients with HTLV. The cytotoxicity appeared to be genetically restricted by HLA determinants B8 and DR3. These investigations are providing new insights into the host factors that influence the pathogenesis of this disease.

Descriptions of these and other findings are given in more detail in the individual project reports that are on file, Office of the Director, Division of Cancer Cause and Prevention.

Other Activities. The Laboratory has been responsible for training intramural and extramural investigators in the techniques for (1) culturing human epithelial tissues and cells and (2) enzyme immunoassays to measure carcinogen-DNA adducts and oncofetal antigens. Members of the staff have also coorganized and/or served on the program committees of both national and international scientific meetings. These meetings include "Use of Human Tissues and Cells in Carcinogenesis Studies," "Control of Growth by Nutrients," "1st International Meeting on Cellular Biochemistry and Function." During this fiscal year, members of the staff have also served as reviewers and on editorial boards of several journals and on intramural and extramural committees, e.g., NIH Handicapped Employees Advisory Committee, Environmental Pathology Committee of the International Academy of Pathology, Cellular Physiology Grant Review Study Section, and Honor B. Fell Division, Tissue Culture Committee.

MARYLAND, UNIVERSITY OF (NO1-CP-15738)

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Current Annual Level: $408,001 (FLAT RATE)

Man Years: 9.17

Objectives: To provide a resource to NCI for the obtainment, transport, and characterization of normal, preneoplastic, and neoplastic tissue from the human bronchus, pancreatic duct, colon, and liver from patients with an epidemiological profile.

Major Contributions:

Bronchus: The contractor provides the following characterization:

1) Morphological and histochemical characterization of human primary lung carcinomas are continuing. Epidemiologic data are studied to determine the relationships between lung tumor type, selected risk factors, and the amount of benzo[a]pyrene (B[a]P) bound to DNA by the same patient's non-cancerous bronchial epithelium.

2) Characterization of tissues by immunocytochemistry has continued using the peroxidase-antiperoxidase method to demonstrate the presence or absence of various antigens. Tumor and normal tissues, abnormal, and preneoplastic tissues are examined for beta human chorionic gonadotropin (HCG), calcitonin, adrenocorticotropic hormone (ACTH), serotonin, alphafetoprotein (AFP), keratin, somatostatin, neuron specific enolase (NSE), calmodulin, and tubulin. Normal and abnormal (but non-neoplastic) adult bronchi contain only mucosubstances, keratin, calmodulin, and tubulin. Keratin is seen only in aldehyde-fixed bronchi, if the epithelium is metaplastic, in the basal layer of ethanol-fixed normal bronchus, epithelium (including bronchial glands), and occasionally in columnar cells that reach the lumen. Calmodulin apparently increases levels at the cell borders. Lung tumors have greater heterogeneity than tumors in the bronchial epithelium. Each marker is found in at least an occasional tumor;

HCG is found in 80% of non-small cell tumors, and keratin is found in 75% of such tumors. Somatostatin is seen in keratinizing areas and more diffusely in a smaller proportion of adenocarcinomas. NSE and serotonin are seen in endocrine tumors only. Appearance of other markers shows less predilection for types of lung tumors, although HCG seems to follow glycogen distribution.

3) Indirect immunofluorescent detection of tubulins was performed on cellular outgrowths of bronchial explants involved and non-involved with tumor. Generally uniform, the non-malignant cells had mostly straight microtubules originating from assembly sites near the nucleus, while the variable tumor cells had irregular microtubular patterns in a mesh-like arrangement.

Colon: All tissues were characterized as described below:

1) Morphological [light microscopy (LM), transmission electron microscopy (TEM), scanning electron microscopy (SEM), and histochemical examinations of normal, premalignant, and malignant human epithelium continue. The comprehensive description of the morphology of normal human colon is still incomplete, and some of the previously reported morphological markers of premalignancy may ultimately be declared normal aspects of different tissue segments. This possibility is suggested in electron microscopy (EM) data showing three colonic cell types (undifferentiated, endocrine, and mucous), in ascending segments, apical vesicles are EM dense on electron microscopic examination, but in the rectum they are EM lucent. By light microscopy, using histochemical stains, there are differences seen in ascending, transverse, and descending colon and rectum. AB-PAS staining of ascending colon reveals many mucous cells with mixed magenta and blue-purple staining; in the rectum, almost all cells stained blue, indicating highly acid mucous. HID-AB staining showed a large proportion of cells in all four regions staining brown-black, indicating high amounts of sulphomucin.

2) Human ascending, transverse, and rectal colonic epithelium from immediate autopsies are being maintained routinely in explant culture and provided to NCI for xenotransplantation.

Pancreas: Pancreatic ducts from immediate autopsies were collected and were perfused for cell isolation and culture. This tissue was discontinued as a neccessary exclusion due to the rising cost of providing the resource and our decreasing ability to fund the full activity. (This contract is flat funded and, as such, encompasses a time-related decreasing level of effort.) All tissues received were characterized as described below.

Pancreatic tissues from immediate autopsy were examined by morphological techniques, histochemistry, immunohistochemistry, and freeze fracture. Pancreatic ducts were maintained using the contractor's organ explant and cell culture techniques. Routine autopsy and surgically derived tissues were examined to elucidate cellular alterations in pancreatic cancer. Previously, 40 cases (18 autopsy cases and 22 surgical cases) were under study including both primary and metastatic tumors. Over 90% of these nonendocrine tumors looked like duct cell adenocarcinomas in routine light microscopy; however, selected cases were under study by electron microscopic mucosubstances, keratin, CEA, AFP, HCG, and calcitonin.

Liver: Samples were collected and portions stored at -70°C. The methods for culturing liver tissue and cells are still under study, as indicated below.

Comparison of methods for the primary culture of human hepatocytes and rat hepatocytes are being continued using different media and substrates, including human liver biomatrix. Electron microscopy of zero-time samples is used to assess the viability of liver tissue at the time of perfusion. Comparisons are being made for optimal cell isolation between perfusion of intact lobes and wedge-shaped sections of lobes. Pieces of liver are also quick frozen in liquid nitrogen for subsequent use in metabolic studies at NCI. Preliminary results indicate that primary cultures of human liver cells can provide a mechanism for studying chemical metabolism and mutagenesis.

Abstraction of Medical Records and Obtainment of Donor Histories: From the beginning of this study, the status of completion is as follows:

	Medical Records Abstracted	Patients Interviewed	Data Coded
Bronchus	439	312	358
Colon	298	251	272
Total	737	563	630

Proposed Course: During the coming year, emphasis will continue on providing this unique resource to NCI.

MARYLAND, University of (NO1-CP-31008)

Title: Resource for Human Esophageal Tissues and Cells from Donors with Epidemiological Profiles

Current Annual Level: $76,495

Man Years: 1.2

Objectives: To provide tissue specimen and cells of human esophagus to the Laboratory of Human Carcinogenesis for carcinogenesis studies.

Major Contributions: Specimens have been collected and characterized by the contractor. An epidemiological profile of the donors has been provided whenever possible. Tissues from the ME sources possess chance viability in organ cultures, which decreases rapidly with time after death. Esophagi collected more than 8 hours post mortem are unlikely to survive in vitro.

Also in this period, the cell banking facility, an integral part of the new contract, has been established. Techniques for primary suspension cultures of cells from normal and malignant esophageal mucosa and for freezing and thawing cell stocks in viable condition are now routine. Cells were obtained from 64 normal human esopohagi and 5 esophageal cancers. Currently, there are 308 vials of frozen stock, including 172 epithelial cell suspensions (166 normal, 6 malignant), 24 3T3 feeder cells, and 112 sarcoma-180 mouse tumor cells for tumor-conditioned medium. Monolayer cultures are being developed according to the methods of Dr. Susan Banks-Schlegel. Explants from 40 "normal" uninvolved and 40 malignant human mucosa were cultured.

Morphological, cytochemical, and immunocytochemical characteristics were determined for each tissue and will be delivered to NCI on request. Assays are also being conducted to determine specific biochemical markers occuring in normal, premalignant, and malignant human esophageal epithelium.

Proposed Course: The current tasks, including the in vitro testing of the quality of the cells now being generated and stored for in vitro carcinogenesis experimentation, will be continued and improved.

GEORGETOWN UNIVERSITY (NO1-CP-31007, successor to NO1-CP-05707)

Title: Collection and Evaluation of Human Tissues and Cells from Donors with an Epidemiological Profile.

Current Annual Level: $56,066

Man Years: 0.96

Objectives: To provide NCI with a source of human lung and bronchial tissues taken at surgery, human alveolar macrophages from fiberoptic bronchoscopy, and bronchial lavage of normal subjects, both smokers and nonsmokers.

Major Contributions: The contract was recompeted in February 1983 (without interruption of the contract activity) for continued acquisition of these resources. In this period, the contractor provided bronchial and peripheral lung samples, pleural fluids, specimen of resected esophagus, and broncho-alveolar cell in lavages from smokers and non-smokers to NCI for ongoing experiments here. Medical histories were collected for each donor and are being held by the contractor until delivery is requested by the NCI.

Proposed Course: To continue and improve provision of the specified tissues and cells to NCI throughout the next contract period.

LITTON BIONETICS, INC. (NO1-CP-15769)

Title: Resource for Xenotransplantation Studies of Carcinogenesis in Human Tissues in Athymic Nude Mice

Current Annual Level: $240,000 (FLAT RATE)

Man Years: 2.43

Objectives: To use an immunodeficient animal model, athymic nude mouse, for 1) long-term survival of human tissue xenografts; 2) to provide a continuing resource of athymic nude mice for these long-term studies; 3) to use human tissues to study the development of preneoplastic and possibly neoplastic lesions induced by carcinogens; and 4) to study the ability of selected agents to modify the effects of carcinogens on human tissues.

Major Contributions: Human bronchus, pancreatic duct, colon, breast, prostate, and esophagus can be maintained for prolonged periods as xenografts, as evidenced by viable-appearing epithelium with normal histology and the incorporation of labeled precursors into epithelial cells of the grafts.

Epithelium-denuded rat tracheas now serve as anchorage for human bronchus cells, which attach to and layer the luminal surface of the tracheas. Esophageal xenografts are characterized by epithelial growth and cyst formation.

Malignant transformation continues to elude observation in xenografts. Explants treated in vitro with carcinogens respond with epithelial abnormalities which, when xenografted, only rarely maintain their transformation in the

nude mouse. Squamous metaplasia occurs in grafts given carcinogens in vivo, but has not become malignant. Emphasis is now being placed on increased immunosuppression as a means of effecting the change.

Proposed Course: 1) Continue long-term testing of the effects of carcinogens and treatment regimens on human tissue xenografts in the athymic nude mouse; 2) Determine tumorigenic potential of human cells exposed to chemical and physical carcinogens in vitro; 3) Xenotransplantation of transformed and non-transformed cells combined with other cells (species and type) with and without treatment with carcinogens, enzymes, or ionizing irradation (cesium source); and 4) Increased emphasis on further immunosuppression of the nude mouse to enhance its xenotransplantation capabilities. In this area, anti-mouse interferon antibodies, coinjection of human fibroblasts, antilymphocyte serum, and various monoclonal antibodies are being explored for this purpose. These efforts are on-going and the results are not yet sufficient for discussion.

NATIONAL NAVAL MEDICAL CENTER (YO1-CP-30257)

Title: Procurement of Human Tissues

Current Annual Level: $15,375

Man Years: 1.0

Objectives: To provide preoperative histories and nontumorous bronchial and colonic epithelium (obtained at the time of surgery for cancer or for benign lesions) to the NIH for the study of carcinogen activation and deactivation; to provide the capability to metabolize carcinogens to mutagens.

Major Contributions: Since the beginning of this new agreement, the National Naval Medical Center (NNMC) has delivered only 14 surgical specimens of human lung to NCI. Epidemiological histories were obtained on all patients and the specimens were properly processed through the Pathology Department of the NNMC. We will use a no-cost extension to recover the inactive period.

Proposed Course: A supplemental no-cost agreement to use the Navy technician for the collection of tissues from Walter Reed Army Hospital via Dr. Zajtchuck is now being formulated. This step will increase the consistency of the tissue yields from these surgeons and, perhaps, stabilize the technical aspects with more experienced management. In addition, specimens obtained from Walter Reed will increase the total volume without added cost.

UNIFORMED SERVICES UNIVERSITY OF HEALTH SCIENCES (YO1-CP-00502)

Title: Hybridoma Resource

Current Annual Level: $87,000

Manyears: 0.75

Objectives: Prepare and screen monoclonal antibodies to cell surface antigens and alkylate DNA for use in studying carcinogen and cocarcinogen action in bronchial epithelium including 1) identification of normal, preneoplastic, and neoplastic brochial epithelial cells, 2) characterization of transforming growth factors secreted by human cancer cells, and 3) measurement of DNA damage in normal and neoplastic bronchial epithelial cells caused by carcinogens and antitumor drugs.

Major Contributions: Mice have been injected with tobacco smoke condensate (TSC) adducted to DNA mixed with protamine, the latter serving as the adjunct. Spleen cells from mice producing antibody were fused with the MAT-sensitive NS-1 cells, cloned, and expanded, and the supernatants were screened for antibody. Antibodies were found to react with BP-DNA adducts along or with TSC. Further characteristics of specificity are being examined. In other studies, O^6-ethylguanosine and N^7-ethylguanosine were complexed specifically with protamine and used to immunize mice. Monoclonal antibodies resulting from this immunization were produced by the techniques described above. These antibodies react with CLZ-DNA. Specificity determinations are under way.

Proposed Course: To develop monoclonal antibodies to a number of DNA adducts formed by environmental carcinogens including those found in tobacco smoke; to produce monoclonal antibodies to the alkylated DNA adducts and surface antigens from chemically treated cells; to analyze cell surface antigens for possible correlations with chemically induced DNA modifications.

VETERANS ADMINISTRATION HOSPITAL (YO1-CP-30205)

Title: Resource for Procurement of Human Tissues from Donor with an Epidemiological Profile

Current Annual Level: $54,000

Man Years: 1.6

Objectives: The general objectives of this interagency agreement are 1) to effect a better morphologic and biochemical characterization of normal, premalignant, and malignant respiratory epithelium; 2) to obtain human tissues for cultures of essentially normal human lung tissues so that they may be studied in their response to carcinogens both in culture and by xenotransplantation into immune-deficient experimental animals; 3) to structure a lung cancer classification based on coordination of input from all collaborations; and 4) to extend the above undertakings to esophagus and colon.

Major Contributions: The contractor obtained tissue samples of lobar, segmental, and sub-segmental bronchi from 288 patients who underwent the following operations:

Right pneumonectomy	22
Left pneumonectomy	22
Right upper lobectomy	78
Right middle lobectomy	12
Right lower lobectomy	34
Left upper lobectomy	26
Lingulectomy	6
Left lower lobectomy	22
Bilobectomy	10
Local excision	56
Total	288

By light microscopy, it has been ascertained that 30 of the 288 patients did not harbor a malignancy but rather had aspergillosis, crypotococcosis, sequestration, bronchiectasis, or granuloma. The pulmonary malignancies were identified as follows:

Squamous cell carcinoma	150
Adenocarcinoma	52
Undifferentiated carcinoma	28
Broncho-alveolar carcinoma	8
Carcinoid	6
Carcinoma in situ	2
Metastatic	6
Lymphoma	2
Hamartoma	2
Thymoma	2
Total	258

Malignant and normal (non-involved) tissues from these sources were provided to the NCI in 42 separate deliveries.

Proposed Course: Based on mutual experiences to date, the contractor's role should continue as follows:

1) Continue obtainment and morphological examination of viable normal and abnormal human tissues from patients with an epidemiological profile

2) Increase obtainments to include more samples of esophageal, gastric, and colonic mucosa

MICROBIOLOGICAL ASSOCIATES, INC. (NO1-CP-05637)

Title: Biochemistry and Cell Culture Resource

Current Annual Level: 0

Man Years: 1.43

Objectives: This contract was terminated February 28, 1983, and is not being recompeted. The purpose of this contract is to provide the Laboratories of Human Carcinogenesis (LHC), Cellular Carcinogenesis and Tumor Promotion, and Experimental Pathology with resources for investigations of chemical carcinogenesis. LHC pursued specifically the mechanisms of human carcinogenesis; accomplishments relating to those efforts are described.

Major Contributions:

Human Bronchus Epithelial and Fibroblastic Cells: The contractor grew and cryopreserved 3T3 cells and bronchial epithelial and fibroblastic cells.

1) 3T3 cells. In order to expand the epithelial populations to a density sufficient for freezing, large numbers of Swiss mouse 3T3 cells were used for feeder layers. The contractor produced, irradiated, and delivered 2.7 x 10^{10} cells (193 deliveries) to the NCI, plus approximately 10-20% of that number for use in the contractor's facility.

2) Bronchial epithelial and fibroblast cells. 449 ampules (352 x 10^6 cells) of bronchial epithelial cells and 185 ampules (293 x 10^6 cells) of fibroblasts from the same bronchial explant of different tissue donors were frozen at various passage levels. Large pieces of intact explant could be cryopreserved and used to initiate new epithelial cultures following storage up to 9 months.

Mesothelial cells: Although mesothelial cells are difficult to grow and clone, cloning efficiencies of up to 20% were obtained, which allowed the initiation of cytotoxicity assays.

Cytotoxicity: Cytotoxicity of cigarette smoke condensate (CSC) 2A1 was examined in human bronchus epithelial (HBE) cells and compared with that observed in normal and the six times more resistant, chemically transformed 3T3 cells (normal 3T3 cells suffer 98% cloning inhibition in 40 µg CSC; transformed 3T3 cells, only 15%). Even with the complication of the 3T3 feeders in HBE cultures, HBE cells were less sensitive than normal 3T3 cells.

Normal human fibroblasts (NHF) and mesothelial cells were similarly resistant to the effects of UV irradiation. However, mesothelial cells were 35 times more sensitive to lethal chrysotile asbestos than human lung fibroblasts.

Mesothelial sensitivity to chrysotile was much greater than to crocidolite asbestos. The sensitivity of normal and DNA repair-deficient fibroblasts to asbestos seemed to correlate with the level of repair deficiency. NHF cells with blocked replication slowly recovered the toxicity of chrysotile, while xeroderma pigmentosum cells, which were found to have twice the membrane negativity of the NHF cells, were unaffected or had increased toxic effects.

NHF cells were also used in studies of formaldehyde, which produced significant enhancement of MNU mutagenicity, due perhaps to inhibition of a methyltransferase enzyme.

Continuous passage of a carcinogen-altered bronchial epithelial cell line was achieved through nine passages. Samples were cryropreserved.

Cell-Mediated Mutagenesis: The lymphoblastoid cell line TK-6 was established as target cells in a cell-mediated mutagenesis and toxicity assay using human bronchus as the metabolizing tissue. Successful dose-response assays were conducted with a direct acting mutagen, MNNG, but results with a promutagen, benzo[a]pyrene (BP), were less successful. The background frequency of TG^r mutants was by growth in CHAT media. An attempt to increase the assay sensitivity involved multiple sampling time and dosing regimens; however, the data were inconsistent, and the task was terminated.

Biochemistry of Carcinogen Metabolism: High pressure liquid chromatography (HPLC) for BP metabolites in media was standardized and 189 samples were analyzed; procedures for isolating BP metabolites on alumina columns were established and metabolites in 203 samples were collected.

Methodologies were studied for separating various organic extractable aflatoxin B_1 (AFB_1) metabolites using both thin layer chromatography and HPLC. The result was successful separation of the major metabolites, including AFQ_1, AFM_1, and AFP_1; separation and quantitative analysis were performed for organic and water-soluble AFB_1 metabolites in 94 samples.

DNA was extracted, and ^{3}H-BaP or ^{3}H-AFB_1 binding levels were determined for 172 tissue samples.

Proposed Course: Contract terminated February 28, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04513-08 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Metabolism of Chemical Carcinogens by Cultured Human Tissues and Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Herman Autrup, Acting Chief, Carcinogen Macromolecular Section, LHC, NCI

COOPERATING UNITS *(if any)*

University of Maryland School of Medicine, Baltimore, MD; Institute for Cancer Research, New York, NY; Cancer Institute, Beijing, People's Republic of China; Department of Surgery, University of Nairobi, Nairobi, Kenya

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human bronchus, colon, duodenum, esophagus, and pancreatic duct cultured either as explants or epithelial cells in chemically defined media provide an excellent in vitro system to study the metabolism of chemical carcinogens, including those found in tobacco smoke and the environment. Several classes of chemical carcinogens, polynuclear aromatic hydrocarbons, N-nitrosamines, hydrazines, aromatic amines, and mycotoxins, can be metabolically activated by human tissues. Fetal human liver, stomach, and esophagus cultured as explants metabolized the same group of compounds. The metabolic pathways leading to the formation of DNA adducts in explants and epithelial cell cultures have been defined for benzo(a)pyrene (BP), 7,12-dimethylbenz(a)anthracene, aflatoxin B1 (AFB), and N,N-dimethylnitrosamine (DMN). The adducts between these carcinogens and DNA in human tissues are essentially the same as those found in experimental animals in which the chemicals are carcinogenic. Inter-individual differences in carcinogen-DNA binding values vary 50- to 150-fold. The role of AFB in liver carcinogenesis has been further studied. We found that when urine samples collected in Murang'a district, Kenya, were analyzed for the presence of 2,3-dihydro-2-(7'-guanyl)-3-hydroxyaflatoxin B1 (AFB-Gua I) by high-pressure liquid chromatography, 8 of 106 samples had a detectable level of AFB-Gua I; its identity was confirmed by photon-counting fluorescence spectrophotometry. These results are an indication of interaction between the ultimate carcinogenic form of AFB and cellular nucleic acids in vivo and further support the hypothesis that AFB may play an important role in the etiology of human liver cancer.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than Principal Investigator) engaged on this Project:

Vincent Wilson	Staff Fellow	LHC, NCI
Kirsi Vahakangas	Visiting Fellow	LHC, NCI
John F. Lechner	Senior Staff Fellow	LHC, NCI
Susan Schlegel	Expert	LHC, NCI
Curtis C. Harris	Chief	LHC, NCI

Objectives:

To determine the metabolic pathways of chemical carcinogens in target tissues of experimental animals and humans. To measure interindividual and intertissue variations in the metabolism of carcinogens.

Methods Employed:

Explant cultures and epithelial cell cultures of human and animal tissues; isolation of cellular macromolecules; high pressure liquid chromatography; enzyme systems; and postlabeling analysis.

Major Findings:

Cultured human bronchial mucosa can enzymatically activate procarcinogens (polyluclear aromatic hydrocarbons: 7,12-dimethylbenz(a)-anthracene (DMBA), 3-methylcholanthrene (MCA), benzo(a)pyrene (BP), 6-nitrobenzo(a)pyrene (6-NO_2 BP), and dibenz(a,h)anthracene (DBA); N-nitrosamines: N-nitrosodimethylamine (DMN), N-nitrosodiethylamine, N-nitrosopiperidine, N-nitrosopyrrolidine (NPy), and N,N'-dinitrosopiperazine; a substituted hydrazine: 1,2-dimethylhydrazine (1,2-DMH); a mycotoxin: aflatoxin B_1 (AFB_1); and an aromatic amine: 2-aminoacetylfluorene) into metabolites that bind to cellular macromolecules including DNA.

The extrapolation of carcinogenesis data among animal species depends in part on qualitative and quantitative differences between metabolic activation and deactivation of procarcinogens. Therefore, the metabolism of BP has been extensively studied in explants of tracheobronchial tissues from experimental animals--hamster, rat, mouse, bovine--and humans. The total metabolism as measured by both organic solvent-extractable and water-soluble metabolites of BP was substantial in the respiratory tract from humans and animal species susceptible to the carcinogenic action of BP. Furthermore, the results suggested that determination of both activation and deactivation pathways is important in assessing carcinogenic risk of a chemical. No qualitative difference in the profile of organosoluble metabolites (tetrols and diols being the major metabolites) was observed among the different species. The metabolism of BP-7,8-diol to BP tetrols was mediated not only by the mixed function oxidase system but also by the prostaglandin synthetase pathway. Addition of arachidonic acid to the culture medium enhanced the production of the BP-tetrols. The prostaglandin synthetase pathway did not activate BP itself.

The binding values of BP to cellular DNA were quite similar in all tissues although slightly higher binding was observed in hamster trachea. Wide inter-individual variation in the binding of BP to DNA was seen in tissues from outbred species. The major BP-DNA adducts in all animal species were formed by interaction of BP diol-epoxide with the 2-amino group of deoxyguanosine. Both stereoisomeric forms of (+)-(7β,8α)-dihydroxy-(9α,10α)-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE I) reacted with deoxyguanosine, the (7R)-form being the most reactive. No difference in the relative distribution of the various adducts was seen among the species, except in the rat (CD, Wistar, and Buffalo) where BPDE-deoxyadenosine adducts accounted for 20% of the total modification. In cultured hamster trachea the persistence of the different adducts was similar. In conclusion, the metabolism of BP is qualitatively similar in tracheobronchial tissues from both humans and animal species in which BP has been experimentally shown to be carcinogenic. The major DMBA-DNA adduct was also formed between the "bay-region" diolepoxide and the 2-amino group of deoxyguanine.

The metabolism of AFB was studied in rat trachea and human bronchus. The major organosoluble metabolite in both species was aflatoxin M_1, but smaller amounts of aflatoxin P_1 and Q_1 were also detected. One major AFB-DNA adduct was formed by addition of AFB-2,3-oxide to the 7-position of guanine. This reaction product is unstable, and the imidazole ring will open to stabilize the molecule. A relatively greater amount of the ring-opened compound was observed in the rat trachea. The major AFB-DNA adduct was similar to the one formed in fetal human liver explants and in rat liver in vivo, an organ susceptible to the carcinogenic action of AFB. In human bronchus, the binding level of BP was generally higher than that of AFB, and no correlation existed between the binding level to DNA of these two carcinogens.

The metabolism of BP was studied in both epithelial and fibroblast cells initiated from the same bronchus specimens. The total metabolism was 3-fold higher in the epithelial than in the fibroblast cells. No qualitative differences in the metabolic profile of BP between the explant culture and the epithelial cell cultures were observed.

Nontumorous esophagus cultured in a chemically defined medium metabolized BP, DMBA, AFB, DMN, and N-nitrosodiethylamine (DEN) to species that reacted with DNA. No detectable amount of radioactivity was associated with DNA after incubation with NPy. The major carcinogen-DNA adducts were: (1) trans addition of (+) BP diol epoxide I at the 10 position to the 2-amino group of guanine; (2) with DMBA, addition of DMBA-3, 4-dihydroxy-1,2-epoxide to the 2-amino group of guanine, (3) with DMN, 7-methylguanine and O-6 methylguanine (O-6 MeG/7-MeG = 0.3); and (4) with AFB, 2,3-dihydro-2-(N-guanyl)-3-hydroxy-aflatoxin B_1.

The mean level of binding of BP in human esophageal DNA was lower than that in bronchus from the same individual and showed a 100-fold interindividual variation. As N-nitrosamines are potential esophageal carcinogens in rats, a comparative study in humans and rats on the metabolism of this group of compounds was performed. Both acyclic and cyclic N-nitrosamines were metabolized by rat esophagus. The highest level of metabolite binding was seen with N-nitroso-benzylmethylamine (BMN), an organotrophic carcinogen for the rat

esophagus. The binding level was about 100-fold higher than in human esophagus.

N-Nitrosoethylmethylamine, another unsymmetrical N-nitrosamine, was preferentially oxidized by rat esophagus in the ethyl group, as shown by higher formation of CO_2 and acetaldehyde from the compound labeled in the ethyl group. The highest binding level to DNA from this compound was observed with the methyl group. No binding to human esophagus was detected. N-Nitrosopyrrolidine was oxidized by both rat and human esophagus in the -position as measured by the formation of 2,4-dinitrophenylhydrazone derivative of 4-hydroxybutanal. Binding of metabolites of NPy to DNA was detected only in rat esophagus. DMN was metabolized by both human and rat esophagus. While most of the radioactivity associated with DNA was found to be incorporated into guanine and adenine, methylation of the guanine was detected by chromatography of the hydrolyzed rat DNA. The results indicate significant quantitative and perhaps qualitative differences between cultured rat and human esophagus in their ability to activate N-nitrosamines.

Metabolism of various carcinogens in cultured human colon and duodenum has been investigated. Nontumorous tissue was collected at the time of either "immediate autopsy" or surgery from patients with or without colonic cancer. After 24 hours in culture, explants were exposed to radioactive-labeled carcinogen for another 24 hours, and the binding to cellular DNA was measured by radiometric methods.

The following carcinogens were converted by human colon to species that bound to DNA: BP, 6-NO_2BP, DMBA, AFB, DMN, DMH, and 3-amino-1,4-dimethyl-5H-pyrido (4,3-b)indole. The latter is a potent fecal mutagen formed by pyrolysis of tryptophan.

The major carcinogen-DNA adducts were identified for BP, DMBA, AFB and were found to be identical to the adducts formed in human bronchus. The mean level of binding of BP was higher in duodenum than in colon. A wide inter-individual variation was observed. A positive correlation in the binding level of BP between bronchus and colon and duodenum from the same individual was seen.

Some quantitiative differences in the metabolic profile of BP were observed between the four organs. A significantly higher amount of BP tetrols and BP 9,10-diols were formed by human bronchus compared to the GI tissues, while a higher level of BP phenols was formed by the latter. The relative distribution of BP-DNA adduct was also similar in the four organs indicating that the metabolism of BP by these four organs is qualitatively similar, but quantitative differences exist. A comparison of conjugation of primary metabolism by normal and tumorous colonic tissues from the same patient showed that sulphate conjugation was the predominant pathway in normal colon while glucuranidatiase was the major pathway in tumorous colon.

Cultured human pancreatic duct could metabolize BP, DMBA, AFB, and DMN as measured by radioactivity associated with DNA.

Explant cultures of human fetal liver, stomach, and esophagus extensively metabolized chemical carcinogens into DNA binding species. The metabolism of

N-nitrosamines--DEN, NPy and BMN--was significantly higher in stomach than in the other tissue from the same fetus. Fetal esophagus did metabolize NPy into a DNA-binding metabolite in contrast to adult esophagus. The major BP-DNA and AFB-DNA adducts in fetal livers were similar to the adducts observed in other adult human tissues. The HPLC profile of organosoluble BP-metabolite was more complex than with adult tissues.

In order to access the role of AFB in human liver carcinogenesis, we collected urine samples in Murang'a district, Kenya, for analysis of AFB-Gua I, a "DNA-repair product." It has previously been shown that food samples collected in this district are known to be contaminated with AFB, and a positive correlation exists between the dietary intake of AFB and the incidence of liver cancer. The urine samples collected at the out-patient clinic of Murang'a district hospital was concentrated on C_{18}Sep-Pak columns, and AFB-Gua I was isolated by high pressure liquid chromatography in two different systems. Eight of 106 samples had a detectable level of a compound whose synchronous fluorescence spectrum was identical to chemically synthesized AFB-Gua I. The spectrum did not show any bathochromic shift when pH was made alkaline. These results are an indication of interactions between the ultimate carcinogenic form of AFB and cellular nucleic acids in vivo and further support the hypothesis that AFB may play an important role in the etiology of human liver cancer.

In order to understand the interaction of complex chemical mixtures, such as tobacco smoke and diesel exhaust, with biological system, the post-labeling method of Randerath has been modified. At least 17 different carcinogen-DNA adducts were formed when tobacco smoke condensate was incubated with DNA in the presence of liver microsomes. The identification of these adducts are presently being attempted.

Significance to Biomedical Research and the Program of the Institute:

As most environmental carcinogens require metabolic activation to exert their carcinogenic effect, the study of their metabolic pathways and the reaction of the ultimate carcinogen with cellular macromolecules in potential human target tissues is important. Extension of these studies to more complex potentially carcinogenic mixtures, such as tobacco smoke condensate is important. The development of controlled culture conditions for human tissues provides a model system for these studies in intact human tissues. The use of explant culture also provides a link between studies in experimental animals and the human situation as the metabolism of the carcinogens can be studied at the same level of biological organization in both species, this information is essential for extrapolation of carcinogenesis data between species. Furthermore, human tissues obtained by immediate autopsy also will allow a comparative study in various organs from the same individual. From this study we hope to be able to identify an easily accessible cell type that can be used for the identification of individuals at high risk of devloping chemically induced cancers.

Proposed Course:

To identify endogenous and exogenous factors that alter the metabolism of environmental procarcinogens. To continue a combined laboratory-epidemiology

study of carcinogen metabolism. To use ultramicroassays of carcinogen metabolism and carcinogen-DNA adducts so that metabolism and repair can be studied in biopsy specimens. To correlate metabolism of chemical carcinogens between different target tissues within a single individual. To compare the metabolism of chemical carcinogens in target tissues and possible "detector" cells, i.e., monocytes and macrophages. A study on the biological effect in relationship to level and type of carcinogen-DNA interation will be continued.

Publications:

Autrup, H.: Carcinogenesis studies in cultured human colon. In Autrup, H. and Williams, G. M. (Eds.): Experimental Colon Carcinogenesis. Boca Raton, CRC Press, 1983, pp. 95-106.

Autrup, H., Bradley, K., Shamsuddin, A. K. M., Wakhisi, J. and Wasunna, A.: Detection of aflatoxin-guanine adducts in urine collected in Mruang'a district, Kenya. Carcinogenesis (In Press)

Autrup, H. and Grafstrom, R. C.: Comparison of carcinogen metabolism in different organs and species. In Hietanen, E. (Ed.): Cytochrome P-450: Biochemistry, Biophysics and Environmental Implications. Amsterdam, Elsevier Biomedical Press, 1982, pp. 643-648.

Autrup, H., Grafstrom, R. and Harris, C. C.: Metabolism of chemical carcinogens by tracheobroncial tissues. In Rice, J., Langenbach, R. and Nesnow, S. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Press, 1983, pp. 473-495.

Autrup, H., Lechner, J. F. and Harris, C. C.: The use of human tissues and cells in carcinogen metabolism and toxicity studies. In Homburger, F. (Ed.): Safety Evaluation and Regulation of Chemicals. Basel, S. Karger AG, 1983, pp. 151-159.

Cohen, G. M., Grafstrom, R. C., Gibby, E. M., Smith, N., Autrup, H. and Harris, C. C.: Metabolism of benzo[a]pyrene and 1-naphthol in cultured human colon and tumor tissues. Cancer Res. 43: 1312-1315, 1983.

Harris, C. C. and Autrup, H. (Eds): Human Carcinogenesis. New York, Academic Press (In Press)

Harris, C. C., Grafstrom, R. C., Lechner, J. F. and Autrup, H.: Metabolism of N-nitrosamines and repair of DNA damage in cultured human tissues and cells. In Magee, P. (Ed.): Banbury Reports. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1982, vol. 12, pp. 1-19.

Harris, C. C., Trump, B. F., Autrup, H., Hsu, I-C., Haugen, A. and Lechner, J.: Studies of host factors in carcinogenesis using cultured human tissues. In Bartsch, H. and Armstrong, A. (Eds.): Host Factors in Human Carcinogenesis. Lyon, IARC Scientific Publication No. 39, 1982, pp. 497-514.

Nebelin, E., Autrup, H., Christensen, B. and Blomkvist, G.: Detection of metabolites of N-nitrosopyrrolidine and N-nitrosomethylethylamine in cultures of human bladder epithelial cells of normal origin. In Bartsch, H., O'Neill, I. K., Castegnaro, M., and Okada, M. (Eds.): N-Nitrosocompounds - Occurrence and Biological Effects. Lyon, IARC Scientific Publication No. 41 (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05130-03 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Carcinogenesis Studies Using Cultures of Human Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

John F. Lechner, Ph.D., Senior Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

University of Maryland School of Medicine, Baltimore, MD; Litton Bionetics, Rockville, MD; Georgetown University School of Medicine, Washington, DC; VA Hospital, Washington, DC; National Naval Medical Center, Bethesda, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7	3	4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Defined methods to grow replicative cultures of normal human bronchial epithelial (NHBE) cells without serum have been developed. These cells can be subcultured several times, will undergo 35 population doublings, and have expected epithelial cell characteristics of keratin, desmosomes, and blood group antigens on their cell surface. NHBE cells inoculated at clonal density will multiply with an average generation time of 28 hr, and the majority of the cells are small, migratory, and have few tonofilaments. Adding human blood-derived serum (BDS) depresses the clonal growth rate of NHBE cells in a dose-dependent fashion. In contrast, 10 representative lines of human lung carcinomas either replicate poorly or fail to grow at all when inoculated at clonal density in serum-free medium; their rates of multiplication increase in direct proportion to the amount of BDS added to the optimized medium. BDS reduces the clonal growth rate of NHBE cells by specifically inducing the normal cells, but not lung carcinoma cells, to undergo squamous differentiation. The differentiation inducing activity was not present in plasma but was found in platelet lysates. In vitro carcinogenesis experiments with normal bronchial epithelial tissue and cell cultures have yielded populations of cells that have abnormal characteristics. These phenotypically altered cells (PACs), which have keratin epithelial cell markers, extended population doubling potentials, abnormal human karyologies and abnormal response to differentiation control by serum and platelet factors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

James C. Willey	Medical Staff Fellow	LHC	NCI
Edward Gabrielson	Medical Staff Fellow	LHC	NCI
Takayoshi Tokiwa	Visiting Scientist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To develop systems to study malignant transformation of human epithelial cells. These studies include the following: (1) develop defined media for replicative epithelial cell cultures from bronchial tissues; (2) study the differentiation control of bronchial epithelial cells in culture; (3) study in vitro carcinogenesis of bronchial epithelial cells using a defined system; (4) evaluate both long-term and rapidly dividing explant cultures as model systems to study in vitro malignant transformation; (5) study the effects of chemical and physical cocarcinogens and promoters on the progession of carcinogen-induced, phenotypically altered cells (PAC) to malignancy; and (6) assess xenotransplantation methodologies in order to attain increased sensitivity for the assay.

Methods Employed:

Human bronchial tissues are obtained from surgery, medical examiner, and "immediate" autopsy donors. Bronchial tissues are dissected from surrounding stroma, cut into 0.5 cm square pieces, and used to establish explant cultures.

Replicative cultures of normal bronchial epithelial cells are developed from explant culture outgrowths. As many as 20 successive outgrowth cultures can be obtained from a single tissue by repeated transferring of the explant. Upon transfer of the explants to new dishes, the outgrowth cultures remaining in the original dishes are incubated in defined, serum-free medium to expand the population and are then subcultured. These cells are used in in vitro carcinogenesis and cell nutrition and differentiation studies and in carcinogen metabolism and DNA repair experiments or are cryopreserved for future use.

Several criteria are used to establish the normal epithelial phenotype of the cells grown in culture. Markers to identify normal epithelial cells in vitro (an important objective for this laboratory) include karyology: polygonal morphology; ciliary activity; scanning electron microscopic morphology; ultrastructural identification of tight junctions; desmosomes and tonofilaments; production of acidic and neutral mucopolysaccharides; immunostaining of keratin, blood group antigens, and type IV collagen; population doubling potential; clonal growth rate; and mitogenic responsiveness to peptide growth factors and hormones.

Long-term explant cultures are being used to scrutinize cellular changes induced by exposure to chemical carcinogens. Subsegmental bronchi are grossly

dissected free of peripheral lung tissue. The bronchi are then cut with a scalpel into 0.2-cm^3 fragments. Four degassed Gelfoam® sponges (2 cm^2) are placed in a 60-mm culture dish containing 3.0 ml of medium supplemented with 1% serum, and four or five bronchial fragments are placed on the surface of each sponge. This culture system is similar to organ culture in that the sponge supports keep the tissue fragments from becoming submerged. The cultures are incubated in a 4% CO_2 air atmosphere at 36.5°C. The media (without and with incorporated carcinogens) are replaced at 4-day intervals. The tissues are incubated for 12 weeks before being dissociated and expanded as pure epithelial cell cultures.

Pure populations of bronchial epithelial cells are also exposed for extended periods to chemical carcinogens. Abnormal, mitotic cells are selected on the basis of aberrant terminal differentiation control and extended population doubling potential.

Growth of carcinogen-induced abnormal cells in athymic nude mice is being tested using several procedures. Optimal conditions are being ascertained by comparing tumor latency time between BALB/c and NIH nude mice. Different doses of tumorigenic lung carcinoma cells are injected subcutaneously, both alone and with irradiated normal human bronchial fibroblasts or with irradiated HT 1080 human sarcoma cells. In addition, frozen/thawed rat trachea are being used as containers. Cells are injected into the trachea lumen, the ends are tied off, and the trachea are implanted subcutaneously. The lumen provides a pseudo-basement membrane for cell attachment.

As a model system for in vivo carcinogenesis, small subsegmental bronchi are implanted subcutaneously into nude mice. After vascularization, carcinogens incorporated into beeswax pellets, absorbed on lycra fibers or in gelatin pellets are inserted into the lumen. Periodically, these tissues are recovered and assessed by pathological examination.

Major Findings:

Human bronchial epithelial cell culture experiments have yielded the following results. (1) A method for routinely initiating replicative epithelial cell cultures of human bronchus was developed. Large pieces of bronchus tissue were initially set up as explant cultures and incubated in a rocking chamber for 2 to 3 days to facilitate reversal of ischemia. The explants were then cut into smaller pieces and explanted into Ca^{2+}-reduced M199 medium containing 1.25% fetal bovine serum and 35 µg protein/ml of bovine pituitary extract. This medium, with a low serum concentration, permits rapid outgrowth of epithelium but retards growth of the fibroblastic cells. Thus, after 5 to 10 days incubation, very few fibroblastic cells were present among the epithelial outgrowth. At this time, the tissue explant was transferred to a new dish for reseeding a second wave of epithelial cell outgrowth. Sequential tissue transfer was repeated up to 20 times over a period of 1 year, and the epithelial cells were not discernably different from the first outgrowth culture. (2) A defined (serum-free) medium for normal human bronchial epithelial (NHBE) cells was developed. Clonal growth dose-response experimentation was used to tailor the concentrations of nutrients and growth factors in MCDB 151 medium to meet the requirements of the normal human

bronchial epithelial cells. Modified MCDB 151 medium (LHC-7) contains transferrin, insulin, epidermal growth factor, hydrocortisone, phosphoethanolamine, ethanolamine, bovine pituitary extract, and T_3. In addition, the Ca^{2+} and Mg^{2+} concentrations were adjusted, and the osmolality was lowered to 280 by reduction of the concentration of sodium, biocarbonate, and HEPES buffer. Further improvements in in vitro conditions and techniques were obtained by modifying the surface of the petri dish. Clonal growth dose-response experiments showed that precoating the surface of the dish with a mixture of collagen and fibronectin improved the rate of cell attachment, plating efficiency, and clonal growth rate. (3) Supplementation with as little as 0.25% fetal bovine blood-derived serum (BDS) resulted in a decrease in clonal growth rate; 8% supplementation completely inhibited growth by inducing terminal squamous cell differentiation. Human lung carcinoma lines were also incubated in LHC-7 medium without and with 8% BDS. The results showed that serum toxicity per se was not responsible for the observed inhibition of NHBE cell growth; all 10 carcinoma lines divided significantly more rapidly ($p < 0.05$) in BDS-supplemented medium except for line A 427, which failed to grow at clonal density in LHC nutrient media. Thus, the growth factor requirements of these lung carcinoma cell lines were significantly different from their normal counterparts. There was significantly less inhibition of NHBE cell growth with plasma-derived serum. On the other hand, platelet factors at concentrations that stimulated fibroblastic cell multiplication also inhibited DNA synthesis and stimulated terminal differentiation of NHBE cells. (4) Incorporation of 1.25% BDS into LHC-7 altered the Ca^{2+} requirement. No significant change in the Ca^{2+} requirement was noted between 60 and 1000 µM in serum-free medium, but 50% inhibition of growth occurred above 700 µM Ca^{2+} with serum supplementation. The calcium concentration of the medium influenced colony morphology and cell shape. The cells were small and tightly polygonal when grown in the absence of normal (1 mM) levels of calcium. Progressive reduction of the calcium concentration to 0.06 mM caused the cells to become less closely associated. (5) Cell density was found to influence the effect of Ca^{2+} on growth. Whereas optimal growth occurred at clonal densities in medium containing 1 mM Ca^{2+}, rapid squamous terminal differentiation occurred when the medium of dividing high-density cultures was changed from 0.1 to 1 mM Ca^{2+}. (6) Mg^{2+} and Ca^{2+} regulation of clonal growth of bronchial epithelial cells caused by a mechanism that was significantly different from that that occurred in lung fibroblasts. The results with lung fibroblasts suggest that the role of Mg^{2+} is more closely related to the intracellular events that determine the maximal clonal growth rate, whereas the results with bronchial epithelial cells suggest that the roles of the two ions are equally important and, to a great extent, that the ions can be substituted for one another. (7) Serum influenced growth factor requirements. Cholera toxin stimulated growth of NHBE cells in media supplemented with 1.25% serum. The absence of serum obliterated cholera toxin stimulation. These results suggest that cholera toxin can neutralize the squamous terminal differentiation-promoting activity of serum. (8) Cultures of human bronchial epithelium have the normal human karyotype and average 35 population doublings in vitro. (9) Epithelial cell markers have been demonstrated. Keratin was shown by both immunospecific Kreyberg and rhodamine B staining. Blood group antigens were also detected by immunospecific staining. However, mucopolysaccharide staining was negative. Quiescent cultures differentiated into cells that had beating cilia.

Ultrastructurally, the content of tonofilaments and desmosomes depends upon medium conditions; numerous tonofilaments and desmosomes are commonly seen only in cells incubated in BDS medium. (10) Normal epithelial explant and cell culture systems for adult bronchial epithelium have now been extensively developed. Thus, in vitro chemical carcinogenesis experiments with human epithelial cells are feasible. Large quantities of epithelial cells can be readily obtained, which will permit experiments using large numbers of cells at risk to be repeated several times.

Carcinogenesis experiments have yielded the following: (1) Explant cultures were maintained for more than 1.5 years. A steady state condition was established whereby dying cells were replaced by cells migrating from the tissue. With time, the outgrowth cultures became predominantly fibroblastic in appearance. After 1.5 years, these explant tissues were transferred, and a new wave of mitotic epithelial cells migrated from the tissue, attesting to long-term viability of tissue. However, the population of epithelial cells ceased dividing after a few successive subcultures. (2) Subsegmental bronchiolar tissues explanted onto Gelfoam® supports were exposed to chemical carcinogens. After 12 weeks of incubation and continuous exposure, control cultures exhibited some squamous metaplasia, but the appearance of the glands was generally unremarkable and cellular atypia was not noted. In contrast, epithelium continuously exposed to Ni^{2+} (10 µg/ml, as $NiSO_4.6H_2O$) exhibited extensive cell growth, amorphous glands were common, and areas of cellular atypia with mitotic cells were frequent. Replicative cultures of epithelial cells were initiated from enzymatically dissociated, exposed, and control tissues. Cultures became quiescent after four (1:3) subculturings. After 4 additional weeks of incubation colonies of mitotic epithelial cells appeared only in the cultures originating from the Ni^{2+}-exposed tissues. These latter cultures expressed aberrant differentiation and growth control characteristics (see below) and are being assessed for tumorigenic properties. (3) Phenotypically altered cells (PACs) were isolated after repeated treatments of bronchial epithelial cell cultures with Ni^{2+}. Colonies of Ni^{2+}-induced PACs arose 3 to 7 weeks after the cultures had become mitotically quiescent. Fourteen of the PAC colonies were isolated from four experiments and expanded. All expressed the keratin epithelial cell markers and exhibited desmosomes in early passage. Chromosomal studies revealed aneuploid karyologies. The clonal growth response to ethanolamine and to serum and Ca^{2+} differ both between the two and compared with that of normal cells. (4) Differentiation stimulation by 4% serum has been found to be useful as a means to select and characterize PACs. Some PACs respond to Ca^{2+} serum and differentiate, whereas most cultures fail to respond to the differentiation-promoting signal. (5) Of 10 human lung tumorigenic cell lines tested, all were found to grow poorly in serum-free LHC-7 medium that was especially developed for optimal growth of normal human bronchial epithelial cells under defined clonal conditions. However, whereas supplementation of LHC-7 medium with serum caused cultures of normal epithelial cells to terminally differentiate, growth of the tumorigenic cell lines in LHC-2 medium was significantly increased by serum supplementation. (6) The tumorigenicity of these isolates is being tested in athymic nude mice. Rat tracheal "container" experiments have been encouraging; phenotypically altered epithelial cells have been detected growing on the lumen after 2 weeks' implantation in the nude mouse. (7) The tumorigenicity assay has been made 10 times more sensitive by the injection of 2 million

irradiated human bronchial fibroblastic or HT 1080 cells with the test cells, i.e., 30,000 tumorigenic lung carcinoma cells, coinjected with irradiated fibroblasts cause tumors to appear more quickly than 1 million injected tumor cells alone. (8) Implantation of carcinogens into subsegmental bronchi that were transplanted into nude mice failed to induce malignant transformation after up to 38 months.

Significance to Biomedical Research and the Program of the Institute:

The extrapolation of experimental animal data to man is a major problem in the study of carcinogenesis. One approach to provide a link is to develop model systems in cultured human tissues for carcinogenesis investigations. Such systems could be used for the identification of individuals who are highly susceptible to chemical carcinogens.

Proposed Course:

Growth conditions for human epithelial cells will be continually improved, and the number of experiments designed to characterize the growth properties of these cells will be increased. Experiments studying the effect of tumor-promoting agents are underway. Experiments are in progress to determine whether carcinogen-treated bronchial epithelial cells lose antigens commonly associated with normal cells and to demonstrate the growth characteristics ascribed to transformed cells. Changes in these properties may be indicative of premalignant transformation. Studies of the effects of multiple carcinogen exposures as well as cocarcinogenesis with physical and viral agents on the development of neoplastic lesions in explant and cell cultures and xenotransplanted tissues will be continued and expanded. Ultimately, the mechanisms through which carcinogens transform epithelial cells will be investigated.

Publications

Lechner, J. F.: Nutrient, hormone, growth factor and substrate interdependent regulation of epithelial cell growth. Fed. Proc. (In press)

Autrup, H., Lechner, J. F. and Harris, C. C.: The use of human tissues and cells in carcinogen metabolism and toxicity studies. In Homburger, F. (Ed.): Safety Evaluation and Regulation of Chemicals. Basel, S. Karger AG, 1983, pp. 151-159.

Harris, C. C., Trump, B. F., Autrup, H., Hsu, I-C., Haugen, A. and Lechner, J.: Studies of host factors in carcinogenesis using cultured human tissues and cells. In Bartsch, H. and Armstrong, A. (Eds.): Host Factors in Human Carcinogenesis. Lyon, International Agency for Research on Cancer (IARC Scientific Publication No. 39), 1982, pp. 497-514.

Lechner, J. F., Haugen, A., McClendon, I. A. and Pettis, E.W.: Clonal growth of normal adult human bronchial epithelial cells in a serum-free medium. In Vitro 18: 633-642, 1982.

Lechner, J. F., Haugen, A., McClendon, I. A. and Shamsuddin, A.: Control of growth and squamous differentiation of normal human bronchial epithelial cells. Differentiation (In Press)

Lechner, J. F., Haugen, A., Tokiwa, T., Trump, B. F. and Harris, C. C.: Effects of asbestos and carcinogenic metals on cultured human bronchial epithelium. In Harris, C. C. and Autrup, H. (Eds.): Human Carcinogenesis. New York, Academic Press. (In Press)

Lechner, J. F., Kaighn, M. E., Groden, J. and German, J.: Bloom's syndrome cells: abnormal serum growth response. Exp. Cell Res. (In Press)

Lechner, J. F., Stoner, G. D., Haugen, A., Autrup, H., Willey, J. C., Trump, B. F. and Harris, C. C.: In vitro human bronchial model systems for carcinogenesis studies. In Webber, M. M. and Sekely, L. (Eds.): In Vitro Models for Cancer Research. New York, CRC Press. (In Press)

McDowell, E., Harris, C. C. and Trump, B.F.: Histogenesis and morphogenesis of bronchial neoplasms. In Shimosato, Y., Melamed, M. and Nettesheim, P. (Eds): Morphogenesis of Lung Cancer. New York, CRC Press, Inc., 1982, pp. 1-36.

Ohnuki, Y., Lechner, J. F., Bates, S. E., Jones, L. W. and Kaighn, M. E.: Chromosomal instability of SV40-transformed human prostatic epithelial cell lines. Cytogenet. Cell Genet. 33: 170-178, 1982.

Trump, B. F., Wilson, T. and Harris, C. C.: Recent progress in the pathology of lung neoplasms. In Peereboom, T. (Ed.): Lung Cancer 1982. Amsterdam, Excerpta Medica, 1982, pp. 101-124.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05133-04 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Epidemiology Studies Using Monoclonal Antibodies to Aflatoxin B1-DNA Adducts

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Curtis C. Harris, M.D., Chief, LHC, NCI

COOPERATING UNITS *(if any)*

Immunology Branch, NCI; Boston University, School of Public Health, Boston, MA; Massachusetts Institute of Technology, Cambridge, MA; Cancer Institute, Beijing, People's Republic of China

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Immunological approaches to measure DNA damage caused by carcinogens may be useful in biochemical epidemiology studies to identify individuals at high cancer risk. Mouse myeloma cells (P3x63) were fused with spleen cells from BALB/c mice immunized with aflatoxin B1-DNA adducts. Hybrid cells were grown in selective medium and tested for production of antibody-secreting hybridomas. Clones secreting monoclonal antibodies binding specifically to aflatoxin B1-DNA adducts have been obtained. These antibodies have been characterized and in conjunction with competitive ultrasensitive enzyme immunoassay used to quantitate aflatoxin B1 modified DNA in liver obtained from rats received doses ranging from 0.01-1.0 mg AFB1/Kg. At this time, the limit of sensitivity is one aflatoxin B1 residue per 1,355,000 nucleotides. These monoclonal antibodies and others are being used to measure aflatoxin B1-DNA adducts in liver samples from individuals at high risk of developing liver cancer.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Herman Autrup	Acting Chief, CMIS	LHC, NCI
Glennwood E. Trivers	Research Scientist	LHC, NCI
Kirsi Vahakangas	Visiting Fellow	LHC, NCI
Dean L. Mann	Senior Investigator	LHC, NCI

Objectives:

Monoclonal antibodies will be used to detect carcinogen-DNA adducts in human tissue specimens and cells and will be used in experiments such as (1) determination of exposure of individuals to chemical agents; (2) adduct distribution in different organs; (3) DNA repair studies; and (4) experimental in vitro carcinogenesis.

Methods Employed:

Spleens from immunized mice are removed 2-3 days after the last immunization and minced in Dulbecco's phosphate buffered saline. Spleen cells (10^8) are mixed with myeloma cells (10^7), fused with polyethylene glycol, and grown in selected medium. Myeloma cells will not grow in the selective hypoxanthine/aminopterine/thymidine (HAT) medium. Since spleen cells will not grow in culture, the only cells that survive are cell hybrids. Hybrid cells are dispersed in 96-well plates and incubated at 37°C. Cell growth after 14 days is recognized as a successful hybrid. Cells can then be cloned with thymus cells with a modified enzyme-linked immunosorbent assay. Cells producing specific antibody are recloned and then expanded and injected i.p. into mice for development of ascites tumor. Monoclonal antibodies from ascites and cell culture medium are then isolated and characterized. The characterization procedures recognize only aflatoxin bound to DNA and not free aflatoxin B_1 (AFB_1)-guanine adducts or six other AFB_1 metabolites. These assays are performed using both enzyme-linked immunosorbent assay (ELISA) and ultrasensitive enzyme radioimmunoassay (USERIA) techniques.

Major Findings:

Immune response to AFB_1-DNA adducts was obtained by injecting methylated bovine serum albumin-AFB_1-DNA conjugate or AFB_1 conjugate emulsified in Freund's complete adjuvant into mice. Hybridoma clones producing monoclonal antibodies against AFB_1-DNA adducts or AFB_1 have been obtained and characterized. Competitive ELISA using these monoclonal antibodies reliably quantitated AFB_1 residue per 1,355,000 nucleotides. The competitive USERIA was determined to be at least 10- to 12-fold more sensitive than the competitive ELISA in analysis of AFB_1-adducted DNA. Using biotinylated monoclonal antibody and an avidin enzyme conjugate, a competitive enzyme immunoassay has also been developed. In addition, monoclonal antibodies to aflatoxin B_2 have been found to react with AFB_1-modified DNA. These antibodies will be useful in confirming results obtained with our initial antibodies.

Significance to Biomedical Research and the Program of the Institute:

Methods are being developed to quantitatively measure carcinogen-DNA adducts in femtomole and less amounts. These methods will be useful in studies of the molecular interactions of carcinogens and cell genome and in measurement of carcinogen-DNA adducts in biopsy specimens from people in high- and low-risk environments for cancer.

Proposed Course:

Studies are underway to make monoclonal antibodies that specifically recognize other carcinogen-DNA products as well as isolated base adducts. These monoclonal antibodies and USERIA are being utilized to search for carcinogen-DNA adducts in human biopsy specimens and to determine (1) their rate of formation and removal and (2) their value in predicting an individual's cancer risk.

Publications:

Groopman, J. D., Haugen, A., Goodrich, G. R., Wogan, G. N. and Harris, C. C.: Quantitation of aflatoxin B_1-modified DNA using monoclonal antibodies. Cancer Res. 42: 3120-3124, 1982.

Harris, C. C. and Autrup, H. (Eds.): Human Carcinogenesis. New York, Academic Press. (In Press)

Harris, C. C. and Cerutti, P. A. (Eds.): Mechanisms of Chemical Carcinogenesis. ICN-UCLA Symposium on Molecular and Cellular Biology. New York, A. L. Liss, 1982.

Harris, C. C., Yolken, R. H. and Hsu, I.-C.: Enzyme immunoassays: Applications in cancer research. In Busch, H. and Yeomen, L. C. (Eds.): Methods in Cancer Research, Vol. 20. New York, Academic Press, 1982, pp. 213-242.

Shamsuddin, A. K. M. and Harris, C. C.: Improved enzyme immunoassays using biotin-avidin-enzyme complex. Arch. Pathol. Lab. Med. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05192-03 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Repair of Carcinogen Induced Damage in Human Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Curtis C. Harris, M.D., Chief, LHC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Pathology, DCBD, NCI; Department of Physiology, Hershey Medical Center, Hershey, PA; Department of Pathology, University of Maryland School of Medicine, Baltimore, MD; Karolinska Institute, Stockholm, Sweden

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The effects of various DNA damaging agents were compared in human skin fibroblasts and human bronchial epithelial and fibroblast cells. The three cell types exhibited the same (1) rate of repair of DNA single strand breaks (SSB) after irradiation, (2) rate of excision repair after UV-irradiation, and (3) level of SSB after exposure to 7,12-dimethylbenz[a]anthracene, benzo[a]-pyrene diol epoxide (BPDE), and N-methyl-N'-nitro-N-nitrosoguanidine. Since formaldehyde is formed in equimolar quantities with methylcarbonium ions during the metabolic activation of N-nitrosodimethylamine, we have recently examined the effects of formaldehyde on the repair of the promutagenic lesion, O6-methyl-guanine, formed following N-nitrosodimethylamine metabolism. Formaldehyde decreases O6-transalkylase activity, inhibits the removal of O6-methylguanine, and, in low concentrations, synergistically potentiates the cytotoxicity and mutagenicity of N-methyl-N-nitrosourea. In high doses, 100 or 130 micromolar formaldehyde is detectably mutagenic itself. Therefore, exposure to formalde-hyde may lead to the dual genotoxic mechanism of both directly damaging DNA and inhibiting repair of mutagenic and carcinogenic lesions caused by alkylating agents and physical carcinogens.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on the Project:

Herman Autrup	Acting Chief, CMIS	LHC, NCI
John F. Lechner	Senior Staff Fellow	LHC, NCI

Objectives:

To understand the mechanism of repair of DNA damage by environmental agents in human epithelial tissues and cells and to investigate the genotoxicity of formaldehyde.

Methods Employed:

Culture of human epithelial and fibroblastic cells; alkaline elution technique for detection of DNA single strand breaks (SSB) and DNA protein crosslinks (DPC); BND cellulose chromatography for measurement of repair replication; ^{3}H-thymidine incorporation in the presence of hydroxyurea for measurement of unscheduled DNA synthesis; isolation of cellular macromolecules; high pressure liquid chromatography.

Major Findings:

Our initial effort was directed at comparing DNA damage and its repair in human epithelial versus fibroblastic cells. DNA SSB induced by 5 krads of X-rays were rapidly rejoined in both bronchial fibroblasts and epithelial cells. Approximately 90% of the SSB were rejoined in the first hour, and most of the remaining SSB were more slowly rejoined over the next 5 hours. Since DNA damage caused by many chemical carcinogens has been shown to be repaired by excision repair, the response of bronchial cells to UV irradiation was studied. The level of presumed excision SSB was very similar in all cell types. Cells incubated with the repair polymerase inhibitor combination of arabinoside cytosine (Ara-C) and hydroxyurea (HU) caused excision-generated SSB to accumulate due to inhibition of the polymerase step of excision repair. The level of SSB accumulated from excision events occurring after UV irradiation was approximately equal in fibroblasts and epithelial cells. Also, the frequency of M. luteus endonuclease-sensitive sites immediately after UV irradiation was similar in both cell types.

Exposure of cells to several chemical carcinogens resulted in DNA SSB in both epithelial and fibroblastic cells. With the procarcinogen 7,12-dimethylbenz-[a]anthracene, a small number of DNA SSB was seen in both cell types. Also, in the case of anti-benzo[a]pyrene diol epoxide (BPDE), a similar response was observed in both cell types. N-Methyl-N'-nitro-N-nitrosoguanidine caused a high number of SSB in both cell types; following post-treatment incubation in medium, most of these SSB were rejoined in both cell types. Similar results were seen with bronchial cells derived from several donors.

Repair replication, as measured by BND cellulose chromatography, was demonstrated at appreciable levels in both epithelial and fibroblastic cells following either UV irradiation or BPDE. A smaller effect was seen with BPDE in fibroblasts as is compared to epithelial cells. The data of several experiments consistently showed the level of repair replication in bronchial fibroblasts to be 38% + 8% of that seen with epithelial cells. On the other hand, the effect on UV-irradiated fibroblasts was more similar, e.g., 82% + 11% to that seen with UV-irradiated epithelial cells. Incubation of fibroblast and epithelial cells with radiolabeled BPDE followed by purification of DNA by BND chromatography demonstrated no significant difference of binding of BPDE to DNA in both cell types. The removal of BPDE-DNA adducts from human epithelial cells is presently under investigation.

Divalent nickel showed no effect on DNA repair replication after BPDE damage or UV irradiation in either cell type. Furthermore, nickel had no effect on the level of excision SSB after UV irradiation. Bronchial cells exposed to either washed or unwashed asbestos showed no appreciable level of DNA SSB with or without polymerase inhibitor, a combination of Ara-C and HU.

Formaldehyde is a common environmental pollutant and a metabolite of demethylation reactions of drugs and carcinogenic N-nitrosamines. It is also a respiratory carcinogen in rats and a potential carcinogenic hazard in humans. Therefore, we have initiated a systematic study of the genotoxicity of formaldehyde in cultured human cells. Exposure to formaldehyde resulted in formation of DPC to an equivalent extent in bronchial cells and skin fibroblasts from normal and xeroderma pigmentosum (XP) group A patients. Post-treatment incubation of the cells in fresh medium rapidly decreased the level of DPC, with an approximate half-life of 2 hours, independent of dose and cell type. After 8-10 hours' incubation, only 5% to 10% of the DPC remained. Incubation of DNA from the formaldehyde-treated cells in alkaline lysing solution indicated that the DPC were relatively stable ($t_{1/2}$ ~ 10 hr). When the DPC were removed with proteinase K in the alkaline elution assay, dose-dependent formation of DNA SSB could be detected in epithelial and fibroblastic cells. DNA SSB were almost completely removed after 4 hours post-treatment incubation, and the rate of removal was similar in both cell types. By use of the polymerase inhibitor combination (Ara-C/HU), DNA SSB could be accumulated in bronchial cells and skin fibroblast but were not accumulated in XP cells. Since XP cells removed DPC as efficiently as normal cells, the involvement of excision repair is unclear. These data together indicate that DPC from formaldehyde may be removed by other mechanisms than excision repair.

Formaldehyde inhibited the rejoining of X-ray induced DNA SSB in bronchial epithelial and fibroblastic cells at concentrations of formaldehyde that did not cause significant DNA SSB. Similar effects were obtained in both cell types. The mechanism of the observed repair inhibition of formaldehyde was further investigated. The "long patch repair" agents, UV irradiation and BPDE, were used to study the effect of formaldehyde on unscheduled DNA synthesis (UDS). Significantly higher concentrations of formaldehyde were required to inhibit UDS than to inhibit rejoining of DNA SSB from X-irradiation. This indicated a higher sensitivity of the ligation step of excision repair to formaldehyde action.

Since formaldehyde is formed in equimolar quantities with methylcarbonium ions during the metabolic activation of N-nitrosodimethylamine, we have recently examined the effects of formaldehyde on the repair of the promutagenic lesion O^6-methylguanine formed following N-nitrosodimethylamine metabolism. Formaldehyde decreases O^6-transalkylase activity, inhibits the removal of O^6-methylguanine, and, in low concentrations, synergistically potentiates the cytotoxicity and mutagenicity of N-methyl-N-nitrosourea. In high doses (100 or 130 μM), formaldehyde is detectably mutagenic itself. Therefore, exposure to formaldehyde may lead to the dual genotoxic mechanism of both directly damaging DNA and inhibiting repair of mutagenic and carcinogenic lesions caused by alkylating agents and physical carcinogens.

Significance to Biomedical Research and the Program of the Institute:

Methodologies developed for and utilized in studies of DNA damage and repair in animal (normal and tumor) cells can be successfully extended to similar investigations in cells cultured from human tissues susceptible to carcinogenesis. These investigations should aid in identifying mechanisms by which chemical and physical agents will damage the genetic material and exert carcinogenic and/or cocarcinogenic properties.

Proposed Course:

Identify endogenous and exogenous agents that will damage DNA and/or affect its repair. To continue comparative studies of human epithelial and fibroblastic cells. To compare the levels of DNA damage (i.e., DNA SSB, DPC, or repair replication) with levels and persistence of DNA adducts caused by chemical carcinogens. To continue correlation of the extent of DNA damage from various agents with biological effects including toxicity, mutagenesis, and transformation assays.

Publications:

Fornace, A. J., Lechner, J. F., Grafstrom, R. C. and Harris, C. C.: DNA repair in human bronchial epithelial cells. Carcinogenesis 3: 1373-1378, 1983.

Grafstrom, R. C., Fornace, A. J. Jr., Autrup, H., Lechner, J. F. and Harris, C. C.: Formaldehyde damages DNA and inhibits repair in human bronchial cells. Science 220: 216-218, 1983.

Harris, C. C., Grafstrom, R. C., Lechner, J. F. and Autrup, H.: Metabolism of N-nitrosamines and repair of DNA damage in cultured human tissues and cells. In Magee, P. (Ed.): The Possible Role of N-Nitrosamines in Human Cancer. Cold Spring Harbor, Banbury Conference, 1982, Vol. 13, pp. 121-139.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05193-03 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Keratin Proteins and Cross-linked Envelopes in Transformed Human Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Susan P. Banks-Schlegel, Expert, LHC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Pathology, DCBD, NCI; U. of Md. School of Med., Baltimore, MD; VA Hospital, Washington, D.C.; Litton Bionetics, Rockville, MD; U. Alabama, Birmingham, AL; U. Pittsburgh School of Medicine, Pittsburgh, PA

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Malignant transformation of epithelial cells was found to be accompanied by significant changes in the array of keratins and in the proportion of cells making cross-linked envelopes. Numerous morphological and biochemical differences have been observed between normal and malignant epithelial cells in culture. Analysis of keratin proteins extracted from a wide variety of tumors and tumor cell lines indicates that these proteins may prove to be a valuable adjunct in diagnostic pathology.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Curtis C. Harris Chief LHC, NCI

Objectives:

The growth and differentiation of normal human epithelial cells will be analyzed and compared with those of their neoplastic counterparts. We will attempt to establish neoplastic epithelial cells in culture and examine the growth and differentiated properties of these cells compared with their normal counterparts. These studies are designed to provide insight into the specific changes that occur during malignant transformation. In particular, initial studies will evaluate the usefulness of analysis of keratin protein patterns and cross-linked envelopes in the characterization of epithelial neoplasms. Defects in the pathway of terminal differentiation will also be assessed.

Methods Employed:

Human tissue is obtained from "immediate" autopsy (i.e., performed within 1 hour of death), autopsy material that is less than 12 hours post mortem, or from surgical specimens. The epithelium and some adherent connective tissue are carefully dissected from the remainder of the tissue. In some instances, the epithelium is separated from the adherent stroma either by heat separation or surgical excision, and the epithelium is analyzed for keratin proteins. In other cases, the epithelium is first cut into explants and used for radiolabeling of proteins. Tumors (minced) were radiolabeled in an analogous manner. When being used for cell culture, the epithelium or tumor is minced and trypsinized to obtain a single-cell suspension. The cells are grown on tissue culture dishes containing a layer of lethally irradiated 3T3 cells. The cells are fed every 3 to 4 days in medium containing 10% fetal calf serum plus various hormonal supplements and growth factors. The morphological and biochemical characterization of the cells is being assessed by a variety of techniques: light and electron microscopy, histochemical staining, immunofluorescent staining, radiolabeling of macromolecules, immunoprecipitation, polyacrylamide gel electrophoresis, autoradiography, and peptide mapping. The terminal differentiation of the cells is triggered by means of ionophore and the extent of terminal differentiation is assessed by cornified envelope formation. When using human tumor material, the epithelium is not separated from adherent stroma. Otherwise, the tumor and normal tissue are handled identically.

Major Findings:

Cancer of the human esophagus and lung occurs worldwide and frequently represents a major cause of death in the population. Therefore, we have chosen to focus our efforts on these two organ systems.

In contrast to the simplified keratin content of bovine, rabbit, and rat esophageal epithelium (composed mainly of a 57 and 46 or 51 kD keratin,

depending on the animal species), human esophageal epithelium contains a quantitatively different array of keratin proteins, ranging in molecular weight from 37 to 61 kD. The pattern of keratin proteins from human esophageal epithelium differs qualitatively and quantitatively from that of human epidermis. Human esophageal epithelium lacks the 63, 65, and 67 kD keratins characteristic of human epidermis, consistent with the absence of a granular layer and an anucleate stratum corneum. Human esophageal epithelium contains a distinctive 61 kD keratin protein, which was either not present or present in only small amounts in human epidermis, and variable amounts of a 37 kD keratin. Whereas the 56, 59, and 67 kD keratins were the most abundant keratins in human epidermis, the 52, 57, and 61 kD keratins predominated in human esophageal epithelium. During in vitro cultivation, both human epidermal and esophageal keratinocytes produced colonies that were stratified; however, the morphologic appearance of these cultured epithelia differed. Only cultured human epidermal keratinocytes contained keratohyalin granules in the outermost layers and a prominent 67 kD keratin on immunoprecipitation. Otherwise, the keratin contents appeared similar. In conclusion, human esophageal epithelium exhibited intertissue and interspecies differences in the pattern of keratin proteins. During in vitro cultivation, human esophageal keratinocytes retained some aspects of their distinctive program of differentiation.

Analysis of keratin proteins extracted from human esophageal tumors revealed dramatic changes in the pattern of keratins. In addition to an overall reduction in the amount of keratin, most tumors were characterized by a complete loss of the major 52 and 61 kD esophageal keratins. The lower molecular weight keratins (48 to 50.5 kD) and the 57 kD keratin were conserved in the transformed phenotype. Injection of these tumors into a nude mouse and analysis of the tumors for keratin proteins revealed an even more dramatic shift in patterns of keratins.

After repeated attempts at establishing esophageal epithelial tumors in culture, we have succeeded in establishing two tumor cell lines. While the cells looked typically epithelial, the colonies that grew out were morphologically very different from those of normal esophageal epithelial cells. Although the pattern of keratin filaments within the cells looked very similar to that of normal esophageal epithelial cells, the pattern of keratins from these tumor cells was remarkably different from that of the normal cell and resembled the in vivo tumor pattern in several respects. Surprisingly, immunoprecipitation of the keratins from one of the tumor cell lines revealed the presence of a 67 kD keratin typical of human epidermis. These data, however, were consistent with the fact that some well differentiated squamous cell carcinomas of the esophagus show signs of keratinization. Another differentiated function, the ability to make cross-linked envelopes, was also dramatically altered in these tumor cells, similar to the in vivo situation. When terminal differentiation of these cells was induced by means of a calcium ionophore, only 20 to 30% of the cells made envelopes. In comparison, 94% of the normal esophageal epithelial cells made envelopes during ionophore-induced terminal differentiation. These cell lines are currently being examined for alterations in the expression of a number of properties commonly ascribed to transformation.

Most human lung tumors arise from the area of the bronchus. They have been classified by the World Health Organization on the basis of their histological

appearance and synthetic product(s) into several different classes. Keratins have been found useful not only for delineating the epithelial nature of the tumor but also as an adjunct in defining the type of tumor present. For instance, well differentiated squamous cell carcinomas, urotheliomas, and mesotheliomas tend to be strongly keratin positive. Adenocarcinomas tend to be weakly positive to negative. Both adenocarcinomas and epidermoid carcinomas of the lung contained keratin proteins, as demonstrated by immunocytochemical and electron microscopic data. However, the amount of keratin varied depending on the tumor type (decreased in adenocarcinomas) and the degree of epidermoid differentiation (decreased in poorly differentiated tumors) (studies performed in collaboration with Drs. Elizabeth McDowell, Benjamin Trump, and Tom Wilson at the University of Maryland). Since the lung tumors were not easily classified on the basis of immunoperoxidase staining or ultrastructural localization for keratin, we analyzed keratin-enriched protein fractions of these tumors by one-dimensional gel electrophoresis to investigate their usefulness in distinguishing these lung neoplasms. Keratin extraction data and keratin immunoprecipitation data revealed that there were distinct qualitative and quantitative differences useful in distinguishing adenocarcinomas from epidermoid carcinomas of the lung.

We have also undertaken an extensive study to evaluate the keratin pattern exhibited by a number of different cell lines derived from a variety of lung tumors, including a mucoepidermoid carcinoma, several adenocarcinomas, large cell carcinomas, mesotheliomas, and small cell carcinomas. The cell lines had been established by Dr. Adi Gazdar at NCI-Navy Medical Oncology Branch, Bethesda, MD. While certain keratins were found to be prevalent in all the tumor cell lines examined, other keratins were unique to a given tumor type. In general, the different tumor types tended to exhibit a distinctive pattern of keratin expression. While human bronchial epithelial cells acquire the ability to form cross-linked envelopes when grown in cell culture, analysis of ability of the various tumor cell lines to form envelopes indicated that only lines derived from tumors containing an epidermoid component displayed an ability to form cross-linked envelopes, thereby maintaining certain aspects of their distinctive phenotype in cell culture.

The biochemical characterizations of the various epithelia and tumors have been compatible with immunological approaches using specific antibodies, as demonstrated in studies performed in collaboration with Dr. J. Said at Cedars-Sinai Medical Center and Drs. G. Pinkus and J. Corson at Harvard Medical School.

Significance to Biomedical Research and the Program of the Institute:

Most human cancers are epithelial in origin. A better understanding of the complex process of neoplasia will require both a full understanding of the normal program of differentiation in human epithelial cells and how it is altered during malignant transformation. Advances in the ability to grow human epithelial cells in culture will undoubtedly facilitate attempts to unravel the mechanism(s) involved in malignant transformation.

Proposed Course:

Studies aimed at understanding the control of differentiation and the sequence of events involved in malignant transformation of epithelial cells will continue.

Publications:

Banks-Schlegel, S. P. and Harris, C. C.: Tissue-specific expression of keratin proteins in human esophageal and epidermal epithelium and their cultured keratinocytes. Exp. Cell Res. (In Press)

Banks-Schlegel, S. P. and Howley, P.: Differentiation of human epidermal cells transformed by SV-40. J. Cell Biol. 96: 330-337, 1983.

Banks-Schlegel, S. P., Vocci, M., Combs, J. and Harris, C. C.: Human esophageal epithelium in cell culture. In Webber, M. and Sekeley, L. (Eds.): In Vitro Models for Cancer Research. Boca Raton, CRC Press (In Press)

Corson, J. M., Weiss, L. M., Banks-Schlegel, S. P., and Pinkus, G. S.: Keratin proteins in synovial sarcoma. Am. J. Surg. Pathol. 7: 107-109, 1983.

Said, J. W., Nash, G., Tepper, G. and Banks-Schlegel, S.: Keratin proteins and carcinoembryonic antigen in lung carcinoma: an immunoperoxidase study of 54 cases with ultrastructural correlations. Human Pathol. 14: 70-76, 1983.

Said, J. W., Nash, G., Banks-Schlegel, S., Sassoon, A. F. and Shintaku, I. P.: Localized fibrous mesothelioma--an immunohistochemical and electron microscopic study. Human Pathol. (In Press)

Said, J. W., Nash, G., Banks-Schlegel, S. P., Sassoon, A., Murakami, S., and Shintaku, P.: Keratins in human lung tumors: patterns of localization of different molecular weight keratin proteins. Am. J. Pathol. (In Press)

Walts, A. E., Said, J. W. and Banks-Schlegel, S. P.: Keratin and carcinoembryonic antigen in exfoliated mesothelial and malignant cells: an immunoperoxidase study. Am. J. Clin. Pathol. (In Press)

Warhol, M. J., Pinkus, G. S. and Banks-Schlegel, S. P.: The localization of keratin proteins in the human epidermis by a post-embedding immunoperoxidase technique. J. Histochem. Cytochem. 31: 517-523, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05291-02 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Adducts in People Exposed to Benzo[a]pyrene

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Curtis C. Harris, M.D., Chief, LHC, NCI

COOPERATING UNITS *(if any)*

Mount Sinai School of Medicine, New York, NY; NCI-Navy Medical Oncology Branch, NCI; LCCTP, DCCP, NCI; University of Maryland School of Medicine, Baltimore, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Exposure to benzo[a]pyrene (BP), a ubiquitous carcinogen, may be unusually high for individuals in certain occupations. Formation of BP-DNA adducts due to human exposure is most likely to be at very low levels that are beyond the sensitivity of radioimmunoassay and chromatographic analyses. Thus, ultra-sensitive enzymatic radioimmunoassay (USERIA) and enzyme-linked immunosorbent assay (ELISA) have been employed to detect and quantitate BP-DNA antigenicity in humans at high cancer risk due in part to BP exposure. DNA isolated from white blood cells of asphalt workers (roofers) and foundry workers and DNA from lung tissue, bronchial washings and alveolar macrophages of lung cancer patients and smokers are being investigated. BP-DNA antigenicity has been detected in several of the high-risk individuals. These data suggest that the activation of BP to its ultimate carcinogen as well as formation of adducts with DNA occurs in humans.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Nuntia Sinopoli	Visiting Fellow	LHC, NCI
Kirsi Vahakangas	Visiting Fellow	LHC, NCI
Dean L. Mann	Senior Investigator	LHC, NCI

Objectives:

Using rabbit anti-BP-DNA antibodies and the most sensitive immunoassays available, BP-DNA antigenicity will be determined in high-risk individuals. Results should help us in further understanding activation and mechanism of carcinogenesis in humans.

Methods Employed:

Twenty-five to 40 ml of peripheral blood was obtained from 28 male volunteers who were active in their occupation as roofers for over 20 years. The blood samples were centrifuged at 100 x g for 15 min, and the "buffy coat" containing white blood cells was separated. The isolated "buffy coat" was homogenized in 5 volumes of HKM:0.25 M sucrose buffer (0.05 M HEPES, pH 7.3; 0.025 M Cl; 0.05 M MCl_2) using a glass homogenizer. The homogenate was centrifuged for 10 min at 300 x g at 4°C. The pellet was suspended in HKM-sucrose buffer containing 0.5% Triton 100 and centrifuged for 10 min at 4°C. The pellet was suspended in HKM-sucrose buffer and recentrifuged. The final pellet was resuspended in 5 ml KHM-sucrose buffer containing 1% SDS and 1 M NaCl. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added, and the mixture was vigorously agitated for at least 20 min followed by centrifugation at 10,000 x g for 10 min. The aqueous epiphase was removed and treated with RNase (100 µg/ml) at 37°C for 20 min. Following RNase digestion, 3 volumes of cold ethanol were added to the solution. DNA was removed by winding onto a glass rod. Residual ethanol was removed by nitrogen and DNA dissolved in water. Purity and quantitation of DNA were determined by absorbance at 260 nm and 280 nm using a Beckman DU8 spectrophotometer. The final volume of DNA solution was adjusted to 1 mg DNA/ml water, and the solution was and rendered single stranded by boiling. Single-stranded DNA was then stored at 4°C until tested. DNA was similarly isolated from lung tissue, bronchial washings, and alveolar macrophages. DNA from foundry workers was received in purified form, ready to be tested.

Competitive enzyme immunoassasys, USERIA and ELISA, were performed on the test DNA samples by using rabbit anti-BPDE-DNA antibody. Polyvinyl U-bottom 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with unmodified DNA (control) and BPDE-modified DNA (1 ng/well for USERIA and 5 ng/well for ELISA). Standard competitive inhibition curves were obtained by mixing serial dilutions of known standard BP-DNA with rabbit antisera. Percentage inhibition of the test samples was determined from the standard curves. All tests and assays were done in duplicate, and the standard deviation was less than 10%.

Major Findings:

Metabolic activation of benzo[a]pyrene (BP) to its ultimate carcinogenic form, 7β,8α-diol-9β,10α-BP epoxide (BPDE), and the binding of BPDE to DNA are important steps in BP carcinogenicity in experimental animals. Since people of certain occupations are exposed to high concentrations of BP, we have used ELISA and USERIA to measure BPDE-DNA adducts in white blood cells from two of these occupational groups. Seven of 28 samples from roofers and 7 of 20 samples from foundry workers were positive for BPDE-DNA adducts (range: 2 to 120 femtomoles BPDE/50 μg DNA. Positive BPDE-DNA samples of bronchial lavage cells were obtained from 3 out of 5 donors. The donors who had positive samples were smokers of either tobacco or marijuana. Control DNA obtained from 2 human lymphocyte cell lines was negative. These results indicate that the metabolic activation of BP and formation of BPDE-DNA adducts occur in humans.

Significance to Biomedical Research and the Program of the Institute:

Demonstration of carcinogen-DNA interaction in human tissue will enable us to better understand the mechanism of carcinogenesis in humans. Although white blood cells may not be the prime target for certain carcinogens, the presence of carcinogen-DNA antigenicity in these cells not only suggests a widespread distribution of the carcinogen but also provides an opportunity to screen high-risk individuals with relatively simple procedures.

Proposed Course:

Since BP-DNA antigenicity suggests the presence of BP-DNA adducts in humans, we are in the process of further documenting this result using biophysical approaches to measure carcinogen-DNA adducts. We are also further characterizing the anti-BP-DNA antibodies using various other antigens. Control groups, e.g., Seventh-Day Adventists who neither smoke or eat meat, will be studied. Once consolidated, these results would form the basis for conducting biochemical epidemiology studies of high-risk individuals.

Publications:

Harris, C. C.: Respiratory carcinogenesis and cancer epidemiology. In Straus, M. (Ed.): Lung Cancer: Clinical Diagnosis and Treatment. New York, Grune & Stratton, 1982, pp. 1-20.

Harris, C. C.: Role of carcinogens, cocarcinogens and host factors in human cancer risk. In Harris, C. C. and Autrup, H. (Eds.): Human Carcinogenesis. New York, Academic Press. (In press)

Harris, C. C., Yolken, R. H. and Hsu, I.-C.: Enzyme immunoassays: Applications in cancer research. In Busch, H. and Yeomen, L. C. (Eds.): Methods in Cancer Research. New York, Academic Press, 1982, pp. 213-242.

Yuspa, S. H. and Harris, C. C.: Molecular and cellular basis of chemical carcinogenesis. In Schottenfeld, D. and Fraumeni, J. Jr. (Eds.): Cancer Epidemiology and Prevention. Philadelphia, W. B. Saunders, 1982, pp. 23-43.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05292-02 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Tumor Promoters and Cocarcinogens on Growth and Differentiation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

James C. Willey, Medical Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

University of Maryland School of Medicine, Baltimore, MD; Litton Bionetics, Rockville, MD; Georgetown University School of Medicine, Washington, DC

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Models were developed using normal human bronchial epithelial (NHBE) cells for studying compounds reputed to be cocarcinogens and/or tumor promoters in animal models. Compounds were tested for cytotoxicity by colony forming efficiency (CFE) and population doubling time (PDT) assays. The compounds were then screened by using concentrations that were maximally mitogenic or that caused no more than a 50% decrease in colony forming efficiency of cells exposed for 6 hours. The cells were observed for morphologic changes under phase contrast light microscopy and scanning and transmission electron microscopy and assayed for changes in ornithine decarboxylase (ODC), plasminogen activator (PA), and aryl hydrocarbon hydroxylase (AHH) activity, and cross-linked envelope (CLE) formation. Twenty-eight compounds have been tested thus far for mitogenicity or cytotoxicity, and most have been characterized by the biochemical assays. As these initial studies are completed, selected compounds will be used for in vitro carcinogenesis experiments and to further investigate their mechanisms of action.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

None

Objectives:

To evaluate biochemical, morphological, growth and differentiation effects of putative carcinogens and tumor promoters in normal human bronchial epithelial cells, and putative inhibitors of tumor promotion; also to compare effects of these compounds on lung carcinoma cell lines.

Methods Employed:

1. Clonal Growth Assays: Outgrowths of NHBE cells were subcultured and 100,000 cells were inoculated per well in 24-well plates in LHC-1 medium minus epidermal growth factor (EGF) (LHC-0) or MCBD-151 medium plus triiodothyronine (T^3) and pituitary extract (PEX) (LHC-4). The effects of compounds on cell population doublings per day (PD/D) were measured using clonal growth rate assays. Five thousand cells were inoculated per dish. Test reagents were added 24 hours after inoculation. After 7 days of incubation the colonies were fixed with 10% formalin and stained with 0.25% aqueous crystal violet. The mean number of cells per clone in 18 randomly selected colonies (9 per replicate dish) was determined for each additive concentration. To derive the growth rate (PD/D), the $\log_2$ of the average number of cells per clone was divided by the number of days in incubation. Student's t-test was used to evaluate the significance of difference between experimental groups.

2. Cross-linked Envelope Assay: Cells were inoculated at 20,000 cells per well onto coated 12-well plates in LHC-0 or LHC-4. Twenty-four hours later, cells per well were calculated using a grid, and media were removed and replaced with media containing the test reagent + 0.8% low gelling temperature agar. After a 6-hour incubation, 2 ml of sodium dodecyl sulfate (20%)-dithiothreitol (10 mmol) was added over the agar. After an additional 4-hour incubation, crosslinked envelopes per well and percent cross-linked envelopes per cell population were then calculated.

3. Morphological Assays: Cells were examined through phase contrast microscopy and transmission and scanning electron microscopy. For quantitation of morphology an Artek® image analyzer was programmed to measure cell areas and number of cells per colony.

4. Biochemical Assays: 70-100,000 cells per well were inoculated onto coated 24-well plates in 1 ml LHC-0 or LHC-4. Twenty-four hours later, media were removed and replaced with 250 µl of fresh medium containing the test reagents. After a further 6-hour incubation, ODC, PA, and AHH activities were assayed.

5. ODC Assay: Media containing the test reagents were removed after 6 hours. Cells were quickly frozen at -70°C. ODC was then quantified by measuring the release of $^{14}CO_2$ from labeled ornithine.

6. Plasminogen Activator Assay: After 5 hours of incubation, 25 µl of plasminogen (final concentration 0.1 U/ml) was added and incubation was continued for 1 more hour. Media were removed and centrifuged on an Eppendorf E-3200 microfuge for 30 seconds to remove cells; 90 µl of medium was incubated with 10 µl of glycyl-L-prolyl-arginyl-[^{14}C-anilide] (5 mmol) for 1 hour at 37°C. The reaction mixtures were then extracted 3 times each with 2 ml of Econofluor II and counted in ^{14}C channel. A standard curve was made by incubating varying concentrations of urokinase with 0.1 U/ml of plasminogen.

7. Aryl Hydrocarbon Hydroxylase Assay: After 6 hours, media containing test reagents were removed and replaced with 1 ml of medium containing ^{3}H-benzo[a]-pyrene (32 µCi/ml) and cells were incubated at 37°C on a rocker for 6 hours. AHH activity was then determined by recovering released $^{3}H_2O$ using an activated charcoal-alumina column.

Major Findings:

Cytotoxicity-mitogenicity data have been obtained for phorbol, 12-O-tetradecanoylphorbol-13-acetate (TPA), teleocidin B, 2,7-dichlorodibenzo-p-dioxin (DCDD), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (10^{-7} M, 10^{-8} M, and 10^{-9} M) and for cigarette smoke condensate (0.1, 1, 10 µg/ml), catachol, benzo[a]pyrene (B[a]P), benzo[e]pyrene (B[e]P), and pyrene (1.5×10^{-6}, 10^{-7}, 10^{-8} M). All of these compounds were present with 0.1% DMSO as carrier, and the results were compared to a 0.1% DMSO control. The effects of teleocidin B, TPA, phorbol, TCDD, and DCDD were each assessed by cellular morphology, clonal growth rate, and enzyme asays, i.e., PA, ODC and AHH. NHBE cells were inoculated into LHC-1 medium minus EGF (LHC-0), or LHC-1 plus PEX and triiodothyronine (LHC-4). Inoculation densities were 5,000 cells/60 mm plate for growth and 100,000 cells/plate for biochemical assays. After 24 hours, media were removed and replaced with either LHC-0 or LHC-4 containing the different concentrations (0.01 to 100 nM) of the test compounds. Teleocidin B and TPA had similar effects on growth, morphology and enzyme activities. At > 1 nM, squamous differentiation ensued; division ceased, cells became larger with small, dense nuclei, and cell boundaries became indistinct. Teleocidin B and TPA caused an elevation of ODC activity in LHC-0 media and a decrease of ODC in LHC-4. Both compounds caused an increase of PA and a decrease of AHH activity in both media. TCDD caused a 15% decrease in cell growth at 100 nM, and increased ODC and AHH in both media but increased PA only in LHC-4. DCDD did not alter growth, but its biochemical effects were similar to, though less marked than, those of TCDD. In summary, TPA and teleocidin B cause similar effects in NHBE cells, TCDD induced both ODC and AHH in these cells, and the cellular response to these agents is altered by composition of the medium. CSC and catachol caused an increase in AHH (138% and 126%, respectively) and had no effect on CLE. CSC had no effect on PA, while catachol caused a slight decrease (79%). B[a]P and pyrene caused a decrease in AHH (87% and 78%, respectively) and an increase in PA (133% and 124%, respectively) and had no effect on CLE. B[e]P had little effect on any of these tests at this concentration.

A group of compounds which enhance intracellular cyclic AMP levels increase ornithine decarboxylase activity and some also increase clonal growth. Many of these compounds were found to increase ODC activity in LHC-0 medium without

increasing clonal growth. Such compounds include cholera toxin 10^{-8} M, isobutylmethylxanthine 10^{-6} M, forscolin 10^{-6} M, L-epinephrine 1.5 x 10^{-6} M, and isoproterenol 1.5 x 10^{-6} M. The β-adenergic hormones were investigated more extensively. A synergistic interaction between undefined factors in PEX, EGF, and adrenergic hormones to stimulate clonal growth of the NHBE cells is supported by our findings. L-epinephrine or isoproterenol do not stimulate clonal growth in the absence of PEX and EGF but in the presence of these compounds clonal growth increases from 0.95 PD/D to 1.2 PD/D. These findings also indicate that since ODC is increased by the β-adenergic hormones in LHC-0 without an increase in clonal growth, the increase in ODC activity is necessary but not sufficient for cell division. Of the 25 compounds thus far tested for effects on clonal growth, one has been found to stimulate the growth of cells in the absence of EGF or PEX. Bombesin at 0.15 µg/ml stimulates an increase in the clonal growth of NHBE cells from 0.4 PD/D to 0.7 PD/D. In contrast to EGF, this increase in growth is not accompanied by an increase in cell migration. Bombesin also increases the colony forming efficiency from 1% to 2% in LHC-0. When cells are grown in the presence of epinephrine, it has been found that the synergistic properties of pituitary extract can be partially replaced by bombesin at 0.15 µg/ml and endothelial cell growth supplement at 20 µg/ml.

We have begun to characterize biochemical and morphological properties of 11 human lung carcinoma cell lines. Initial findings are that the cancer lines A549, HUT-292, and A1146 are relatively resistant to TPA. Whereas NHBE cells have a 50% inhibition of growth at 5 x 10^{-10} M, HuT 292 and A1146 were resistant to these differentiating effects of TPA at concentrations up to 10^{-8} M. The A549 cancer cell line was not affected by TPA up to a concentration of 10^{-6} M. The initial biochemical findings are that the HUT-292 cancer cell line has a much lower PA activity than NHBE cells. Characterization of biochemical properties of NHBE cells at different levels of squamous morphological differentiation have revealed that more basal-appearing cells have a lower level of PA and a higher level of ODC.

Some biochemical characteristics of cultured normal human mesothelial cells have been determined. Normal mesothelial cells have a much lower level of PA than NHBE cells. The level of PA in mesothelioma cells is not different from that in the normal mesothelial cells. TPA at 10^{-7} M causes a squamous morphological change in normal mesothelial cells but, in contrast to NHBE cells, this squamous morphological change is reversible upon removing the TPA.

Significance to Biomedical Research and the Program of the Institute:

We have initiated investigations into the relevance in human systems of a number of phenomena seen to be associated with the cell transformation process in animal models. The system being used is very flexible since the medium is defined and allows one to observe effects of cocarcinogens under reproducible conditions between repeated experiments on tissue from one individual and from different individuals. These investigations should help to identify both environmental and host factors determining susceptibility to cancer and some of the mechanisms relevant to carcinogenesis in humans.

Proposed Course:

Identify biochemical, morphological, and growth effects of putative cocarcinogens and tumor promoters on NHBE cells and select compounds which demonstrate effects potentially relevant to human carcinogenesis for further investigation. Further investigation will include observation of effects on cancer cell lines and ability of putative tumor promotion inhibitors to prevent effects seen in normal cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05293-02 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Repair and Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

George H. Yoakum, Senior Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

Department of Biochemistry, Johns Hopkins University School of Public Health, Baltimore, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.33	1	.33

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Adaptation of the protoplast fusion method for high frequency transfection in human cells provides a method for application to general problems in human somatic cell genetics. The ability to transfect genes at frequencies greater than 1 per 1000 is sufficient to attempt isolation of single copy genes from human genomic libraries linked to selectable markers and to screen for human oncogenes in normal human epithelial cells.

Transfection of subgenomic fragments into appropriately selected human cell recipients provides a means to rapidly study the regulation, expression, and pathobiological effects of individual viral genes. The gene transfer recipient culture (GTC2) carrying a subgenomic fragment of hepatitis B virus (HBV) that contains only the core antigen gene (HBc gene) was used to determine some of the factors that regulate the expression of the HBc gene. We found that growth of HBc+ GTC2 cells in LHC4 medium with 5% serum and treatment of the cells with 5'-azacytidine stimulated expression of the HBc gene. Expression of the HBc gene in GTC2 cells is cytopathologic, producing highly lethal effects when gene expression peaks. Subsequently, we found that a hepatocellular carcinoma (Alexander cells), which has carried the entire HBc genome since isolation from a chronically infected patient, responds to the same factors for production of the HBc gene product. In addition, there is a temporal relationship between the expression of the HBc gene and cytopathology for both GTC2 and Alexander cells. This is consistent with a primary role for the HBc gene product in the cytopathology found in human liver during HBV infection. Such information may be useful for development of therapeutic regimens for chronically infected patients and may provide insight about the biological nature of the virus at the genetic and molecular levels. In addition, the initial steps toward isolation of the xeroderma pigmentosum type A (XP-A) gene(s) have been completed, and transfection of normal human bronchial primary cultures with oncogenic viral complements has resulted in partial or total transformation of these cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations (other than the Principal Investigator) engaged on this Project:

Brent Korba	Senior Staff Fellow	LHC, NCI
Curtis C. Harris	Chief	LHC, NCI
Herman Autrup	Acting Chief, CMIS	LHC, NCI
John F. Lechner	Senior Staff Fellow	LHC, NCI

Objectives:

The primary goals of this research project are development and application of a genetic approach to problems of human carcinogenesis at the molecular level. Two research programs selected to initiate this approach focus on (1) The mechanism of HBV pathology and its role in carcinogenesis and (2) the genetic, molecular, and enzymatic mechanism(s) of human DNA-repair processes, their relationship to carcinogenesis, and potential for cancer prevention.

Methods Employed:

We have developed a method to transfect a variety of human cell types, i.e., carcinoma cells, transformed fibroblasts, and normal fibroblastic and epithelial cells. Introduction of exogenous genes (human or viral) in vitro to human cells is essential to development of effective research programs in human carcinogenesis at the genetic and molecular levels. By adapting the protoplast fusion method of transfection for transfer of pXV2-derived plasmids, we can stably transfer the HBV HBc gene into human cells at frequencies greater than 10^{-3} units. This transfection procedure permits transient expression of transferred genes in 70-90% of the recipient cell culture for 6-12 days after the procedure. Thus, initial observations of the acute biological effects of the transfected gene provide a prescreen of subgenomic viral segments with cytopathologic potential before selection is initiated to isolate stable transfectants.

Construction of various plasmids required for this study will employ similar methods to those previously described for recombinant DNA technology. The growth of normal and mutant human fibroblasts and epithelial cells will follow methods previously established within the LHC.

Major Findings:

The two primary areas of progress are (1) development of a method to transfect human cells at high frequency and (2) application of this method to (a) determine the role of the HBc gene in the cytopathology of HBV infection for human liver, (b) isolate neo^{+}/UV^{R} (ultraviolet light resistant) XP-A cells transfected with human genomic libraries, and (c) transfect normal human cells with plasmids carrying vHras and vKras oncogenic viral DNA complements.

1. Hepatitis B Carcinogenesis: Transfection of human cells with a $pSV2gpt^{+}$ plasmid constructed to carry a subgenomic fragment of HBV containing only

the HBc gene permitted observation of the cytopathologic effects of HBc gene expression immediately after the procedure. We isolated a human carcinoma cell line stably transfected with the HBc^+ gene fragment by selection of transfected cell cultures for gpt^+ expression. The gpt^+/HBc^+ cell line was used to determine that growth in serum-free medium and 5'-azacytidine treatment stimulate the production of HBc gene product (HBcAg). Subsequently, we found that HBc gene expression in a hepatocellular carcinoma cell line carrying the entire HBV genome is stimulated by these conditions. This cell line has carried the HBV genome since its isolation from a chronically infected patient. The temporal relationship of the cytopathologic response to HBc gene expression is similar for both cell types, indicating a primary role for HBc gene expression in the cytopathology of HBc-infected human liver.

2. XP-A Gene Isolation: Microcarrier bead cultures of XP-A cells were grown to 2-5 x 10^8 cells/culture. The protoplast fusion method was adapted for transfection of human genomic libraries to cells growing on microcarrier beads. Gene isolation began with the construction of pSV2 neo^+/HeLa genomic libraries from 20-30 kbp partial BamHI digests of nuclear DNA. The primary E. coli HB101 transformants were stored at -20°C in glycerol. After growth and amplification, the HB101/pSV2 neo^+/$HeLa^+$ cells were fused with 2-5 x 10^8 XP-A cells growing on microcarrier beads. After 72 hours, the cultures were selected for neo^+ expression by growth in G418 (neomycin). The viable cells were removed from microcarrier bead cultures, washed, and seeded onto 100 mm dishes for selection. After ultraviolet (UV) irradiation, several UV-resistant colonies grew to confluence. The isolation of neo^R UV^R XP-A recipient cells indicates that these cells may carry functional recombinants with DNA-repair gene(s) linked to the neo^+ marker.

3. Transfection of Primary Cultures of Human Cells: Transfection of primary human bronchial cultures with plasmids carrying either the vKras or vHras oncogenic complementary DNA results in alteration of cell growth properties. These experiments have demonstrated that epithelial cells become "serum resistant" in LHC4-growth medium at a frequency of 10^{-4} to 10^{-5} units. We have isolated "potentially transformed" cells after transfection with vHras but not vKras. The origin of these cells (i.e., epithelial, fibroblastic), the presence of vHras gene, and production of p21 transforming factor in these cells is still being tested.

Significance to Biomedical Research and the Program of the Institute:

The development of a method to efficiently transfect a variety of human cell types is of general significance to biomedical research programs that employ human somatic cell genetics and molecular biology. The initial application of these procedures to problems related to various aspects of human carcinogenesis has produced a unique insight into the biological role of the core antigen gene of HBV. This information is immediately applicable to understanding the basis of problems associated with chronic HBV infection and carcinogenesis. The gpt^+/HBc^+ cell line produced by transfection analysis of the HBc^+ gene provides a unique opportunity to determine at the molecular level the details of DNA methylation as a control factor for expression of a gene with proven biological significance. In addition, the use of this procedure to isolate the XP-A gene(s) proves its general applicability to

gene-isolation projects. The alteration of normal human bronchial epithelial and/or fibroblastic cells by transfection with plasmids carrying a retroviral oncogenic complementary DNA proves the applicability of this procedure for oncogene testing and isolation.

Proposed Course:

1. The HBV carcinogenesis program will proceed as follows: (a) the effect of HBc^+ gene expression will be studied to determine the factors that regulate HBc^+ gene expression; (b) we will determine the role of the methylation site in HBc^+ promotor and structural genes in regulating HBc gene expression; (c) we will transfect primary human liver cells with tandem duplicates of the HBV genome for long-term carcinogenesis experiments.

2. The XP-A gene isolation project will continue with southern blot analysis of neo^+/UV^R XP-A recipient cells. Identified bands will be recloned and tested by transfection analysis for frequencies of UV^R colonies. This will permit identification of an $XP\text{-}A^+$ fragment of human genomic DNA. Extensive restriction analysis, DNA sequencing, and genetic analysis of UV^R XP-A fragments will be conducted.

3. The transfection of normal bronchial primary human cultures will be used to study the role of oncogenic virus complements in human cells in vitro.

Publications:

Yoakum, G. H.: Amplification of DNA-repair genes using plasmid pkC30. In Wu, R. (Ed.): Methods in Enzymology. New York, Academic Press, 1983, Vol. 101., pp. 138-155.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05321-01 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Carcinogen-DNA Adducts by Synchronous Luminescence Spectroscopy

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Kirsi Vahakangas, Visiting Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

Insitute of Occupational Health, Helsinki, Finland; Department of Pharmacology, University of Oulu, Oulu, Finland; University of Maryland School of Medicine, Baltimore, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Sensitive methods to detect carcinogen-DNA adducts are needed to study the mechanism of carcinogenesis as well as to measure exposure to carcinogens. Synchronous luminescence spectroscopy is a more sensitive method than to conventional luminescence methods. Compared to USERIA (ultrasensitive enzymatic radioimmunoassay), its sensitivity may appear to be at the same range if large quantities of test DNA are available. By this method, benzo[a]pyrene and aflatoxin-DNA adducts were detected in various tissues of persons with known exposure to these carcinogens, but also in some persons without known exposure. According to the preliminary data the relationship between the luminescence intensity and the amount of carcinogen-DNA-adduct seems to be in linear correlation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affilitations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Curtis C. Harris	Chief	LHC, NCI
Herman Autrup	Acting Chief, CMIS	LHC, NCI
Dean Mann	Senior Investigator	LHC, NCI

Objectives:

Benzo[a]pyrene (BP)- and aflatoxin B_1 (AFB)-DNA adducts are assayed in various tissues from people exposed to BP (e.g., smokers, foundry workers) and AFB (e.g., people eating food contaminated by aflatoxin). In addition to qualitative information, the possibilities of using this method for quantitation are being studied.

Methods Employed:

DNA is isolated from cells or tissues by a method which include extractions by organic solvents, RNase and proteinase treatments and ethanol precipitations. Isolated DNA is dissolved either in water or in buffer solution and stored at 4°C for measurement.

The device for synchronous luminescence spectroscopy contains the following units: (1) xenon lamp; (2) fluorescence spectrophotometer; (3) photon-counting unit; (4) amplifier; (5) recorder. Because of the photon-counting unit, the system is more sensitive than the regular fluorescence spectrophotometer.

In conventional luminescence spectroscopy a luminescent substance is excited at fixed wavelength while the intensity distribution pattern of emission or emission spectrum is monitored by scanning the emission wavelength. An excitation spectrum can be obtained by scanning excitation wavelength while the emission is monitored at a fixed wavelength. In synchronous luminescence spectroscopy (as first suggested by Lloyd, Nature 231, 64, 1971), both excitation and emission wavelengths are changed simultaneously while keeping a constant wavelength interval ($\Delta\lambda$) between them.

For both BP-DNA and AFB-DNA adducts, $\Delta\lambda$ of 34 nm is used. For both only one peak is seen in the synchronous spectrum, the maximum of the peak being at 378 nm for BP-DNA and at 425 nm for AFB-DNA.

Major Findings:

BP-DNA adducts have been found by this method in lymphocytes from peripheral blood of coke oven workers, placental DNA from both smokers and nonsmokers, and alveolar macrophages. According to preliminary data the relationship between the luminescence intensity and the amount of BP-DNA adducts in the sample seems to be in linear correlation. Because the amount of BP-diol epoxide molecules in the sample is detected, the concentration of DNA in the sample determines the sensitivity. At the concentration of 20 mg per ml of

sample, 10 fmoles per microgram of DNA can be detected (equivalent to 1 adduct per 300,000 nucleotides). AFB-DNA adducts have been found in liver from people living in geographical areas where AFB is known to contaminate food products to a significant extent.

Significance to Biomedical Research and the Program of the Institute:

Sensitive methods to detect carcinogen-DNA adducts are needed to settle the relationship between the carcinogen exposure, actual binding to DNA, persistence of the adducts, and cancer formed in these same individuals.

Because the amount of adducts reflects not only the amount of carcinogen entered in the body but also the amount of activated metabolites formed within the body, the level of adducts is probably a far more accurate measure of the true exposure to the carcinogen than, for example, the detection of carcinogen in the air.

Proposed Course:

The method described here is one of several methods used in our laboratory to detect carcinogen-DNA adducts. The results gained by this method will be compared with those of other methods.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05322-01 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Hepatitis B Virus in Hepatocellular Carcinoma

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Brent Edward Korba, Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A subgenomic fragment of hepatitis B virus (HBV) has been inserted into the vector pSV2gpt. This fragment carries an intact core antigen gene. Using a protoplast fusion method of transfection developed in this laboratory, this plasmid has been stably introduced into a human mucoepidermoid carcinoma cell line which exhibits many of the properties associated with normal epithelial cells. Cells expressing the HBV core antigen at a low basal level exhibit a marked cytopathic response. Treatment of these cells with 5'-azacytidine, as well as changes in culture conditions, raises the level of expression to a lethal level. There appears to be a correlation between elevated expression of the core antigen gene and loss of cytosine methylation in DNA at a specific site in the promoter region. Factors that regulate core antigen gene expression in transfected cultures also cause similar effects in an hepatocellular carcinoma cell line which has carried several copies of the HBV genome since its isolation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

George Yoakum	Senior Staff Fellow	LHC, NCI
Herman Autrup	Acting Chief, CMIS	LHC, NCI
John Lechner	Senior Staff Fellow	LHC, NCI
Vincent Wilson	Staff Fellow	LHC, NCI
Curtis Harris	Chief	LHC, NCI

Objectives:

To determine the relationship between heptitis B virus (HBV) and hepatocellular carcinoma. Studies are directed toward the development of a model system using cultured human epithelial cells into which HBV DNA and/or chemical carcinogens can be introduced. These experiments will eventually be extended to the study of these agents in primary cultures of normal human hepatocytes.

Methods Employed:

The protoplast fusion method of transfection has been modified to allow for stable transfer of pSV2-derived plasmids into human cells at frequencies greater than 10^{-3}. This procedure permits transient expression of transferred genes in 70 to 90% of the recipient cell culture for 6 to 12 days. Subsequent selection for dominant markers produces a population containing stably integrated plasmid DNA. For these studies a fragment of HBV containing the core antigen (HBcAg) gene (HBc gene) was inserted into the BamH1 site of pSV2gpt using standard recombinant DNA techniques. HuT 292, a mucoepidermoid carcinoma cell line, was used as a recipient since these cells exhibit many of the properties associated with normal epithelial cells.

Expression of the HBc gene was followed using a commercially available radioimmune assay kit for detection of the HBV e antigen (HBeAg). Since HBeAg is reported to be a proteolytic product of HBcAg, this kit quantitatively cross-reacts with HBcAg. Indirect immunofluorescent staining using a high titer human antiserum against HBcAg was also used. Presence of integrated viral DNA sequences was detected by Southern blot analysis of chromosomal DNA extracted from isolated nuclei. Morphology and cytopathic responses of cultured cells to HBcAg were followed using light microscopy and histochemical staining in clonal growth assays.

Major Findings:

Transfection of HuT 292 cultures with plasmids containing a subgenomic fragment of HBV encoding for HBcAg (pKYC200) was used to determine some of the factors that regulate the expression of the HBc gene and to explore the effects of expression of the HBc gene on human epithelial cells separate from the rest of the HBV genome. Within 48 hours following transfection, HuT 292 cultures containing pKYC200 (GTC2) displayed a marked cytopathic response (i.e., vaculation, granulation). Cultures receiving the vector pSV2gpt appeared identical to the parental cultures. Selection of GCT2 cultures for the gpt^{+}

marker produced a cell population which expressed the HBc gene at a virtually undetectable level in RPMI 1640 medium with 10% fetal calf serum (FCS). Growth of these cultures in a modified MCDB 151 medium (LHC4) with 5% FCS significantly elevated the level of HBc gene expression. Treatment with 5'-azacytidine further stimulated expression in either medium. In each of the latter cases, an increased cytopathic response was observed. Expression of the HBc gene peaks approximately 10 to 15 divisions after 5'-azacytidine treatment at which time the cultures experience a biological crisis. The surviving cells, representing less than 0.1% of the precrisis culture, no longer produce detectable levels of HBcAg when allowed to grow to confluence. Subsequently, it was found that an hepatocellular carcinoma cell line (PLC/PRF/5), which carries several complete copies of the HBV genome, responds similarly to growth in LHC4 medium and 5'-azacytidine treatment. Preliminary analysis of integrated HBV DNA from GTC2 cells indicates that loss of DNA methylation at a specific site approximately 50 base pairs upstream from the AUG start codon of the HBc gene is directly correlated with increased expression of this gene.

Significance to Biomedical Research and the Program of the Institute:

Hepatitis B virus, in addition to being the major cause of viral hepatitis, has been epidemiologically linked to hepatocellular carcinoma and acquired immune deficiency syndrome (AIDS). To understand the mechanism of HBV pathology during acute and chronic disease processes, it is essential to separate the various viral genetic elements and to study their biological effects and molecular biology in a model cell system in vitro. This project demonstrates the value of such an approach by revealing the importance of the core antigen in the cytotoxic response of cells to infection with HBV, a role previously unassigned to any specific HBV gene. The methylation state of chromosomal DNA has been implicated as a general controlling factor in carcinogenesis. The system described here provides a unique opportunity to study, at the molecular level, the role of DNA cytosine methylation in controlling the expression of a specific gene of established biological importance in human cells.

Proposed Course:

The project at this point has been directed toward the development of techniques to stably introduce HBV DNA into cultured human cells. Future plans are directed toward a further characterization of the factors that regulate HBc gene expression and its biological consequences. In addition, the transfection of primary cultures of normal human hepatocytes with cloned HBV DNA, in collaboration with Dr. Sun Tsung-tang of the Cancer Institute, Chinese Academy of Medical Science and Dr. I-C. Hsu of the University of Maryland School of Medicine, will initiate a long-term study of HBV-related carcinogenesis in vitro. These studies include the treatment of HBV-transfected cultures with chemical carcinogens, particularly aflatoxin B_1.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05323-01 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Detection of DNA Polymorphisms Associated with Genetic Predisposition to Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Bruce M. Boman, Medical Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Classic genetic linkage studies have been used to predict the genetic risk of developing diseases. Until recently, disease-associated polymorphisms could be assessed only by analyzing gene products, e.g., cell surface histocompatibility antigens or blood group substances commonly referred to A,B,H. Advances in molecular biology have now made it possible to measure genetic polymorphisms at the DNA level. This approach utilizes restriction enzyme catalyzed endonucleolytic cleavage and DNA hybridization with gene-specific probes to detect base-pair substitution and fragment length polymorphisms. The potential of this technology is exemplified by the recent identification of individual DNA polymorphisms associated with diabetes mellitus and hemoglobinopathies. A similar molecular approach using specific DNA probes, e.g., oncogenes or ectopic hormones, can be used to potentially detect and characterize DNA polymorphisms in individuals who are oncogenetically predisposed.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Curtis C. Harris	Chief	LHC, NCI
Vincent Wilson	Staff Fellow	LHC, NCI
George Yoakum	Senior Staff Fellow	LHC, NCI

Objectives:

To study possible DNA polymorphisms associated with the genetic predisposition to cancer through the application of molecular biological technology. Studies are directed to characterize DNA polymorphisms of cellular genes such as oncogenes or ectopic polypeptide hormones and to determine whether any specific polymorphism is associated with a predisposition to cancer.

Methods Employed:

This laboratory has developed molecular biological technology to measure gene polymorphisms at the DNA level. This approach utilizes restriction enzyme catalyzed endonucleolytic cleavage and DNA hybridization with gene-specific probes to detect base-pair substitution and fragment length polymorphisms. To study gene polymorphisms in humans it was necessary to develop methodology to isolate high molecular weight DNA from surgical tissues. We have also isolated DNA from cultured normal and malignant human cell lines. The secretion of ectopic polypeptide hormones by these cell lines is determined by radioimmune assay and immunofluorescence staining. Specific DNA probes for cellular oncogenes and ectopic polypeptide hormones will be used to detect and characterize possible gene polymorphisms present in the various isolated DNA samples.

Major Findings:

The described project is still in its early stages with no major findings as yet.

Significance to Biomedical Research and the Program of the Institute:

The identification and characterization of specific gene polymorphisms associated with the predisposition to cancer have two potential possibilities in regard to oncology research programs: (1) knowledge of any polymorphisms could lead to further understanding of the genetic mechanisms underlying processes of carcinogenesis; and (2) polymorphisms could serve as markers to identify individuals in the normal population who are at risk of developing cancer.

Proposed Course:

To identify polymorphisms associated with the predisposition to cancer we plan to utilize DNA samples isolated from three sources: (1) normal lung parenchyma

from lung cancer patients; (2) cultured human lung cancer cell lines; and (3) cultured normal fibroblastic cell lines from individual members of families that have a high incidence of lung cancer. Isolated DNA samples from normal cultured human epithelial and fibroblastic cell lines will serve as controls.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05324-01 LHC

PERIOD COVERED

January 9, 1983 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Suppression of Human Lung Carcinoma Malignancy in Malignant & Normal Hybrid Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Edward W. Gabrielson, Medical Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies in other laboratories have shown that fusion of malignant cell lines with intraspecific normal cells often results in suppression of tumorigenicity in stable hybrids. This suppression is sometimes transient, leading to speculation that the normal cell contributes genes that suppress malignancy, and when these genes are lost by further passage in tissue culture, malignancy is reexpressed as a recessive trait.

We have recently begun to study the expression of malignancy in hybrids of malignant and normal cells of human bronchial epithelial origin (cells derived from the same tissue as well as the same species).

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Bruce M. Boman	Medical Staff Fellow	LHC, NCI
John Lechner	Senior Staff Fellow	LHC, NCI
Curtis C. Harris	Chief	LHC, NCI

Objectives:

Malignant human lung carcinoma cell lines carried in culture will be tested for the ability to have malignancy suppressed by fusion with normal human bronchial epithelial cells. Chromosomes, and eventually genes, from the normal cell responsible for any observed suppression will be identified.

Methods Employed:

Human lung carcinoma cell lines are carried in culture in our laboratory, and we have derived hypoxanthine phosphoribosyl transferase (HPRT)-lacking and ouabain-resistant mutants for use in hypoxanthine-aminopterin-thymidine (HAT) selection after fusion. Techniques for culture of normal human bronchial epithelial cells have been previously developed in this laboratory.

Fusions will be done with polyethylene glycol, and hybrids selected in a HAT medium (to kill the nonhybridized malignant parent) with oubain (to kill the nonhybridized normal parents). Tumorigenicity of hybrid clones will be assayed in athymic nude mice.

Major Findings:

This project is in its early stages, and we have no major findings.

Significance to Biomedical Research and the Program of the Institute:

Recessive genes responsible for malignancy in human lung carcinoma lines can be recognized and identified with this system.

Proposed Course:

Hybrid cell lines which are not tumorigenic in nude mice will be carried in culture for extended periods of time and periodically reexamined for tumorigenicity. Comparisons of tumorigenic and nontumorigenic hybrid cell lines will be done by examining the karyotypes, biochemical studies, and examining the expression of cell surface antigens.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05325-01 LHC

PERIOD COVERED

November 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemical Carcinogenesis and 5-Methylcytosine Patterns in DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Vincent L. Wilson, Ph.D., Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The pattern of 5-methylcytosine residues in mammalian DNA has recently been found to be crucial to the control of genetic expression. Decreases in DNA 5-methylcytosine content are known to alter the level of differentiation of cells in culture. Thus changes in DNA 5-methylcytosine patterns may be critical to the process of carcinogenesis. Human tumor DNAs will be probed for DNA methylation pattern alterations in selective DNA sequences and genes. Since chemical carcinogens have been shown to decrease genomic 5-methylcytosine levels in BALB/3T3 cells, DNA from carcinogen-treated human epithelial cells will also be probed for changes in 5-methylcytosine patterns.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Curtis C. Harris	Chief	LHC, NCI
John F. Lechner	Senior Staff Fellow	LHC, NCI
Bruce M. Boman	Medical Staff Fellow	LHC, NCI

Objectives:

To determine the relationship between the changes in 5-methylcytosine patterns in DNA and the carcinogenesis process. It is known that tumor cells contain altered methylation patterns in some genes and DNA sequences as compared to normal tissue. It is not known, however, if these changes in 5-methylcytosine patterns initiate carcinogensis, occur during carcinogenesis, or are the result of this multistep process. Studies are directed to determine the correlation between the ability of chemical carcinogens to inhibit the formation of 5-methylcytosine and carcinogen-induced oncogenic transformation. Specific genes and DNA sequences that may be more susceptible to carcinogen-induced decreases in 5-methylcytosine are also sought. These susceptible DNA sequences may be the same areas observed to be undermethylated in tumor cells. The methylation patterns of human tumor DNAs will be probed in the search for demethylated genes and/or DNA sequences that may be specific for the tumor type or tissue of origin.

Methods Employed:

This laboratory has developed and utilized human bronchial tissue and epithelial cell culture as a model for carcinogenesis studies. This system also provides a model for the study of the effects of chemical carcinogens on the methylation patterns in the DNA of normal human epithelial cells. DNAs are isolated from carcinogen-treated epithelial cultures, restricted with Hpa II, Msp I and other enzymes sensitive to cytosine modification, and probed with specific DNA sequences and genes. The genomic levels of 5-methylcytosine will also be monitored in treated cells by [^{3}H-6]-uridine labeling of dividing cells, the DNA isolated, digested to individual bases, and the ratio of 5-methylcytosine to the total cytosine and 5-methylcytosine will be determined. The time course of these effects will also be followed since previous work has determined that the genomic level of 5-methylcytosine in some mammalian cells is decreased maximally by 48 hours post carcinogenic treatment. Epithelial cell DNA methylation patterns will be compared to those of various carcinoma cell lines and human tumors. High molecular weight DNAs will be isolated from human tumors, subjected to the same enzyme restriction, gel electrophoresis, as above, and probed for alterations in methylation patterns in specific genes and DNA sequences.

Major Findings:

This is a new project. Recent findings have determined that methylation patterns in DNA are not only important to gene expression, but changes in

these patterns take place during differentiation and in vitro senescence. Thus the ability of chemical carcinogens to alter 5-methylcytosine patterns in DNA may provide clues to the carcinogenic action of these agents. Previous studies have determined that the alkylation of DNA by alkylating carcinogens inhibits the enzymatic modification of cytosine residues. The abilities of some aromatic hydrocarbon carcinogens to initiate decreases in genomic 5-methylcytosine levels in BALB/3T3 cells have also been demonstrated.

Significance to Biomedical Research and the Program of the Institute:

A basic understanding of the mode of initiation of carcinogenesis by chemical agents should provide clues to the genetic mechanisms taking place during this multistep process. The elucidation of the effects of carcinogens on DNA methylation patterns may advance the understanding of the basic processes involved in tumor formation. Since the majority of human tumors are of epithelial origin, an understanding of the 5-methylcytosine pattern changes in selective genes may provide further clues to the genetic alterations which take place during carcinogenesis.

Proposed Course:

Future studies will depend on the results of the above studies. There are two areas which may be profitable to pursue. The first would be to develop a model system which would allow for the determination of the ability of chemical carcinogens to alter the methylation of a single or limited number of sites in a known DNA sequence, and correlate this to an observable biological effect. The second area would be to study the level of expression of genes or specific DNA sequences determined to be undermethylated in tumor and/or transformed epithelial cells. The pattern of methylation of these genes or DNA sequences may also be monitored for changes during the transformation process in normal epithelial cells as well.

Publications

Wilson, V. L. and Jones, P. A.: DNA methylation decreases in aging but not in immortal cells. Science 220: 1055-1057, 1983.

Wilson, V. L. and Jones, P. A.: Inhibition of DNA methylation by chemical carcinogens in vitro. Cell 32: 239-246, 1983.

Wilson, V. L., Jones, P. A. and Momparler, R. L.: Inhibition of DNA methylation in L1210 leukemic cells by 5-Aza-2'-deoxycytidine as a possible component of chemotherapeutic action. Cancer Res. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05326-01 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

HLA Antigens: Structure, Function and Disease Association

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Dean L. Mann, Senior Investigator, LHC, NCI

COOPERATING UNITS *(if any)*

Neonatal & Pediatric Medicine Branch, NICHHD; Metabolism Branch, DCBD, NCI; Laboratory of Immunoregulation, NIAID; Clinical Center, NIH; Arthritis & Rheumatism Branch, NIADDKD; Washington Univ. School of Med., St. Louis, Mo.

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☑ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies were performed to elucidate the association of HLA antigens with disease conditions. Antisera, detecting specific HLA-DR determinants, were used in immunoprecipitation studies to define molecular characteristics of the antigen-bearing proteins and in in vitro immune response studies in order to determine the effect of these sera on cellular interactions. HLA-A, B, C, DR, MT and MB antigen frequencies were found to be restricted in Pima Indians. There were differences in HLA antigen frequencies in patients with psoriatic arthritis depending on the degree of psoriasis or arthritis. HLA alloantigen frequencies were determined in families with multiple cases of two different diseases, the variant form of 21-hydroxylase deficiency and systemic lupus erythematosus. Linkage of the 21-hydroxylase deficiency syndrome to HLA genes is not unlike that seen for the congenital disease, suggesting more than a one-gene defect in this disease. In families with multiple cases of systemic lupus erythematosus, common HLA haplotypes were seen more frequently than in diseased individuals. Takayasu's arteritis, a frequent disease in Japan and rare in the U.S., was found to be associated with the MT3 antigen which has a higher frequency in Japanese than in the North American Caucasians. Immunoprecipitation studies with HLA-DR alloantisera demonstrated that some antigenic determinants are on the alpha chain of the HLA-DR dimer and others on beta chains. The studies also suggest that there are at least three HLA-DR genes. Alloantisera directed against the HLA-DR determinants activate suppressor cells in in vitro immune response studies, demonstrating an immunoregulatory function of these molecules. The first definitive demonstration of HLA-associated immune response genes was documented in studies of in vitro insulin response in diabetic patients.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

None

Objectives:

To determine function, structure, and disease association of major histocompatibility complex genes and/or their products. The studies are directed at the elucidation of genetic associations and potential genetic control of the immune response as it relates to disease process and etiology. Once markers are identified in the disease population, functional and biochemical studies are being performed in order to clearly define the genetic regulation of disease processes as it relates to immunologic response.

Methods Employed:

Standard HLA typing was performed using the microcytotoxicity technique. The technique for HLA-A, B, C was recently described by Amos and Poole. The method for typing the B lymphocytes for HLA-DR determinant was originally described by Mann et al. A total of 19 allelic determinants controlled by the HLA-A locus, 26 alloantigens of the HLA-B locus, 6 alloantigens of the C locus, 10 alloantigens of the DR locus, and 6 MT antigens were tested for in the populations. HLA typing was performed by the Laboratory of Immunology, Department of Surgery, Uniformed Services University for the Health Sciences under an interagency agreement. The HLA-DR-bearing molecules were isolated as follows. Cell membrane proteins were labeled by growing the cells in ^{35}S-methionine. Alloantibodies were reacted with the cells lysed with non-ionic detergent and the antigen-antibody complexes isolated by adherence to Staphylococcal A proteins. The antigen-antibody complexes were dissociated and the antigens were analyzed by two-dimensional polyacrylamide gel electrophoresis. Two types of functional studies were performed. In the study to determine the effect of antisera directed against HLA-DR determinants on immune response, peripheral blood monocytes were isolated by surface adherence and exposed to antibodies bearing specific HLA-DR determinants. The cells were washed and added to cultures of cells stimulated with tetanus toxoid or SKSD. Responses were measured by ^{3}H-thymidine incorporation. T cells were isolated from this system and reintroduced into autologous cultures. The results demonstrated the induction of suppressor T cells by monocytes coated with anti-HLA-DR antibodies. Peripheral blood lymphocytes were obtained from patients with diabetes receiving insulin. These lymphocytes were stimulated with graded doses of the insulin components and ^{3}H-thymidine incorporation was measured in order to determine immune responsiveness. HLA typing was performed in the same individuals. Associations of the HLA types and responses to insulin components were examined for significance by statistical methods.

Major Findings:

This project has led to several significant observations. The severity of diseases such as psoriasis and psoriac arthritis, which have demonstrable HLA association, now specifically appears to depend on combinations of different HLA alloantigenic determinants. The results indicate that the multiple genes within the complex have influence on or control disease severity. Gene interaction in the major histocompatibility complex is further demonstrated in studies in families with systemic lupus erythematosus. It has been postulated that a single recessive allelic gene linked to the major histocompatibility complex was responsible for the development of congenital adrenal hyperplasia. Our studies have demonstrated that the attenuated form of this disease is also linked to the human major histocompatibility complex. Our studies suggest the possibility that there is more than one gene within the major histocompatibility complex that influences the severity of the disease. Immunoprecipitation studies using antisera directed against HLA-DR alloantigenic determinants have demonstrated that there are multiple genes within this particular region which control the expression of HLA-DR heavy (α) and light (β) chains, the dimeric product of this region. These studies suggested that the HLA-DR polymorphism is carried on the β chain while the more restricted MT and MB specificities are carried on the α chain of this molecule. Antisera reacting against these proteins can induce suppression in in vitro immune response studies. These results are highly indicative that the products of the major histocompatibility complex not only act as immune response genes in recognition of the antigenic determinants but that antibodies developed against these determinants can induce suppression. These results suggest a feedback mechanism for the control of immune responsiveness. Studies with the diabetic population exposed to insulin have demonstrated that the human immune system can recognize small differences in amino acid composition and that an immune response against these differences can be developed. This is the first demonstration that human immune response genes are in fact linked to the major histocompatibility complex in man.

Significance to Biomedical Research and the Program of the Institute:

A variety of diseases have been demonstrated to be associated with the human major histocompatibility complex. These associations and their influence in the disease process are not well understood. Further defining HLA association with disease severity demonstrates that the associations are complex and that the disease entity in and of itself probably results from gene interaction within this major histocompatibility complex. Furthermore, the demonstration that the human immune system can recognize small alterations in amino acid composition of a potential immunogen demonstrates the exquisite sensitivity of the human immune response. Alterations in immune response are well documented in patients with cancers. Whether these alterations occur as a result of or as part of the disease process and disease susceptibility remains to be determined. However, detailed analysis of the genes and gene products within the major histocompatibility complex will further our knowledge as to the role of these genes in the human immune response and in responses that may be related to carcinogenesis.

Proposed Course:

This project heretofore represents the combined effort of a variety of investigators, both clinical and laboratory. This project is now being directed at families where there are multiple cases of disease, particularly cancer, in a single family. Attempts will be made to correlate the combination of genes within the major histocompatibility complex with disease. These studies will be designed to examine alteration in immune response in in vitro assays in an attempt to correlate the susceptibility of disease risk with altered immune responsiveness.

Publications

Broder, S., Uchiyama, T., Muul, L., Muchmore, A., Blaise, R. M. and Mann, D. L.: Activation of regulatory cells by antibodies to human Ia-like antigens. In Fauci, A. and Bellicux, R. (Eds.): Human B-cell Function, Activation, and Immunoregulation. New York, Raven Press, 1982, pp. 289-297.

Chrousous, G. P., Loriaux, L. D., Mann, D. and Cutler, G. B.: Late onset of 21-hydroxylase deficiency in an allelic variant of congenital adenal lympoplasia characterized by attenuated clinical expression and different HLA haplotype associations. Hormone Res. 16: 193-200, 1982.

Chrousos, G. P., Loriaux, L. D., Mann, D. L. and Cutler, G. B.: Late onset of 21-hydroxylase deficiency mimicking Idiopathic Hirsutish or polycystic disease: An allelic variant of congenital virilizing adrenal hyperplasia with a milder enzymatic defect. Ann. Intern. Med. 96: 143-148, 1982.

Ensroth, A. F., Mann, D. L., Johnson, A. H., Knowler, W. C., Pettitt, D. J. and Bennet, P. H.: HLA and B-lymphocyte alloantigens in Gila River Indians. Tissue Antigens 21: 198-207, 1983.

Gerber, L.-H., Murray, C. L., Perlman, S. G., Mann, D. L., Decker, J. L., Barth, W. F. and Nigra, T. P.: Human lymphocyte antigens characterizing psoriatic arthritis. In Farber, E. M., Cox, A. J., Wall, L. and Jacobs, P. H. (Eds.): Psoriasis. New York, Grune and Stratton, 1982, pp. 289-291.

Karr, R. W., Kannapell, C. C., Stein, J. A., Fuller, T. C., Duquesnoy, R.-J., Rodey, G. E., Mann, D. L., Gebel, H. M. and Schwartz, B. D.: Demonstration of a third structurally distinct human Ia beta chain by 2 dimensional gel electrophoresis. J. Exp. Med. 156: 652-657, 1982.

Karr, R. W., Kannapell, C. C., Stein, J. A., Gebel, H. M., Mann, D. L., Duquesnoy, R. J., Fuller, T. C., Rodey, G. E. and Schwartz, B. D.: Molecular relationships of the human B cell alloantigens MT2, MB3, MT4, and DR5. J. Immunol. 128: 1809-1818, 1982.

Karsh, J., Klippel, J. H., Mann, D. L., Reinertsen, J. L., Montsopoulos, H. M., Johnson, A. H. and Decker, J. L.: Histocompatibility antigen combinations in rheumatoid arthritis. Clin. Exp. Rheum. 1: 11-15, 1983.

Lisse, J. R., Kuberski, T. T., Bennett, P. H., Knowler, W. C., Grofton, J. P., Colin, A. and Mann, D. L.: High risk of sacroiliitis in HLA-B27 positive Pima Indian men. Arthritis Rheum. 25: 236-238, 1982.

Mann, D. L., Mendell, N., Kahn, C. R., Johnson, A. H. and Rosenthal, A.: In vitro lymphocyte proliferation response to therapeutic insulin components: Evidence for genetic control by the human major histocompatibility complex. J. Clin. Invest. (In Press)

Muchmore, A. V., Decker, J. M. and Mann, D. L.: Evidence that antisera that react with products of the human HLA-DR locus may block in vitro antigen-induced proliferation by inducing suppression. J. Immunol. 128: 2063-2066, 1982.

Muchmore, A., Megson, M., Decker, J., Knudsen, P., Broder, S. and Mann, D. L.: Inhibitory activity of antisera to human Ia-like determinants: Comparison of intact and pepsin digested antibodies. J. Immunol. (In Press)

Nashel, D. J., Leonard, A., Mann, D. L., Gucicion, J. G., Katz, A. L., and Sliwinski, A. J.: Ankylising spondylitis and systemic lupus erythematosus: a rare HLA combination. Arch. Int. Med. 142: 1227-1228, 1982.

Reinertsen, J. L., Klippel, J. H., Johnson, A. H., Steinberg, A. D., Decker, J. L., and Mann, D. L.: Family studies of B lymphocyte alloantigens in systemic lupus erythematosus. J. Rheumatol. 9: 253-262, 1982.

Rosenthal, A. S., Mann, D. L. and Kahn, C. R.: Genetic control of the immune response to insulin in man and animal. In Gupta, P. (Ed.): Immunology of Diabetes in Man and Animal. New York, Plenum Press. (In Press)

Volkman, D. J., Mann, D. L. and Fauci, A. S.: Association between Takayasu's arteritis and a B-cell alloantigen in North Americans. New Eng. J. Med. 306: 464-465, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05327-01 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Monoclonal Antibodies to Carcinogen-DNA Adducts

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Dean L. Mann, Senior Investigator, LHC, NCI

COOPERATING UNITS *(if any)*

Department of Pathology, Uniformed Services University for the Health Sciences, Bethesda, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Monoclonal antibody technology provides an important and useful tool in the study of population exposure to carcinogens. Carcinogens are known to adduct to human DNA. The presence of these carcinogen DNA adducts can be detected by monoclonal antibodies directed against specific products. Following the mouse model systems, constitutents of tobacco smoke condensate as well as metabolites of nitrosoureas adducted to DNA have been used as immunogens. Protamine has been used as a carrier in the immunization in order to avoid problems with assay techniques that involve other animal proteins. Antibodies directed against carcinogen DNA adducts have been produced in the immunized mice. Spleen cells from these mice have been fused with mouse myeloma proteins and clones of cells producing antibody against the immunogen have been isolated. This laboratory has also demonstrated that humans may develop antibodies to potential carcinogens to which they are exposed. It is therefore important to develop a system of production of human monoclonal antibodies. To this end, a number of established myeloma and B cell lines have been screened for the capability to be fused with human B cells. A model system has been established whereby chronic lymphocytic leukemia cells have been fused to a human myeloma cell line. The product of this cell line was the predominant cell surface immunoglobulin found on the chronic lymphocytic leukemia cells. This immunoglobulin was then used to produce a monoclonal antibody in mice and the antibody selected on the basis of restricted reactions to the specific immunoglobulin. Thus an anti-idiotype monoclonal antibody has been produced. The results demonstrate the capability of using human materials, B cells, and myeloma cell lines to produce monoclonal antibodies.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Curtis C. Harris	Chief	LHC, NCI
Glennwood E. Trivers	Research Scientist	LHC, NCI

Objectives:

The objectives of this project are to produce monoclonal antibodies which would react against chemical compounds known to be associated with or are carcinogenic in animal model systems. The chemicals under study are those found in the environment and also drugs that are known to produce cancer in man. We are attempting to perfect techniques to produce human monoclonal antibodies by fusing peripheral blood B lymphocytes from individuals sensitized to a particular compound with a human myeloma cell. Once the antibodies are developed they will be used to screen populations exposed to environmental chemicals as well as DNA from individuals treated with chemotherapy.

Methods Employed:

Standard hybridoma technology is being used in this study with variations and innovations developed in this laboratory to produce human monoclonal antibodies. In the mouse system, the carcinogen DNA adducts are combined with protamine and injected into mice. After repeated immunizations, the sera from the mouse are screened for antibodies to the immunogen. Once antibodies develop, the spleens are removed and fused to a HAT-sensitive mouse myeloma cell line. The established fusion product is cloned by limiting dilution and, after an appropriate period of growth, culture supernatants are tested for antibodies to the immunogen. Clones with selected and specific reactivity are then expanded in tissue culture systems and injected into the peritoneal cavity of mice in order to produce ascites fluid and high-titered antibody. Analogous techniques are used in the human system. The variation that has been applied in this laboratory is to isolate peripheral blood B lymphocytes and to use these lymphocytes as fusion partners to produce monoclonal antibodies. The assay systems for antibody production are the ELISA and USERIA techniques. These techniques employ the use of a enzyme conjugated anti-immunoglobulin directed against the monoclonal antibody which in turn detects the antigen under study. Appropriate dilutions of the monoclonal antibody are made, and the conjugated anti-immunoglobulin and substrate are added as a means of detection. The USERIA assay employs a radioactive substrate to increase the sensitivity of the methods of detection. Antibodies detecting specific cell surface antigens are assayed for using the fluorescence-activated cell sorter. The monoclonal antibody is exposed to the cell, the excess removed by washing, and a fluorescent-conjugated anti-immunoglobulin is added. All cells showing a forward light scatter pattern are examined for fluorescence.

Major Findings:

Successful immunizations have been achieved with the tobacco smoke condensate (TSC) constitutents enzymatically conjugated to DNA and with O^6ethyl and

N^7-methyl guanosine derivatives. Clones of cells have been derived from fusions that produce antibodies specific for the O^6 and N7 derivatives. Quantities of antibodies are currently being produced in ascites form in the mice. Clones of cells have been derived from fusions of the spleens from mice showing antibody to TSC to DNA. One of the clones appears to be specifically reacting against benz(a)pyrene DNA while other clones appear to be detecting other chemical substances in the DNA modified by the TSC. A prototype of the human system has been developed. Under study are several individuals from one family with chronic lymphocytic leukemia (CLL). Surface immunoglobulins of different heavy chain types have been demonstrated on the CLL cells from the different family members. The object of the experiment was to attempt to isolate the immunoglobulin being produced by the CLL cell using hybridoma technology. Cells from a patient were fused with a human nonproducing myeloma cell line. The resulting fusion cell was cloned and cultured supernatants were assayed for the IgM immunoglobulin. Cells from the patient were used to immunize mice and antibody derived from this immunization was produced by fusion of spleen cells from the mouse with a mouse myeloma cell line. Antibodies from clones of this fusion were selected to react specifically with the IgM being produced by the human-human fusion. An antibody has been developed which appears to react exclusively with the IgM protein produced by the CLL cell lines. Furthermore, this monoclonal antibody reacts with the CLL cells from the patient. This demonstrates the capability of producing human-human fusion products with the resultant production of a human immunoglobulin protein and the further development of a mouse monoclonal antibody which is detecting an apparent idiotype on the surface of these cells.

Significance to Biomedical Research and the Program of the Institute:

The use of monoclonal antibody production technology is a powerful tool in biochemical epidemiology. The production of antibodies specific for chemicals, drugs or their metabolites that are associated with carcinogenesis can be applied to studies which populations are screened for exposure to these environmental carcinogens. In addition, these monoclonal antibodies can be used to attempt to define specific compounds in complex materials known to be associated with carcinogenesis. Antibodies with individual specificities to a particular compound can be used to isolate the compounds and to identify specific chemicals which may be adducted to DNA. In addition, these antibodies can be used to isolate segments of DNA which have specific carcinogen complexes in order to determine the potential alteration in gene expression in cells exposed to chemical carcinogens.

Proposed Course:

The project will continue to attempt to develop a variety of monoclonal antibodies against carcinogen DNA adducts. In turn, these antibodies will be used to study DNA from populations known to be exposed to these potential carcinogens in the environment, either in the workplace or in the general population. With the appropriate inhibition techniques, sera from patients exposed to these potential chemical substances (carcinogens) will be assessed for presence of antibodies to these compounds produced by the host. Systematic screening of exposed populations as well as control populations will be performed. Attempts will be made to develop human monoclonal antibodies using B cells from

individuals who have demonstrable antibody to carcinogen DNA adducts. In vitro sensitization techniques will be developed and explored in these studies. The monoclonal antibodies developed against the IgM idiotype will be tested in other family members of the proband. If reactions are found with other CLL cells in this family the study will be broadened to examine CLL cells from a population with this disease.

Publications

Blattner, W. A., Greene, M-H., Goedert, J. J. and Mann, D. L.: Interdisciplinary studies in the evaluation of persons at high risk of cancer. In Harris, C. C. and Autrup, H. A. (Eds.): Human Carciongenesis. New York, Academic Press (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05328-01 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunologic Studies of Human T-cell Lymphoma Virus

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Dean L. Mann, Senior Investigator, LHC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Tumor Cell Biology, NCI; Environmental Epidemiology Branch, NCI; Metabolism Branch, NCI; Laboratory of Immunology, Department of Surgery, Uniformed University for Health Sciences, Bethesda, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A human C-type retrovirus has been found to be associated with certain adult T-cell malignancies. Lymphocytes from these patients were established in long-term tissue culture using the human T-cell growth factor. Studies were carried out to examine the cell surface markers of cells infected and with producing the human T-cell lymphoma virus (HTLV). HLA typing was performed on these cells. The virus was transferred to human umbilical cord blood lymphocytes by coculture. Cytotoxic lymphocytes were developed against an autologous cell line established from one of the patients. Cell lines established from patients with the adult T-cell leukemias and lymphomas expressed more than the expected two alloantigens controlled by the HLA-A or HLA-B locus. Alloantisera detecting these altered determinants were confined to the HLA-AW19 cross-reactive group and the HLA-B5 cross-reactive group. A monoclonal antibody detecting a polymorphic epitope on HLA alloantigens of these two cross-reactive groups was developed by Dr. Bart Haynes. This monoclonal antibody was found to react with all cells infected with HTLV and producing products of this virus. Human umbilical cord lymphocytes infected with the virus by coculture also expressed altered HLA alloantigenic determinants. These alterations mirrored those seen with tumor cell lines established from patients. Thus, the appearance of neoantigens suggests an association of HTLV provirus replication and HLA alloantigenic expression. Cell surface markers were examined on cell lines established from patients and cell lines established by coculturing with the human T-cell lymphoma virus. These cells were predominantly OKT4+ (helper phenotype). These cells also expressed increased HLA-DR and an antigen detected by the Tac antibody which detects the receptor for T-cell growth factor. A cytotoxic T-cell line, established from a patient with the HTLV-associated disease, killed autologous cells and another cell line, but was not cytotoxic for other cell lines established from patients with HTLV. The cytotoxicity appeared to be genetically restricted by HLA determinants B8 and DR3.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Samuel Broder Associate Clinical Director DCT, NCI

Objectives:

To study the cell surface phenotypes, both HLA and lymphocytes markers, in cell lines established from patients with the human T-cell lymphoma virus. This study is designed to determine whether a certain subpopulation of lymphocytes is infected with the virus and to examine the effect of viral infection on the expression of cell surface antigens. Studies are performed to develop a cytotoxic T-cell line directed specifically at HTLV-infected cells and to examine the possibility that there is an HLA genetic restriction to the development of cytotoxic T cells.

Methods Employed:

HLA typing was carried out using alloantisera detecting 16 allelic antigens of the HLA-A locus, 26 alloantigens of the HLA-B locus, and 6 alloantigens of the HLA-C locus. HLA-DR antigens were detected by alloantisera detecting 10 determinants in this series as well as 6 alloantigens controlled by another locus within the HLA-DR region. Other cell surface markers were examined using the fluorescence-activated cell sorter and monoclonal antibodies directed against cell surface antigens that define subtypes of lymphocytes and/or specific functional subsets of T cells. Indirect immunofluorescence was employed in these studies. Cytotoxic T cells were generated by culturing peripheral blood lymphocytes from a patient with the human HTLV-associated malignancy with a cell line established from the patient's cells. These cytotoxic cells were cloned by limiting dilution and restimulated with the irradiated tumor cell line. Cytotoxicity studies were carried out using a 4-hour chromium-release assay. Cold target inhibition was used to examine the specificity of the reactions of these cytotoxic T cells.

Major Findings:

Alterations in the expression of the HLA alloantigens were found in cells expressing the proviral products of HTLV. Peripheral blood lymphocytes from patients with this disease did not demonstrate an altered HLA alloantigenic expression. In addition, there appears to be a restricted phenotype of HLA in individuals with this particular disease. Once cells are placed in culture the virus is transcribed and altered HLA alloantigens are expressed. The monoclonal antibody 4D12 developed by Dr. Haynes by immunization of mice with an HTLV-infected cell line, HUT-78, has been an important adjunct to our demonstration of altered HLA expression in HTLV-infected cells. This monoclonal antibody reacts with an epitope common to the HLA-AW19 cross-reactive group and the HLA-B5 cross-reactive group of antigens. Reaction of this monoclonal antibody was consistent with all HTLV-positive cell lines. All HTLV cell lines demonstrated a phenotype which suggests infection of a subset

of T cells (helper induced). In addition these cells express increased HLA-DR determinants as well as the Tac antigen, the presumptive receptor for T-cell growth factor. Cell lines established by coculturing and infecting newborn umbilical cord blood lymphocytes also showed altered HLA alloantigen expression, reactivity with the monoclonal antibody 4D12, and cell surface phenotypes characteristic of the tumor cell lines established from patients with this disease. Cytotoxic T cells generated from a patient with the HTLV-associated disease were restricted in their reactivity to the patient's autologous tumor cells as well as tumor cells from only one other patient. The cell lines established from 14 other individuals with HTLV-positive leukemias and lymphomas were not killed by the cytotoxic T-cell line. The two cell lines that could be killed by the cytotoxic T-cell line shared the HLA-A1, B8, DR3 antigenic determinants. This sharing of cytotoxicity suggests a genetic restriction to the cytotoxic reactions.

Significance to Biomedical Research and the Program of the Institute:

The isolation of a type C retrovirus and its demonstrated association with human T-cell malignancies by Dr. Gallo and his associates has been an important advance in our understanding of neoplastic disease processes. With the isolation and capability of transferring this virus to other human cells, it is now possible to study mechanisms of regulation of malignant transformation. One important feature of malignant transformation is the particular type of cell that the virus can infect as well as alterations which may occur in the expression of cell surface antigenic determinants with viral infection. The observation that altered HLA antigen expression occurs with viral infection raises the interesting possibility that control of infection and tumorigenesis may be related to HLA antigen expression. The coincident appearance of altered HLA expression with viral replication strongly suggests viral regulation of HLA gene expression or that the virus encodes for proteins which bear HLA alloantigenic determinants. It has been documented in other studies that the human T-cell lymphoma virus can infect individuals without producing any neoplastic disease process. The alteration in expression of HLA alloantigens may be a mechanism for control of tumorigenesis in that the viral replication induces the expression of an antigen which may be recognized as self or as foreign depending on the HLA type of the individual, and thus result in immunological control of replication and disease process. The demonstration that cytotoxic T cells can be generated from a patient with this malignancy and that there is a restriction in this cytotoxicity suggests a mechanism like this may be in some way controlling a human malignancy.

Proposed Course:

Studies examining the HLA phenotypes and other cell surface markers in HTLV-infected cell lines and in cell lines established by coculture will continue. Attempts will be made to isolate the envelope protein produced by the provirus. We will attempt to produce a monoclonal antibody against this protein specifically directed to detect a cross-reactive determinant on HLA antigens and this viral product. This monoclonal antibody, HLA alloantisera, and the 4D12 monoclonal antibody will be used to test populations where the HTLV virus is endemic. These studies will be designed to elucidate the possible differences in an HLA epitope as it relates to viral infection and tumorigenesis. Cloned

responsive T-cell lines will be developed from patients with the HTLV-associated malignancy or relatives of these patients which demonstrate HTLV infection but where the tumor is not present. These cell lines will also be used to test populations for the recognition of specific viral products. We intend to examine the nature of the immune response in vitro to these viral products. This population study will examine the possibility that there is an HLA restriction to viral infection and tumorigenesis.

Publications:

Gallo, R. C., Mann, D. L., Broder, S., Ruscetti, F. W., Maeda, M., Kalyanaraman, V. S., Robert-Guroff, M., and Reitz, M. S.: Human T cell leukemic-lymphoma virus (HTLV) in T--but not B--lymphocytes from a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA 79: 5680-5683, 1982.

Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Leibowitch, J., and Popovic, M.: Isolation of human T cell leukemia virus (HTLV) in acquired immune-deficiency syndrome (AIDS). Science 220: 865-868, 1983.

Mann, D. L., Popovic, M., Murray, C., Neuland, C., Strong, D. M., Sarin, P., Gallo, R. C., and Blattner, W. A.: Cell surface antigen expression in newborn cord blood lymphocytes infected with HTLV. J. Immunol. (In Press)

Mann, D. L., Popovic, M., Sarin, P., Murray, C., Reitz, M., Strong, D. M., Haynes, B. F., Gallo, R. C., and Blattner, W. A.: Cell lines producing T-cell lymphoma virus (HTLV) have altered HLA expression. Nature (In Press)

Mitsuga, A., Matis, L. A., Megson, M., Bunn, P. A., Murray, C., Mann, D. L., Gallo, R. C., and Broder, S.: Generation of an HLA restricted cytotoxic T-cell line reaction against cultured tumor cells from a patient infected with human T cell leukemia/lymphoma virus (HTLV). J. Exp. Med. (In Press)

Popovic, M., Lange-Wantzin, G., Sarin, P. S., Mann, D. L., and Gallo, R. C.: Transformation of umbilical cord blood T-cells by human T-cell leukemia/lymphoma virus (HTLV). Proc. Natl. Acad. Sci. USA. (In Press)

Popovic, M., Sarin, P. S., Robert-Guroff, M., Kalyanaraman, V. S., Mann, D. L., Minowada, J., and Gallo, R. C.: Isolation and transmission of human retrovirus (human T-cell leukemia virus). Science 219: 856-859, 1983.

responsive T-cell lines will be developed from patients with the HTLV-associated malignancy or relatives of these patients which demonstrate HTLV infection but where the tumor is not present. These cell lines will also be used to test populations for the recognition of specific viral products. We intend to examine the nature of the immune response in vitro to these viral products. This population study will examine the possibility that there is an HLA restriction to viral infection and tumorigenesis.

Publications:

Gallo, R. C., Mann, D. L., Broder, S., Ruscetti, F. W., Maeda, M., Kalyanaraman, V. S., Robert-Guroff, M. and Reitz, M. S.: Human T cell leukemic-lymphoma virus (HTLV) in T--but not B--lymphocytes from a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA 79: 5680-5683, 1982.

Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Leibowitch, J. and Popovic, M.: Isolation of human T cell leukemia virus (HTLV) in acquired immune-deficiency syndrome (AIDS). Science 220: 865-868, 1983.

Mann, D. L., Popovic, M., Murray, C., Neuland, C., Strong, D. M., Sarin, P., Gallo, R. C. and Blattner, W. A.: Cell surface antigen expression in newborn cord blood lymphocytes infected with HTLV. J. Immunol. (In Press)

Mann, D. L., Popovic, M., Sarin, P., Murray, C., Reitz, M., Strong, D. M., Haynes, B. F., Gallo, R. C. and Blattner, W. A.: Cell lines producing T-cell lymphoma virus (HTLV) have altered HLA expression. Nature (In Press)

Mitsuga, A., Matis, L. A., Megson, M., Bunn, P. A., Murray, C., Mann, D. L., Gallo, R. C. and Broder, S.: Generation of an HLA restricted cytotoxic T-cell line reaction against cultured tumor cells from a patient infected with human T cell leukemia/lymphoma virus (HTLV). J. Exp. Med. (In Press)

Popovic, M., Lange-Wantzin, G., Sarin, P. S., Mann, D. L. and Gallo, R. C.: Transformation of umbilical cord blood T-cells by human T-cell leukemia/lymphoma virus (HTLV). Proc. Natl. Acad. Sci. USA. (In Press)

Popovic, M., Sarin, P. S., Robert-Guroff, M., Kalyanaraman, V. S., Mann, D. L., Minowada, J. and Gallo, R. C.: Isolation and transmission of human retrovirus (human T-cell leukemia virus). Science 219: 856-859, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05340-01 LHC

PERIOD COVERED

January 9, 1983 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transformation of Human Bronchial Epithelial Cells by RNA Tumor Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Edward W. Gabrielson, Medical Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Tumor Virus Genetis, NCI, Bethesda, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

There has been much interest concerning the role of genes that transform rodent cells in human neoplasia. In our laboratory, human bronchial epithelial cells have been infected by Kirsten sarcoma virus with an amphotropic murine leukemia virus helper. This viral infection results in epithelial cell colonies which do not differentiate in response to serum as do normal epithelial cells. These transformed cells are currently being further characterized. Temperature-sensitive mutants of Kirsten sarcoma virus, and other RNA viruses with transforming genes, are being studied in transformation assays.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

John Lechner	Senior Staff Fellow	LHC, NCI
Curtis C. Harris	Chief	LHC, NCI

Objectives:

The role of Kirsten sarcoma virus ras gene and other transforming genes carried by retroviruses in in vitro transformation of human bronchial epithelial cells will be studied.

Methods Employed:

The methods and media for culturing normal human bronchial epithelial cells have been previously developed in this laboratory. Dr. Robert Bassin is providing RNA tumor viruses with amphotropic murine leukemia virus helper, demonstrated to be infectious for our human bronchial epithelial cells. A standard virus infection assay with variable multiplicities of infection is done in the presence of polybrene. Cells are subcultured after infection and observed under various media conditions. Colonies with transformed characteristics are selected and subcultured for further characterization.

Integration of the ras gene into the host genome will be assayed by Southern blot/DNA hybridization techniques, and the Kirsten sarcoma virus ras gene product will be identified by immunoprecipitation and electrophoresis, and by immunofluorescence with monoclonal antibody to the gene product provided by Dr. Mark Furth.

Major Findings:

Kirsten sarcoma virus with amphotropic murine leukemia virus helper infection of human bronchial epithelial cells results in epithelial cell colonies which grow in the presence of 5% fetal bovine serum (serum contains a factor which causes differentiation of normal bronchial epithelial cells). These transformed cells also have extended growth, but no cell lines have yet been isolated which grow indefinitely. The presently achieved efficiency of transformation in this system is about 0.2% that of transformation of mouse NIH 3T3 cells by the same virus.

Significance to Biomedical Research and the Program of the Institute:

The role of viral transforming genes in human epithelial cell transformation will be studied.

Proposed Course:

Transformed cell lines will be cloned and characterized for their in vitro growth properties and tumorigenic potential. Chemical carcinogens will also

be examined in this system for potential co-carcinogenic effects. Others, e.g., viral strains with different transforming genes, will be studied for their transforming effect on bronchial epithelial cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05341-01 LHC

PERIOD COVERED

October 1, 1982, to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Model Systems for the Study of Physical Carcinogens at the Cellular Level

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

John F. Lechner, Ph.D., Senior Staff Fellow, LHC, NCI

COOPERATING UNITS *(if any)*

Georgetown University School of Medicine, Washington, DC; Veterans Administration Hospital, Washington, DC; University of Maryland School of Medicine, Baltimore, MD; Litton Bionetics, Kensington, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Amosite asbestos was found to be 100 times more cytotoxic for human mesothelial cells and 10 times more cytotoxic for normal human bronchial epithelial cells than for normal human bronchial fibroblasts. Chrysotile was the most cytotoxic fiber tested; glass fibers were also very cytotoxic for mesothelial cells. Focal hyperplasia and epidermoid metaplasia were observed in explants of human bronchial tissue two weeks after a single exposure to amosite asbestos; both intracytoplasmic and intranuclear asbestos fibers were seen by X-ray micro-analysis in these lesions. Two subculturings after amosite asbestos exposure of phenotypically altered mesothelial cells arose. The control cultures ceased growth during the fourth subculture, and the amosite asbestos-exposed cultures continued to multiply. The exposed cells had a near-normal model number of chromosomes through the sixth subculturing. At the ninth subculture, 80% of the metaphases had dicentric chromosomes and the model number had increased to 77.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this project:

Susan P. Banks-Schlegel	Expert	LHC	NCI
Edward Gabrielson	Medical Staff Fellow	LHC	NCI
Takayoshi Tokiwa	Visiting Scientist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To study the carcinogenicity of asbestos fibers in human mesothelial and bronchial epithelial in vitro systems. These studies include the following: (1) develop defined media for replicative mesothelial cell cultures; (2) evaluate cytotoxicity of asbestos fibers in mesothelial and bronchial epithelial cells; (3) evaluate the effects of asbestos fibers on progression of chromosome rearrangements in mesothelial cells; and (4) evaluate asbestos as a cocarcinogen for cultured bronchial epithelial cells.

Methods Employed:

Human bronchial tissues are obtained from medical examiner and "immediate" autopsy donors. Replicative cultures of normal bronchial epithelial cells are developed from explant culture outgrowths. Upon transfer of the explants to new dishes, the outgrowth cultures are incubated in defined, serum-free medium to expand the population, then subcultured. Mesothelial cells are obtained from pleural effusions obtained from donors without cancer. The fluid is centrifuged, and the pelleted cells are resuspended and inoculated into 100-mm culture dishes at a ratio of one dish per 50 ml of pleural fluid. The cells are dissociated using trypsin when the cultures attain subconfluency. The cultures are further expanded and either cryopreserved or used according to experimental protocols.

Several criteria are used to establish the identity of the cells grown in culture. Markers for bronchial epithelial cells include karyology; polygonal morphology; ultrastructural identification of tight junctions, desmosomes, and tonofilaments; production of acidic and neutral mucopolysaccharides; immunostaining of keratin; blood group antigens and type IV collagen; population doubling potential; clonal growth rate; and mitogenic responsiveness to peptide growth factors and hormones. Mesothelial cells are identified as mesothelial cells by several criteria including immunofluorescent staining with anti-keratin antibodies, a variable cell morphology depending on the presence (fusiform) or absence (cobblestone) of EGF and hydrocortisone in the growth medium, histochemical staining for hyaluronic acid-mucin, the presence of long, branched microvilli, and the normal human karyotype of giemsa-banded metaphases.

Fiber cytotoxicity is assessed using clonal growth dose-response assays. Sixty-mm dishes are inoculated at clonal density. Twenty-four hours later, the medium is replaced with medium containing increasing concentrations of

fibers. After three days of exposure, the fiber-treated and control cultures were rinsed twice with medium, then reincubated in fiber-free medium. Ten days postinoculation, the colonies are fixed in 10% formalin and stained with 0.25% crystal violet.

Bronchial tissue is exposed to amosite asbestos by pipetting fiber suspensions onto the epithelial surface. The explants are then submerged in medium in a stationary position for 2 hours before culturing in a rocked, controlled atmosphere chamber. The culture medium is replaced with fresh medium without fibers the next day and then at 2-day intervals. The tissues are periodically examined by light and electron microscopy.

Replicative cultures of mesothelial cells are exposed to amosite asbestos by including the fibers (2 µg/ml) into the growth medium. After 4 days of incubation, the medium is replaced with medium without fibers and at 4-day intervals thereafter. Two weeks later, the cells are trypsin dissociated and subcultured. The following day, the cultures are reexposed to amosite asbestos. Control cultures are carried in parallel and are either unexposed or exposed to glass fibers. Giemsa banding of mesothelial metaphases is conducted to monitor chromosomal rearrangements post-asbestos exposure.

Major Findings:

Investigations into the mechanism of asbestos carcinogenesis have shown the following: (1) Amosite asbestos (100 to 1000 µg/ml) caused focal epithelial hyperplasia and atypical squamous metaplasia in human tracheobronchial explants. (2) Amosite fibers were shown by both scanning and high-voltage transmission electron microscopy to penetrate cultured epithelial cells. Short fibers (< 12 µ) were found in the cytoplasm of the cells within 6 hours, whereas longer fibers incompletely entered the cells. The epithelial cells did not show marked cell surface activity, and only small membrane sleeves around noncoated fibers were observed at the points of asbestos penetration. (3) To measure toxicity, asbestos (UICC samples, 0.1 to 100 µg/ml) were added to human bronchial epithelial cells that had been subcultured 24 hours previously at clonal density. When compared to glass fibers, asbestos caused a statistically significant ($p < 0.05$) decrease in cell population doubling rate. Chrysotile was approximately 10-fold more cytotoxic than either amosite or crocidolite. A similar order of toxicity was observed when human bronchial fibroblastic cells were used; however, these cells tolerated approximately 100-fold more fibers for the equal level of cytotoxicity. Both intracytoplasmic and intranuclear asbestos fibers were seen by X-ray microanalysis in the hyperplastic lesions. (4) Conditions for replicative cultures of mesothelial cells were developed, including serum-free media. The cells contain keratin and hyaluronic acid-mucin. Early passage cells multiply with a clonal growth rate of 0.85 population doublings per day and exhibit a colony-forming efficiency greater than 35% when inoculated into fibronectin-, collagen (Vitrogen®)-, BSA-coated dishes. As assessed by Giemsa banding, the cells remain chromosomally normal until senescence (35 population doublings in culture). 5) Asbestos fibers are 10-fold more cytotoxic for mesothelial cells than for bronchial epithelial cells. (6) Phagocytosis of asbestos fibers by human mesothelial cells proved to be rapid; fibers were observed penetrating the cells within 2 hours after exposure. The fibers were engulfed

end-first, and a sleeve of membrane surrounded the stalk of longer fibers (> 20 μ) and then migrated up the fibers until they were engulfed. (7) Asbestos-exposed mesothelial cells had a near-normal model number of chromosomes through 6 successive subcultures; however, chromosome rearrangements were noted. Dicentrics were found in 10% of the fourth passage metaphases; at the sixth passage, more than half of the metaphases contained dicentrics. At the ninth subculture, 80% of the metaphases had dicentric chromosomes, and the model number had increased to 77.

Significance to Biomedical Research and the Program of the Institute:

Although asbestos fibers have been epidemiologically associated as a cocarcinogen for human malignancies other than mesothelioma, these fibers are considered to be complete carcinogens for mesothelial cells. In fact, no other consequential etiologic agent other than fibrous structures, i.e., zeolites, ceramics, and occasionally, glass, has been identified as a causative agent for pleural and peritoneal mesothelioma. Mesothelioma is a rarely encountered malignancy. However, the latency period for this disease averages 40 years, and with the marked increase in the use of asbestos during and since World War II, an epidemic of mesothelioma has been predicted for the latter part of this century. Carcinogenesis studies with animals have shown that mesothelioma can be caused by intrapleural or intraperitoneal injections of asbestos. The extrapolation of experimental animal data to man is a major problem; one approach is to develop model systems in cultured human tissues for carcinogenesis investigations.

Proposed Course:

Growth conditions for human mesothelial cells will be continually improved. Experiments are in progress to determine whether asbestos-treated bronchial epithelium loses properties commonly associated with normal cells and demonstrates the growth characteristics ascribed to transformed cells. Changes in these properties may be indicative of premalignant transformation. Investigations into the effects of single and multiple exposures as well as cocarcinogenesis with other physical and viral agents on the development of neoplastic lesions in bronchial explant and mesothelial cell cultures will be continued and expanded. Ultimately, the mechansims through which asbestos transforms cells will be investigated.

Publications:

Haugen, A. and Harris, C. C.: Asbestos carcinogenesis: Asbestos interactions and epithelial lesions in cultured human tracheobronchial tissues and cells. Recent Results Cancer Res. 82: 32-42, 1982.

Haugen, A., Schaefer, P., Lechner, J. F., Stoner, G. D., McDowell, E. M., McClendon, I. A., Trump, B. F. and Harris, C. C.: Cellular ingestion, toxic effects, and lesions observed in human bronchial epithelial tissue and cells cultured with asbestos and glass fibers. Int. J. Cancer 30: 265-272, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05348-01 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemical Carcinogenesis in Respiratory Epithelium

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Andrew J. Saladino, M.D., Senior Scientist, LHC, NCI

COOPERATING UNITS *(if any)*

Department of Pathology, University of Maryland, Baltimore, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human respiratory epithelial cell cultures have been utilized as models for studying effects of putative cocarcinogens and tumor promotors in vitro. This study focused on effects of specific aldehydes and peroxides including acetaldehyde, formaldehyde, benzoyl peroxide, and hydrogen peroxide. Administration of benzoyl peroxide resulted in small cells with increased cytoplasmic density. Benzoyl peroxide and formaldehyde inhibited cell proliferation at low concentrations (56 micromolar and 210 micromolar, respectively) and enhanced ornithine decarboxylase activity. Acetaldehyde had surprisingly little effect on growth rates up to 30 mM. At this concentration it resulted in squamous-appearing cells with depressed DNA/RNA synthesis, depressed aryl hydrocarbon hydroxylase activity, and depressed ornithine decarboxylase activity. Hydrogen peroxide and sodium formate, a by-product of formaldehyde metabolism, caused enhanced aryl hydrocarbon hydroxylase activity. These observations provide useful information in the assessment of the acute effects of these test compounds in human tissues and in the comparison with expected effects observed in animal models.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Curtis C. Harris	Chief	LHC, NCI
John Lechner	Senior Staff Fellow	LHC NCI
James Willey	Medical Staff Fellow	LHC, NCI

Objectives:

We wish to characterize and quantify the acute metabolic and morphologic responses of human respiratory epithelium, maintained in vitro, to certain specific aldehyes and peroxides. These compounds have been selected because of a possible role in the causation of cancer in animal models. No such information concerning human tissues is presently available. It is the intent of these studies to give insight into the necessary events that may be associated with human respiratory cocarcinogenesis and tumor promotion. Cell changes are viewed in the context of altered cell growth, turnover, and differentiation.

Methods Employed:

Methods developed in the Laboratory of Human Carcinogenesis for long-term maintenance and study of human respiratory epithelium in chemically defined media have been utilized. Standard procedures were used for subculturing and metabolic assays. Cell outgrowth from tracheobronchial explants were enzymatically dissociated and replated onto 60 mm dishes for clonal growth assays, into 35 mm 6-well plates for study of DNA/RNA synthesis, and into 16 mm 24-well plates for other biochemical-metabolic studies. For clonal growth assays, low density (5000 cells per dish) dispersed and anchored single epithelial cells were exposed to test agents for 6 hours, allowed to form colonies over the next 7 to 10 days, fixed, stained, and counted to obtain a characteristic population doubling time. Cell areas and cytoplasmic densities were also assessed with the aid of a computerized image analyzer. For DNA/RNA studies, intermediate density (20,000 per well) anchored cells were exposed for 6 and 24 hours to test agents, then measured for tritiated thymidine and orotic acid incorporation. For biochemical-metabolic studies, high density (100,000 per well) anchored cells were exposed for 6 hours to test agents and then assayed in one of the following ways: (1) aryl hydrocarbon hydroxylase (AHH) activity: after 6-hour exposure to test agent, cells were exposed to the substrate tritiated benzo(a)pyrene, and the amount of the metabolic product (tritiated water) per milligram cell protein was measured; (2) ornithine decarboxylase (ODC) activity: after 6-hour exposure cells were exposed to ornithine-^{14}C and the amount of $^{14}CO_2$ product measured per milligram of cell protein; (3) plasminogen activator (PLG) activity: after 6-hour exposure, conversion of plasminogen to plasmin was measured by hydrolysis of a synthetic ^{14}C analide. Compounds studied include acetaldehyde, benzoyl peroxide, formate, formaldehyde, and hydrogen peroxide.

Major Findings:

Specific levels of acute toxicity have been identified. Clonal growth inhibition proceeds in a dose-dependent fashion. Human bronchial epithelium (HBE) was highly sensitive to benzoyl peroxide, moderately sensitive to formaldehyde and hydrogen peroxide, and very insensitive to acetaldehyde and formate. No compound studied was mitogenic. Morphologic and metabolic changes have been observed following exposure to these compounds. Morphologic changes include the production of small, dense cells by benzoyl peroxide and the production of large squamous-appearing cells by acetaldehyde. Cells also tended to be smaller than control cells after exposure to formaldehyde and hydrogen peroxide. Metabolic changes included enhancement of AHH by hydrogen peroxide and formate and enhancement of ODC activity by formaldehyde and benzoyl peroxide. Acetaldehyde in high concentrations (30 mM) consistently reduced both AHH and ODC activity. Preliminary data suggest suppression of DNA and RNA synthesis by benzoyl peroxide and acetaldehyde.

Significance to Biomedical Research and the Program of the Institute:

The majority of human cancers are thought to be associated with exposure to toxic substances in our environment, and epidemiologic evidence supports a multistage progression of human lung cancer. Although much information has been derived from animal in vivo and in vitro models, the responses of human tissues and the relevance of animal model findings to human carcinogenesis are largely unknown. The HBE model system under study is now beginning to provide useful information with regard to cellular handling of toxic substances and permanency of aberrations of cell proliferation and differentiation, especially with regard to aldehydes and peroxides commonly encountered in our environment or as metabolic intermediates. Such patterns of change are of great interest in the synthesis and testing of various hypotheses relating to human respiratory cancer causation.

Proposed Course:

Light and electron microscopic assessment of subcellular changes are now underway. Minor changes in our protease assay procedure should allow assessment of changes induced in this area. Additional parameters such as membrane damage (as judged by liberation of arachidonic acid) and the formation of epithelial envelopes are next to be addressed. Reactive aldehydes, which are also the natural products of membrane lipid peroxidation such as malonaldehyde, hydroxynonaldehyde, and hexonal as well as shorter chain aldehydes such as glutaraldehyde, acreolein, and methyl glyoxal, will be studied in an attempt to define predictable patterns of growth and metabolic responses.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05349-01 LHC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Enzyme Immunoassays for Ectopic Hormones

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Glennwood E. Trivers, Ph.D., Research Scientist, LHC, NCI

COOPERATING UNITS *(if any)*

INSERN, l'Unite' de Radioimunologic Analytique, l'Institut Pasteur, Paris, France

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ectopic hormone activity of primary and metastatic neoplastic lesions occasionally characterizes tumors of all types and is frequently detected in patients with lung carcinomas. Human chorionic gonadotropin (HCG) and adrenocorticotropic hormone (ACTH) are detected, respectively, in approximately 80% and 40% of all lung tumors examined, and HCG is found in nearly 100% of small cell lung carcinomas. Clinical analysis of ectopic hormones suggests their progressive appearance may signal cellular and behavioral changes in response to host or therapeutic influences on the course of paraneoplastic diseases. Ectopic hormones have been studied by histochemistry, immunocytochemistry, radioimmunoassay (RIA), and enzyme immunoassay (EIA). In this effort we are developing ultrasensitive enzyme radioimmunoassays (USERIA) for the more important ectopic hormones. We are currently studying ACTH, HCGB, prostaglandin G, and the unestablished calf thymus extract, thymosin alpha-1, recently thought to be associated with acquired immune deficiency syndrome (AIDS) and perhaps Kaposi's sarcoma.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Curtis C. Harris Chief LHC, NCI

Objectives:

To expand the capabilities of USERIA for ectopic hormones (and related substances) to allow measurement of the limited quantities of hormones that are frequently available in frequent tissue, serum, and plasma samples and that may exist in cell culture medium; ultimately, to study these substances in clinical and experimental conditions that involve exposure to known and suspected carcinogens.

Methods Employed:

Initially, the conditions are determined for binding purified hormones to wells of microtiter plates. Bound hormone molecules are measured in non-competitive EIA using antisera or hybridoma supernatants from commercial or experimental sources. EIA conditions are optimized for conversion to the radiolabeled substrates in the USERIA. The standard curves performed with RIA, EIA, and USERIA are compared, and the differences are established for the amounts of the hormone measured by each assay in biological samples.

Major Findings:

The hormones employed bind poorly, if at all, to polystyrene, but they bind very efficiently to polyvinyl chloride; the larger molecules bind most efficiently, the smallest (prostaglandin) bind too poorly to be useful. With a 50% inhibition of 5.6 pg and a detection limit of 1 pg, the competitive USERIA for ACTH exhibited approximately 6- and 15-fold greater sensitivity than EIA and RIA, respectively, in standard curves, while using 4 and 3 times less sample. In assays of ACTH in human and rat biological samples, USERIA was 4 and 12 times more sensitive than EIA and RIA, respectively. ACTH in unpurified biological samples could not be measured with USERIA, which demonstrated sensitivity superior to EIA and RIA after sample purification (chromatography), even though the amount they measured was increased by purification. The latter finding renders the constraint of purification an essential one for the removal of hormone-like materials and molecules of the albumin type that will bind hormones.

The USERIA for HCG_B and thymosin α-1 are nearing completion of the first stage (establishment of standard curves) and both appear to have the potential for greater differential sensitivity than currently achieved for ACTH.

Significance to Biomedical Research and the Program of the Institute:

A more sensitive assay for the chosen hormones would make possible earlier detection of the ectopic events and could thereby provide a new dimension to

our ability to determine the relevance of the hormones to tumor initiation and progression. Of equal importance is the potential for studying ectopic responses to chemical carcinogens in vivo and in vitro, in tumors with the phenotype, and in cell cultures sensitive to tumorigenic agents.

The currently successful chemotherapy with nitrosoureas and other carcinogenic anticancer compounds are also relevant to this project: (1) an increasing number of humans continue to be necessarily subjected to substances with long-term side effects, including production of secondary tumors; (2) a percentage of the long-term survivors, particularly young adults and children, will quite likely suffer the therapy-induced tumors. This project has the potential to take some advantage of these otherwise undesirable circumstances for the study of hormones and their contribution to the success of the human cancer in the initiation phase. Hormone levels might also relate to the accumulating toxic and carcinogenic levels of these compounds during long-term treatment.

Proposed Course:

Assay of ACTH will involve biological samples from (1) a cross section of normal human subjects including smokers and nonsmokers and (2) a variety of patients with varying illnesses, including cancer of several types. We will study the possibilities of increasing the sensitivity of the USERIA for ACTH. The first phase of the development of USERIA for HCG_B and thymosin alpha-1 will be completed.

Publications:

Rougeot, C., Trivers, G. E., Harris, C. C. and Dray, F.: ACTH immunoassays: development of ultrasensitive and specific procedures for the measurement of biological materials. In Jeelan, J. (Ed.): Enzyme Immunoassay. Proceedings of the 22nd International Symposium on Immunoenzymatic Technique. Amsterdam, Elsevier/North-Holland. (In Press)

Trivers, G. E., Harris, C. C., Rougeot, C. and Dray, F.: Development and use of ultrasensitive enzyme immunoassays. In Conn, P. M. (Ed.): Methods in Enzymology. San Diego, Academic Press, Vol. 103. (In Press).

ANNUAL REPORT OF

THE LABORATORY OF MOLECULAR CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

The Laboratory of Molecular Carcinogenesis (LMC) plans, develops, and conducts a research program designed to (1) clarify the molecular biology of carcinogenesis; (2) elucidate the fundamental nature of the interaction of carcinogenic agents, especially chemical, with biological systems in the induction of cancer; (3) identify those environmental and endogenous factors which relate to and modify the carcinogenic process; and (4) clarify the metabolic regulatory processes which are related to carcinogenesis.

The goal of the Laboratory of Molecular Carcinogenesis is to understand the molecular basis of carcinogenesis with the view toward identifying susceptible populations and preventing human cancer. The research program is designed to understand the molecular basis by which carcinogenic agents cause malignant transformation, and to identify and characterize those exogenous and endogenous factors involved in carcinogenesis. The Laboratory seeks to clarify the metabolic interaction of exogenous and endogenous agents in the living organism at the molecular, cellular, and organism levels and seeks to understand the consequences of these interactions in terms of cell regulation and carcinogenesis. The processes are studied in biological preparations and cells from experimental animals and humans.

In the last several years, the course of the Laboratory research program has been markedly affected by the powerful new techniques of molecular biology and immunology. We have added younger staff highly experienced in DNA recombinant and related techniques, protein chemistry, and hybridoma technology. The power and precision of these techniques have had a highly positive influence on the progress of many of the projects of the Laboratory.

Cell Genetics Section - Studies (1) the isolation and characterization of cellular genes (oncogenes) and their products responsible for the expression of the transformed state, (2) the effects of DNA methylation and Z-DNA conformation on gene expression, rearrangement and mutation, (3) the cancer-prone genetic diseases, and (4) the cellular and molecular mechanism of initiation and promotion of cell transformation by chemicals and irradiations.

Research in the past decade has provided much fundamental information on the very early steps of carcinogenesis, such as the metabolism of carcinogens and the binding of carcinogens to DNA. However, the crucial processes of cell transformation, which take place subsequently, are still unclear. This Section attempts to identify the genes and their products responsible for the initiation and maintenance of cell transformation by chemical carcinogens and irradiation.

The nature of the genes and proteins responsible for the expression of the neoplastic phenotype of chemically transformed human cells has been investigated by using molecular cloning and other new techniques in molecular biology. A new polypeptide recognized in a chemically transformed human cell line has been identified as a mutant of β-actin. The presence of the mutated β-actin was

correlated with the expression of the transformed phenotype in the variants of the transformed line and its hybrids with normal human fibroblasts. A mutation (GC to AT transition point mutation) in the β-actin molecule resulted in several defects in the function of β-actin, such as increased instability, reduced incorporation into cytoskeletal elements, and decreased ability to polymerize in vitro. These defects in the β-actin molecule were associated with the disruption and loss of the structural organization of the cytoskeleton, such as the actin cable network in the transformed cells. The cytoskeletal structure is responsible for cell shape and the motility and fluidity of the cytoplasmic membrane. Alteration of this structure is one of the most striking and universal characteristics of neoplastic cells. The results suggest that a mutation in β-actin leads the cells to express transformation by disrupting the cytoskeletal structure and its function.

Genomic rearrangement can be directly responsible for oncogenic transformation; the structural requirements for this rearrangement need to be investigated. A specific site of genomic rearrangement has now been correlated with undermethylation in the chicken genome. This has been done by comparing members of a family of repeated DNA sequences in which an undermethylated region occurs. Recombination was associated with a short tandemly repeated region contained within a longer repeated sequence element. Recombination generates a specific deletion encompassing the tandem repeats and a much larger segment of 3' flanking DNA. DNA sequencing provided a complete description of this recombination site and of the undermethylated segment. This DNA segment will be used in gene transfer experiments to test further the influence of cytosine methylation on rearrangement. On the other hand, the ability of eucaryotic cells to establish a methylation pattern within newly introduced DNA segments may be important to the repression of oncogenes carried into cells by transforming viruses. Little is known about establishment of de novo methylation or its sequence requirements. A "natural" gene transfer system is being used to investigate this. Mitochondrial DNA lacks the cytosine methylation typical of nuclear DNA. Occasionally, fragments of mitochondrial DNA enter the nucleus by an unknown mechanism and become integrated into the nuclear genome. This provides a unique opportunity to study de novo methylation. A segment of chicken mitochondrial DNA that also occurs in nuclear DNA has been cloned. Its methylation pattern in mitochondrial and nuclear genomes is being compared and the DNA sequence of any newly methylated sites is to be determined.

It is likely that the conformational state of DNA is involved in the regulation of expression of the eukaryote genome. We were the first to find, in a variety of eukaryotic genomes, a large copy number of a dT-dG alternating sequence, a sequence with a potential to adopt Z-DNA conformation. Although most studies on Z-DNA in the past have focused on dG-dC alternating sequences, we emphasized the importance of the dT-dG alternating sequence based on its ubiquitousness in the genomes of various species. Recently, the conversion of dT-dG alternating sequences from B-form to Z-form was found to be dependent on the degree of negative supercoiling of the flanking DNA. Because isomerization between the topological forms of DNA has been implicated in DNA replication, transcription, repair, and recombination, dT-dG alternating sequences may play an important role in these fundamental molecular processes. Experiments are now underway to develop an assay system to detect Z-DNA in biological systems, and to test the hypothesis that such a Z-DNA sequence may be involved in the regulation of gene expression, mutation, and in carcinogenesis.

As one of the gene sources for studying the regulation of gene expression, families of actin genes were isolated from human fibroblasts by using molecular cloning techniques. Actin families are well-conserved through evolution. Their expression is very specific to the type of tissues in spite of the high similarity of the encoded amino acid sequences. One cardiac muscle actin gene and two smooth muscle actin genes, aorta type and stomach type, have been isolated and characterized. They contain DNA sequences which completely match the amino acid sequences of the corresponding actin proteins. They also have the necessary consensus sequences at the boundary between exon and intron sequences, as well as other sequences known to be required for transcription and translation. A cysteine codon was found in all of the muscle actin genes at the position of the first amino acid following the methionine codon; this codon was not found in nonmuscle actin genes. Since no known actin protein starts with a cysteine, it is likely that post-translational removal of both cysteine and methionine accompanies muscle actin synthesis. This observation has interesting implications for actin gene function, regulation, and evolution. A new intron site was found at the position of amino acid 84 in both types of smooth muscle actin genes. This intron location has never been found in any actin gene family of any species. Comparison of the intron location in all actin genes in all species examined indicates that both deletion and insertion of the intron have been involved in the evolution of the actin gene family. To study the regulation of expression of actin genes, a shuttle vector consisting of Bovine Papiloma virus DNA, pBR322 DNA, and an insert of actin genes with the entire coding sequences and 2-3 kb of additional sequences on the 3' and 5' end has been constructed. Actin gene expression is currently being studied in cells transfected with the constructed plasmid DNA.

Human cancer-prone genetic diseases are being studied in order to identify groups of people with an increased susceptibility to environmental carcinogenesis, and to discover the clinical consequences as well as the molecular basis of their cellular hypersensitivity. Patients with xeroderma pigmentosum (XP) and ataxia telangiectasia (AT), diseases with ultraviolet (UV) and X-ray hypersensitivity, respectively, and with the dysplastic nevus syndrome of hereditary cutaneous melanoma (DNS) are being studied. Detailed examinations of the clinical features of affected individuals are being made. A large retrospective XP literature case report study and a prospective registry of XP patients is underway. A possible new form of XP with defective DNA repair without neoplasia was found. Clinical diagnostic features of DNS are being refined. Testicular abnormalities in AT are under study. Cultures of skin and blood from XP and AT family members were established. Effects on cell survival, mutagenesis, DNA synthesis and repair, histone synthesis and chromosome integrity after treatment with DNA damaging agents are being studied. DNS lymphoblastoid cell lines were found to be UV-hypermutable thus implicating DNS as a second hypermutable human disease (XP was the first recognized). We are using DNA mediated gene transfer to attempt to clone the genes responsible for UV hypersensitivity in XP cells. We have identified an XP cell line that is a highly proficient recipient of cloned genes.

Psoralen plus long wavelength ultraviolet radiation (UV-A) is being investigated as a model system for clinically relevant photochemical carcinogenesis. Used experimentally for treatment of psoriasis and mycosis fungoides, psoralen plus UV-A has been found to be mutagenic and carcinogenic. We have developed an in vitro assay to measure the effects of UV-A mediated psoralen-DNA binding on human lymphoid cells. The studies indicate that the low doses of psoralen plus UV-A received by patients' leukocytes during therapy may result directly in decreased DNA synthesis in their circulating lymphoid cells. This system may

be exploited to destroy circulating neoplastic cells by irradiation of extra corporeal blood cells during leukophoresis. These studies are aimed at understanding the mechanism of cell damage induced by psoralen plus UV-A so as to control the toxicity to human cells during therapy and to identify individuals with abnormal sensitivity.

The possible involvement of mutation in the transformation process is being examined by two approaches. First, the pattern of stable alteration of the structure of cellular genes induced by treatment with chemical carcinogens was investigated by comparing the DNA sequences of the actin genes isolated from a human fibroblast line transformed by a single treatment with 4-nitroquinoline-1-oxide (4NQO) with that of normal genes. One point mutation was found in the aorta type smooth muscle actin gene which is dormant in the cells used and therefore has not been selected for its survival. No mutation was detected in the coding sequence of another dormant gene, stomach type of smooth muscle actin gene. In the pseudo -actin gene, which has been dormant for many generations, there were many mutations, predominantly with base substitution point mutation. One-base insertion and one-base deletion mutations were also frequently found. Large insertions or deletions were not found. At the level of amino acid sequences, one mutation corresponding to a one-base substitution point mutation was found in -actin, expression of which is essential for survival of cells. Gene rearrangement or polymorphism of these actin genes was not detected. These results suggest that the most frequent type of alteration of DNA sequence induced by 4NQO-treatment (or spontaneously) is base substitution point mutation and, less frequently, one-base insertion or deletion. Secondly, the possibility that chemically-induced cell transformation results in genetic instability which increases the probability of neoplastic progression, was tested by comparing the mutation rates of normal diploid human fibroblasts and a chemically transformed human fibroblast line. When the two genetic loci, i.e., the hypoxanthine guanine phosphoribosyl transferase locus, a X-linked recessive locus, and the Na^+-K^+ ATPase locus, an autosomal codominant locus, were used, the mutation rates of the normal and transformed cells did not differ significantly. Thus, it was concluded that increased mutation-rates do not necessarily occur following chemical transformation. The structure activity relationship of tumor promoting agents is being examined by using new and unusual derivatives of promoters. Enhancement of transformation of Balb/3T3-A31-1-1 cells is an indicator of promotor potential. Aplysiatoxin (AP) strongly enhanced carcinogen-initiated cell transformation, whereas debromoaplysiatoxin (DBAP) did not. DBAP differs from AP only by a lack of a bromine residue in its hydrophilic region. The hydrophilic region has been considered to be less important than the hydrophobic region for tumor promoting activity. However, AP and DBAP had an equal potency to inhibit the binding of phorbol-12, 13-dibutyrate (PDBu) and epidermal growth factor (EGF) to the cells. The addition of serum to the assay buffer abolished the inhibitory effects of DBAP on both PDBu- and EGF-bindings but did not affect AP or TPA activity. The factor in serum which abolish DBAP effects was purified and identified as a protein of approximately 70,000 molecular weight. The promoting activity of DBAP in carcinogen-initiated mouse skin was shown to be weak compared to that of AP. Thus, the weak promoting activity of DBAP in vivo was predicted from the in vitro transformation assay system. Another new type of promoter, teleocidin B, showed 100-fold greater activity in enhancing cell transformation than TPA, one of the strongest promoters

previously known. The extreme activity of teleocidin B was ascribed to its remarkable resistance to metabolic inactivation within the cells. These results indicate that both a new type of interaction of promoting agents at hydrophilic regions with proteins in serum and the metabolism of promoter within cells exert an influence upon the promoting activity of some promoters and the structure-activity relationship of promoters. When catechol, a mouse skin cocarcinogen, was given to the cells, transformation was induced at high frequencies whereas catechol alone, carcinogen alone or postcarcinogen treatment with catechol did not induce transformation. Thus, cocarcinogenesis was observed in vitro similar to that observed in vivo in earlier studies.

Metabolic Control Section - Studies (1) the metabolic activation and detoxification of the polycyclic hydrocarbons (PCH) and other carcinogens and drugs and the relationship of this metabolism to individual sensitivity and susceptibility to carcinogenesis; and (2) regulation and structure of the genes for the enzymatic system primarily responsible for the metabolic activation and detoxification of PCH and other chemical carcinogens.

This section studies the molecular events of malignant transformation induced by chemical carcinogens, mainly those of the PCH class. The aim is to understand the enzymatic conversion of carcinogens to either detoxified forms, or to active carcinogenic forms. Higher organisms have systems for the detoxification and elimination of foreign chemical compounds, including carcinogens. These systems primarily involve microsomal cytochrome P-450 mixed-function oxygenases, but also include epoxide hydratase and conjugating enzymes. The vast majority of foreign compounds are processed by these enzyme systems. The mixed-function oxygenases are influenced by a variety of environmental factors such as drugs, pesticides, and carcinogens, and are influenced by the nutritional and hormonal state of the animal. The age, sex and genetic makeup also determine enzyme activity. Work in this laboratory provided the key studies which showed that this enzyme system was responsible for the activation of PCH procarcinogens to their ultimate carcinogenic forms. A primary goal is to define the enzymatic mechanism by which polycyclic hydrocarbons are activated either to carcinogenic forms or to detoxified products. As these enzymes are characterized, and as sensitive methods are developed for their assay, it may be possible to characterize an individual's enzymatic makeup with respect to carcinogen metabolism and to understand the relationship between this metabolism and individual susceptibility to PCH carcinogenesis.

The approach is to identify and fully characterize the enzymes responsible for carcinogen activation and metabolism. In addition, we seek to understand the molecular biology and regulation of this system both at the genetic and epigenetic levels. We plan to assess the types and amounts of these enzymes in human populations using molecular, biological, immunological, and metabolic approaches. We will carry out multi-leveled investigations of the carcinogen metabolizing enzyme systems, continuing our use of HPLC to study carcinogen metabolites, using monoclonal antibodies (MAb) and enzyme inhibitors to study the properties of the enzymes and using recombinant DNA and other molecular biological techniques to study the structure and regulation of the genes for the enzymes of carcinogen metabolizing systems.

The primary enzyme interface between environmental chemicals and higher organisms is the mixed function oxidase system. The various forms of cytochrome P-450 constitute a major part of this system, and are the major receptors for a wide variety of drugs, carcinogens, and other environmental chemicals. Our goal is to identify and characterize these isozymes in different tissues, species, population, and individuals. A major and unique approach to this problem is to develop a library of monoclonal antibodies (MAbs) which are highly specific for individual cytochromes P-450. Thus far, we have developed panels of MAbs to five different cytochromes P-450: two rabbit forms, and three rat forms. The latter MAbs have been prepared to the major cytochrome P-450 in livers of rats treated with 3-methylcholanthrene (3MC), phenobarbital (PB), and pregnenolone-α-16-carbonitril (PCN). The MAbs produced in each case are classified in three groups based on their interaction with cytochrome P-450: (1) those that bind, precipitate, and inhibit enzymatic activity; (2) bind and precipitate, but do not inhibit activity; and (3) those that only bind, but do not precipitate or inhibit activity.

We have used inhibition of enzyme activity by the MAb 1-7-1, prepared to 3MC-induced rat liver cytochrome P-450, to detect the cytochromes P-450 recognized by this MAb. The MAb not only inhibited the aryl hydrocarbon hydroxylase (AHH) activity of MC-induced rat liver microsomes, but also the AHH activity of human placenta. Among placentas from different individuals, the extent of inhibition of AHH by this MAb was virtually complete. Inhibition of 7-ethoxycoumarin deethylase (ECD), however, exhibited a large degree of individual variation. Placentas from both dizygotic and dichorionic monozygotic twins were examined for both absolute amounts of AHH and ECD, and for their degree of inhibition by MAb 1-7-1; high intra-pair concordances were observed, relative to that found in unrelated individuals. The MAb-sensitive activities were also found in human lymphocytes, but not in liver and monocytes. The cytochromes P-450 responsible for these activities in liver and monocytes are therefore antigenically distinct from the enzymes in placenta and lymphocytes.

The results demonstrate the value of MAbs for defining antigenic site relatedness for different enzymatic functions of P-450s, and for identifying and quantifying the amount of a particular enzyme activity in a tissue that is dependent on specific cytochromes P-450. This study may be a prototype for the use of MAbs in phenotyping and mapping of the P-450s responsible for specific metabolic reactions and thus be useful in determining the relationship of P-450 phenotype to individual differences in drug metabolism and carcinogen susceptibility.

MAB-sensitive activities in several animal tissues, species, and strains were also examined as models for the study of cytochrome P-450 in humans. The AHH activities in liver from MC-treated rats, C57BL/6 mice, guinea pigs, and hamsters were all greatly inhibited. Hepatic ECD activity was inhibited in rats, partially inhibited in C57BL/6 mice, and unaffected in guinea pigs and hamsters. Neither hepatic AHH nor ECD was inhibited in the relatively non-responsive mouse strain DBA/2. The pulmonary activities behaved differently from the hepatic activities in that both activities were inhibited by MAb in both C57BL/6 and DBA/2 mice. Studies of the types of cytochromes P-450 in different animal models may lead to a greater understanding of their multiplicity, genetic control, and relationship to drug and carcinogen metabolism.

As an extension of our enzyme inhibition studies, we are developing radioimmunoassays (RIAs) with MAbs to specific cytochromes P-450. An RIA based on the MAb 1-7-1 has detected: 1) the elevation in level of MC-induced cytochrome P-450 in the livers of MC-treated rats, relative to untreated and PB-treated rats; 2) similar differences for guinea pig and C57BL/6 mice but not for hamster and DBA/2 mice; 3) tissue-dependent differences in 3MC-induced rats; liver has higher levels than lung or kidney. The RIA data are in general agreement with the enzyme inhibition results. Development of quantitative and sensitive RIAs based on several MAbs should greatly aid in the detection and phenotyping of cytochromes P-450 in tissues and individuals, and is a major goal of our laboratory.

Monoclonal antibodies have also proven useful for immunopurification of cytochromes P-450. A Sepharose-MAb 1-7-1 immunoadsorbent tightly binds some cytochrome P-450 from liver microsomes of MC-treated rats. The enzyme appears on SDS gel electrophoresis as two bands of MW 52,000 and 54,000. The MAb interaction with the former species is relatively acid-labile. The immunoadsorbent also binds with comparable affinity a lesser amount of a polypeptide of MW 50,000 from liver microsomes of control and PB-treated rats. These results demonstrate the utility of MAb-based immunoadsorbents for purification of individual cytochromes P-450. The various isozymes may then be analyzed structurally and enzymatically.

We have analyzed a series of flavone compounds for their specificity toward different forms of cytochrome P-450. Many of these compounds have extraordinary inhibitory or stimulatory activity towards different forms of cytochrome P-450. One of these, L-Maackianin, was found to have activity opposite to that of 7,8 benzoflavone, an inhibitor we discovered several years ago. Thus, we have two inhibitors with unique specificity to different P-450's. These inhibitors may yield useful information on the subtle differences in cytochromes P-450 engaged in carcinogen activation and detoxification. They also will be valuable in anti-tumorigenesis research. Since they are naturally occurring, they may play a significant role in modulating carcinogenesis in humans in the natural environment.

We have adopted recombinant DNA and related molecular biological techniques to assess, with greater precision, the molecular mechanisms of regulation of P-450 gene expression, the multiplicity of P-450s, and the structural-functional relationships among the P-450s. This work also has great potential for increasing our understanding of biochemical individuality in human carcinogenesis. We have characterized in much greater detail the structure of the P-450 gene whose isolation we reported last year. We have constructed subclones of the different domains of this gene and of adjacent sequences. Using these as hybridization probes, we have discovered repetitive DNA in the intervening sequences nearest the 5' and 3' ends of the gene. The repetitive sequences in these two locations are different. We have also obtained preliminary evidence that the large intervening sequence nearest the 5' end of the gene contains sequences that code for a second mRNA that encodes a peptide of about 70 kilodaltons. We have also successfully cloned and characterized cDNAs corresponding in sequence to three different MC-induced cytochromes P-450 (MC-P-450s). Including the single cloned P-450 cDNA described last year, we now have recombinant plasmids containing cDNAs complementary to four distinct MC-P-450s. Two of the new recombinants correspond to the mRNAs for the two major forms of MC-P-450. The third corresponds

to a minor form. Using these cloned cDNAs as hybridization probes, the two major MC-P-450 mRNAs were found to be 2000 and 2700 base pairs long, while the minor form mRNA was 2000 base pairs. We have found that both major MC-P-450 mRNAs are undetectable in liver from control animals but are strongly induced and are among the most abundant mRNAs found in MC-induced liver. The minor MC-P-450 mRNA is present in low levels in controls and is induced to 10-fold by MC. The kinetics of induction of these three MC-P-450 mRNAs are now being studied. By hybridization selection and translation, we find that these mRNAs encode 55 or 59 kilodalton peptides that are recognized by polyclonal antibodies to MC-P-450. These peptides are now being characterized further, using the P-450-specific monoclonal antibodies prepared in this laboratory. We have also used the three newly cloned MC-P-450 cDNAs as hybridization probes to isolate the corresponding genes from a libraty of the rat genome. At present two of these genes have been successfully isolated and are being characterized by restriction enzyme mapping and R-loop analysis; the isolation of the third gene is in progress.

Nucleic Acids Section - Studies (1) interaction of chemical and physical carcinogens with nucleic acids and their actions on the functions of DNA, (2) the relationship between defects in repair of cellular DNA and human cancer, (3) chemically produced alteration of the repair of DNA, and (4) the interaction of DNA with proteins believed necessary for cellular transformation.

The study of human cells defective in repairing damaged DNA was extended, with the rationale that DNA-repair deficient cells are more susceptible to the adverse effects of carcinogens (cell killing, mutagenesis, sister chromatid exchange, and malignant transformation) than their repair-proficient counterparts. A group of human tumor (19) and SV40-transformed (7) strains deficient in the repair of O^6-methylguanine (O^6-MeG, a modified DNA base made by certain methylating agents) was identified earlier in this project. Such strains were called Mer^-. Like SV40 and Rous sarcoma virus, adenovirus was found to produce Mer^- strains. An activity was present in 8 Mer^+ strains but not in 11 Mer^- strains that demethylated O^6-MeG in DNA, thereby producing guanine and repairing this damaged base. After the reaction, the methyl group was found bound to a 22,000 M.W. protein, presumably the O^6-MeG-DNA methyltransferase. Using methyl group transfer as a stoichiometric measure, Mer^+ strains were judged to contain on the average 60,000 methyltransferase molecules per cell. A group of 5 Mer^+ cell strains was found sensitive to cell killing by MNNG. This group was found able to repair approximately one-third as much O^6-MeG as Mer^+ Rem^+ cell strains.

To provide a basis for somatic cell genetic studies of DNA repair, hybrids between cells having the various DNA repair phenotypes were produced after preparing cells with selectable markers by transfection with plasmids. Primary fibroblasts and established cell lines from various strains of mice show differences in their ability to respond to agents that produce O^6-MeG.

Studies with hydroxyurea indicate that human tumor cells and normal human fibroblasts depend upon ribonucleotide reductase(s) to different extents when repairing UV-damaged adenovirus.

The gene products of oncogenic viruses play a role in cellular transformation. A conclusive evaluation of the role of individual oncogene products has been difficult because the isolation and purification of the protein from tumor-bearing

animals are extremely difficult. The production of specific antibodies is also hindered by the limited availability of the purified proteins. Recently, however, recombinant DNA techniques have made possible the production of large quantities of gene products. Using a temperature-sensitive gene expression control system, it has been possible to produce in E. coli a significant yield of polyoma middle T antigen (MT). The vector system consisted of the lambda strong promoter, PL, which is regulated by the temperature-sensitive CI gene expression. The MT cDNA was fused with the O-gene of lambda DNA. The MT produced in the E. coli specifically reacted against MT specific monoclonal antibodies. The isolated MT protein will be used in further studies of its biological activities and for production of specific antibodies. The vector system constructed in the present study can be used to produce many other oncogene products of viral and cellular origins. To study the control mechanisms of oncogene expression, the control regions of metallothionein or mouse cell DNA fragments were joined with HSV thymidine kinase or MT structural genes. The expression of these genes is assayed after induction by heavy metals or other means. These studies will give insight to the molecular mechanisms of gene expression.

Protein Section - Studies (1) protein-nucleic interactions which may regulate or coordinate the production of cellular proteins, (2) specific alterations in DNA sequences which may modify control functions or lead to altered levels of regulatory proteins, and (3) mechanisms which modify genetic information in DNA by post-transcriptional processing of RNA.

The regulated expression of the genetic information encoded in DNA depends on specific protein-nucleic acid interactions in chromatin. Two major approaches were used to study the role of chromosomal proteins in maintaining the structure and regulating the function of chromatin. The first approach involved production of antibodies against chromosomal proteins and use of these antibodies to study the organization of the proteins in chromatin. Using this approach, in the last year, it was found that: 1) poly (ADP-ribose) polymerase is associated with only part of the nucleosomes; 2) a cross-linked complex of poly ADP-ribose and histones occurs in vivo; 3) large storage pools of HMG proteins are found both in the nucleus and cytoplasm of amphibians. The HMG proteins are involved in maintaining the chromatin in a transcribed state; 4) antibodies against the folding domain of histone H_5 cross-react with the folding domain of the minor histone $H_1°$ but not of the major histone H_1. Monoclonal antibodies against different antigenic sites of $H_1°$ were used to study the role of this histone in cell differentiation; and 5) chromatin can be fractionated by immunoaffinity chromatography. The second approach involves the use of specific cloned DNA sequences to purify proteins associated with such sequences. Plasmid Blur-8, containing the highly repetitive alu sequence, was used to detect a component isolated from HeLa cells which binds specifically to the alu sequence. Various segments of a cytochrome P-450 enzyme gene have been subcloned into pBR322. The subcloned plasmids were used for studying the chromatin structure of this gene and for assaying for proteins with specific affinity for various regions of the gene. Using these clones, it will be possible to compare the proteins associated with repetitive DNA sequences to those associated with unique DNA sequences.

A shuttle vector plasmid has been constructed which can be used in mammalian cells to study gene stability, rearrangement, and recombination. The plasmid contains sequences derived from a bacterial plasmid from SV40 virus, and also a marker gene, galactokinase, which can be scored in the appropriate bacterial host. This construct replicates in mammalian cells and bacteria. An experi-

mental protocol was designed in which mammalian cells were infected with the plasmid, replication permitted, and then the plasmid DNA extracted from the cells. After purification and elimination of residual infectious DNA, the plasmid was introduced into a bacterial host which permitted the detection of the presence or absence of a functional galactokinase gene. With this assay it was possible to assess quantitatively the stability of the plasmid in the mammalian cells. It was found that approximately 1% of the progeny plasmids had lost a functional marker gene. The defective plasmids contained point mutations, deletions, and insertions of cell DNA. Many of the mutational events occurred early in infection and were found with different methods of DNA infection.

Bacterial plasmids have been used to study repair and mutagenesis of benzopyrene diol epoxide (BPDE)-damaged DNA in E. coli. In nontargeted experiments the plasmids were randomly modified by BPDE and introduced into E. coli strains which differed in their capacity for repair and mutagenesis. By measuring the survival of bacteria containing plasmids and mutagenesis of a plasmid gene, it was possible to identify host cell functions for error-free repair and for mutagenesis. It was found that repair functions can be distinguished temporally from mutagenic activities after induction of the SOS response. In "targeted" experiments, a specific fragment of the plasmid from a nonessential marker gene was modified with BPDE and ligated back into the plasmid. The survival curves of these constructs were virtually identical to those of the randomly modified plasmids, suggesting that the principal determinant for survival of BPDE-damaged DNA is the simple presence of the carcinogen rather than secondary mutational events in essential functions. Mutants were found in the targeted regions but not in another nontargeted gene, indicating that mutagenesis is targeted. A collection of these mutants has been sequenced and transitions, transversions and frame shift mutations identified.

By means of a histochemical method which detects certain phosphoproteins and sialoglycoproteins on gels, a non-casein phosphorylated glycoprotein was found in mouse colostral milk that was not seen in mature milk. Electrophoretic assays of proteins synthesized in mammary gland explants in the presence of ^{32}P indicated that the phosphoprotein found in colostrum was made in the gland. Proteins in colostral milk from normal and tumor bearing mice were examined. Qualitative and quantitative differences were observed. Normal human colostral and mature milk were separated by one and two dimensional gel electrophoresis on polyacrylamide gels and examined for the presence of phosphoproteins and sialoglycoproteins using the histochemical method. Several modified proteins not seen in mature milk were detected in colostral milk.

The first higher eucaryotic opal suppressor tRNA gene has now been isolated by molecular cloning and sequenced. It is apparently present in a single copy in the chicken genome and is not part of a cluster of tRNA genes. The tRNA is encoded in an 87 bp segment without intervening sequences and it specifies a tRNA with an anticodon that reads the termination sequences and it specifies a tRNA with an anticodon that reads the termination codon UGA. This gene has an unusual 5' internal promoter region but encodes the normal 3' internal promoter. In vitro transcription experiments indicate that the gene has an active but weak promoter. Gene copy number and promoter strength provide an explanation for how a low level of this specialized tRNA is maintained in the cell.

Protein phosphorylation is important in many cellular processes including oncogenic transformation of human cells. tRNAs, like the opal suppressors that apparently insert phosophoserine directly into protein, have acquired new interest and significance. The structure and function of human and rabbit opal suppressor genes are being investigated through molecular cloning. The human and rabbit genomes contain DNA sequences that hybridize to the cloned chicken opal suppressor gene, which is being used as a probe to isolate the corresponding genes from human and rabbit DNA libraries. Subcloned DNA sequences flanking the chicken tRNA gene have been prepared and will be used to characterize a larger segment of the mammalian genomes and to identify evolutionarily conserved regions integral to gene expression.

Transfer RNA^{Phe} lacking the Wye base occurs in neuroblastoma tissue. It was found that this tRNA is used more extensively in protein synthesis than the corresponding $tRNA^{Phe}$ which occurs in normal tissues with a fully modified Wye base. These data suggest that preferential usage of a hypomodified tRNA in protein synthesis facilitates the synthesis of certain proteins in tumor cells.

The two-dimensional electrophoretic analysis of DNA described last year has been extended to the study of repeated families of DNA sequences in the rat and mouse genomes. It was possible to delineate the relations between families defined by different restriction enzymes by means of certain features of the display. Among these were the horizontal and vertical co-linearity of the spots generated by the DNA fragments, as well as the presence of diagonal streaks which frequently connected the spots. By analysis of these patterns, it was concluded that the murine repeated DNA sequences defined by three restriction enzymes (BglI, BamHl, and EcoRI) contained common elements. Analogous findings were made for the rat genome. It was found that the DNAs isolated from normal and transformed mouse 3T3 cells did not differ.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04496-06 LMC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Role of Chromosomal Proteins in the Structure and Function of Chromatin

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Michael Bustin Visiting Scientist, LMC, NCI

COOPERATING UNITS *(If any)*
Department of Biophysics, Kings College, London; Department of Biochemistry, Georgetown University, Washington, D.C.; Department of Membrane Biology, German Cancer Center, Heidelberg

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	3.0	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin is studied. Microinjection of antibodies to histones and nonhistone proteins into the cytoplasm and nucleus of somatic cells and of amphibian oocytes indicate that transcribed regions in the genome contain histones and HMG-17 and that HMG-1 and 2 are found in the cytoplasm of somatic cells and the ooplasm of amphibian oocytes. The large pool of these proteins which bind to single stranded DNA suggests that they are required for chromatin assembly. Polyclonal antibodies to 5 M urea-soluble proteins and monoclonal antibodies to histone H1° have been elicited. The antigenic determinants to which the monoclonal antiH1° bind have been identified. The monoclonal antibodies were used to map the orientation of the molecule in chromatin. Chromatin has been fractionated by immunoaffinity chromatography on sepharose column to which chromosomal protein HMG-17 was covalently bound.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than Principal Investigator) engaged on this Project:

Leo Einck	Staff Fellow	LMC, NCI
Ella Mendelson	Visiting Fellow	LMC, NCI
Barbara Dunn	Guest Researcher	LMC, NCI

Objectives:

To understand the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes. To explore the possibility that neoplastic transformation is associated with defined alterations in either the type or the organization of chromosomal components.

Background Information and Research Strategy:

The chromatin fiber, which is the backbone of chromatin and chromosomes, is a dynamic structure. Thus, it is difficult to determine the structure-function relation of defined chromosomal components. This question was approached by purifying chromosomal proteins, eliciting specific antibodies to these, and using the antibodies as probes for studying the in situ arrangement of the defined chromosomal components at various stages of chromatin organization. So far, it has been possible to purify the antigens, elicit antibodies, and adapt and develop various immunochemical techniques for detecting and quantifying the binding of specific antibodies to chromatin and chromosomes. Immunoelectron microscopy and immunofluorescent techniques are used to study the organization of histones and defined nonhistone chromosomal proteins in the interphase, transcribing, and replicating chromatin fiber and in metaphase and polytene chromosomes.

An alternative approach is to purify proteins which bind to specific cloned DNA sequences. In this approach, cloned DNA sequences are labelled either by nick translation or by end-labelling. The retention of specific DNA fragments on nitrocellulose filters after addition of cellular protein extracts is an assay for the presence of proteins specific for particular DNA sequences. Their presence is verified by electrophoresis of the DNA fragments eluted from the nitrocellulose filters. The filter assay can be used to monitor purification of the DNA binding protein which is done by conventional techniques.

Currently, the research effort is concentrated in the following areas: 1) Studying the involvement of chromosomal proteins in transcription by microinjecting functional antibody fragments into living cells; 2) defining antigenic determinants of H_1° by using monoclonal antibodies; 3) fractionation of chromatin by immunoaffinity chromotography; 4) studies on the antigenicity of 5 M urea soluble chromatin proteins; and 5) purification of proteins specific for repetitive DNA.

Methods Employed:

Microinjection, enzyme linked immunoassays, immunofluorescence, immunoreplica, enzyme digestions, electrophoresis, centrifugation, chromatin preparation, monoclonal antibodies, autoradiography, DNA cloning, restriction analysis, nitrocellulose filter assays, radiolabelling and immunoaffinity chromatography.

Major Finding:

1) Studies on the function of chromosomal proteins by microinjection of functional antibodies and antibody fragments into living cells. The major goal of these studies is to understand the in vivo function of chromosomal proteins. We have approached this question by microinjecting antibodies against defined chromosomal proteins into living cells and studying the effect of such injection on various cellular functions. The antibodies which were injected were fluoresceinated and their movement after microinjection into the cytoplasm or nucleus of the cell was followed using fluorescence microscopy. Using this approach we have found that antibodies to histones and to chromosomal protein HMG-17 inhibited transcription, while antibodies to chromosomal proteins HMG-1 and HMG-2 did not. Antibodies to HMG-1 and HMG-2 have also been injected into the giant nuclei present in the oocytes of several amphibian species. Following microinjection, the lateral transcription loops of transcriptionally active lampbrush chromosomes present in these nuclei retracted, suggesting that HMG-1 and 2 are present at or near the transcribable regions. Using immunofluorescence and immunoblotting techniques it was possible to demonstrate that this "nuclear" protein is found both in the nucleus and ooplasm. The presence of a cytoplasmic pool of HMG proteins suggests that this protein is needed for assembly into chromatin of the DNA which is very rapidly synthesized during embryogenesis. Furthermore, since HMG proteins bind to single stranded DNA, it is possible that during rapid replication it is necessary to have a relative large proportion of the DNA as single strands.

2) Definition of antigenic determinants in chromosomal proteins. Histone H_5 is an erythrocyte-specific histone which brings about condensation of the chromatin. A peptide region in this protein named GH_5 probably is responsible for stabilizing the basic nucleosome structure. In cells committed to differentiation, or in which DNA synthesis stops, a new histone called $H_1°$ is induced. We have elicited antibodies to peptide GH_5 and demonstrated that it cross-reacts immunologically with protein $H_1°$. By trypsin digestion of $H_1°$, an analogue of peptide GH_5 was isolated from $H_1°$. The antibody cross-reacts with this peptide. Thus, antisera which have been elicited against the chicken erythrocyte specific peptide GH_5 can be used to study the commitment of cells to differentiation. We have produced five monoclonal antibodies by immunization of mice with H_5. All five clones are directed against the GH_5 region. Two of these cross-react with $H_1°$, while three do not. Since the sequences of $GH_1°$ and GH_5 are known, we can pinpoint the exact region against which the antibodies are directed. Competition experiments indicate that the antibodies are directed against non-overlapping determinants. Immunofluorescence studies reveal that only the monoclonal antibodies which crossreact with $H_1°$ stain the nuclei of somatic cells. Thus, the monoclonal antibodies will be suitable for studying the organization of $H_1°$ in chromatin. Indeed, the antibody binding is markedly dependent

on the conformation of chromatin, which in turn is dependent on ionic strength. Thus, the availability of an antigenic site to antibody binding can reflect changes in chromatin structure associated with a particular protein segment.

3) Fractionation of chromatin by immunoaffinity chromatography. Superimposed on the repeated nucleosome structure of chromatin there is heterogeneity in chromatin structure and function. The presence of minor histone variants and small amounts of nonhistone chromosomal proteins indicate that there is compositional heterogeneity among various regions in chromatin. Antibodies against defined chromosomal components can be used to fractionate chromatin according to the antigenic composition of different regions. We have prepared an affinity column of antibodies to chromosomal protein HMG-17. This protein seems to be associated with transcribable regions in the genome. Polynucleosome stretches containing between 3 and 10 nucleosomes were applied to these columns. About 10% of the input DNA bound to the columns. The proteins which were present in the bound nucleosomes containing, or in the vicinity of, protein HMG-17 are hyperacetylated. This finding supports the notion that transcribable chromatin regions are hyperacetylated.

4) Antigenicity of 5 M urea soluble chromosomal proteins. In addition to association with histones, the DNA in eukaryotic cells is associated with a large number of proteins commonly known as nonhistone proteins. It is thought that this heterogeneous group of proteins contains regulatory molecules and various enzymes involved in the controlled process of gene expression. The nonhistone proteins can be fractionated into several groups according to the conditions in which they are extracted from chromatin. Since each of the groups is still highly heterogeneous in their content of chromosomal proteins, immunological methods have been used to study the tissue, developmental and oncogenic specificity of the fractions. We have purified and characterized a protein fraction extractable from purified chromatin by 5 M urea at low ionic strength. Analyses of the fraction by electrophoresis in polyacrylamide gels reveal tissue specificity among various human tumor lines. This specificity is not reflected in the immunological reaction. Apparently, all the cells contain a few common components which are antigenically dominant and obscure any putative immunological differences between the proteins present in the chromatin in different cells.

5) Purification of proteins associated with specific DNA sequences. A variety of evidence suggests that in some cases specific proteins are associated with specific DNA sequences. Such specific proteins may regulate the expression of a particular DNA sequence. The detection and study of such proteins is hampered by lack of suitable assays and by the fact that they are probably present in the cell in very small amounts. We are attempting to purify a protein (or proteins) which may have enhanced affinity for the highly repetitive human Alu DNA sequence. Towards this goal, we have propagated plasmid Blur-8, which contains the Alu sequence, and restricted it so as to generate a series of fragments of different molecular size. The fragments were then end-labelled, mixed with an extract prepared from HeLa nuclei, and passed through a nitrocellulose filter. The amount of Blur-8 DNA bound was compared to the amount control pBR322 DNA (similarly restricted) bound. The specificity for the Alu fragment was further ascertained

by eluting the DNA fragments bound to the nitrocellulose filter. The results indicated a 8-10 fold enrichment in Alu fragment in the fraction retained on the nitrocellulose filter. We conclude that the HeLa nuclear extract contains one or more proteins which bind preferentially to Alu DNA sequences.

Significance to Biomedical Research and the Program of the Institute:

Understanding the mechanism of gene regulation and its relation to neoplasia requires knowledge of the structure of chromatin and chromosomes. The approach developed in this laboratory is presently the only approach in which specific probes for well-defined, purified, chromosomal components are used to study the organization of these components in intact chromatin and chromosomes. As such, a unique opportunity has developed whereby certain structural aspects of these nucleoproteins can be visualized and directly related to functional stages of the genome. The immunological techniques developed for the study of the in situ organization of proteins in chromatin and chromosomes are applicable to studies on damage and repair in the genome as a result of binding of carcinogens or X-ray and UV exposure.

Proposed Course:

Studies devoted to understanding the structure-function relation of chromosomal proteins will be continued. In the forthcoming year we will concentrate our efforts on: 1) Understanding the role of protein $H_1°$ in chromatin; 2) using immunoaffinity chromatography to fractionate chromatin; and 3) studying the nature of the protein which binds to Alu sequences.

Publications:

Allan, J., Smith, B. J., Dunn, B. and Bustin, M.: Antibodies against the folding domain of histone H_5 cross-react with $H_1°$ but not H_1. J. Biol. Chem. 257: 10533, 1982.

Bustin, M., Dunn, B., Gillette, R., Mendelsohn, E. and Soares, N.: Antigenic determinants of chromosomal proteins HMG-1 and HMG-2. Biochemistry 21: 6773, 1982.

Kurth, P. D., Reisch, J. and Bustin, M.: Selective exposure of antigenic determinants in chromosomal proteins upon gene activation in polytene chromosomes. Exp. Cell Res. 143: 257-267, 1983.

Malik, N., Bustin, M. and Smulson, M.: Antibody to poly (adenosine diphosphate-ribose) polymerase and its use in chromatin analysis. Nucleic Acid Res. 10: 2939-2950, 1982.

Wong, M., Kanai, Y., Miwa, M., Bustin, M. and Smulson, M.: Immunological evidence for the in vivo occurrence of a cross-linked complex of poly ADP-ribosylated histone H_1. Proc. Natl. Acad. Sci. USA 80: 205, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04516-07 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular and Molecular Effects of Psoralen Plus Ultraviolet Light

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

K. H. Kraemer Research Scientist LMC NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Psoralen plus long wavelength ultraviolet radiation (UV-A) is being investigated as a model system for clinically relevant photochemical carcinogenesis. Used experimentally for treatment of psoriasis and mycosis fungoides, psoralen plus UV-A has been found to be mutagenic and carcinogenic. We have developed an in vitro assay to measure the effects of UV-A mediated psoralen-DNA binding on human lymphoid cells. Parameters monitored include the rate of DNA synthesis, induction of DNA-psoralen cross-links, induction of sister chromatid exchanges, alterations in the rate of cell proliferation and survival, and in immune reactivity. These studies indicate that the low doses of psoralen plus UV-A received by patients' leukocytes during therapy may result directly in decreased DNA synthesis in their circulating lymphoid cells. This system may be exploited to destroy circulating neoplastic cells by irradiation of extracorporeal blood cells during leukophoresis. These studies are aimed at understanding the mechanism of cell damage induced by psoralen plus UV-A so as to control the toxicity to human cells during therapy and to identify individuals with abnormal sensitivity.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

None

Objectives:

Humans are exposed to chemicals which may interact with ultraviolet radiation to become carcinogenic. 8-methoxypsoralen (8-MOP), a compound which is found in many plants, plus high intensity long wavelength ultraviolet radiation (UV-A) is being used experimentally to induce remissions in psoriasis and in mycosis fungoides. The combination of 8-MOP plus UV-A produces DNA-8-MOP binding and has been shown to induce mutations in bacteria and in mammalian cells and to cause skin cancer in mice and in humans. Individuals with some cancer-prone genetic diseases may be at increased risk from this treatment. We are developing an in vitro model system to assess clinically relevant photochemical carcinogenesis. 8-MOP plus UV-A treatment of extracorporeal circulating blood cells during leukophoresis may be useful in destroying neoplastic cells while minimizing systemic toxicity.

Methods Employed and Major Findings:

We previously demonstrated that circulating lymphoid cells of some psoriasis patients receiving 8-MOP plus UV-A therapy had a significant reduction in DNA synthesis. We have developed an in vitro assay system to approximate some of the conditions of 8-MOP plus UV-A exposure of human lymphoid cells during in vivo therapy. The assay has been used with fresh lymphocytes and with long-term lymphoblastoid cell lines. These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells.

We have developed a simple microtiter assay to measure lymphoblastoid cell survival after treatment with 8-MOP plus UV-A or other DNA damaging agents and have automated the end-point analysis of this assay.

In the lymphoblastoid cells, as much as a 50% inhibition of DNA synthesis following 8-MOP plus UV-A treatment was associated with 100% survival. Greater inhibition of DNA synthesis resulted in an exponential decrease in cell survival. Similarly, measurements of 8-MOP-DNA cross-linking by the alkaline elution technique revealed a dose dependent increase in cross-link induction above a threshold of approximately 50% inhibition of DNA synthesis. The formation of detectable cross-links was also correlated with decreased cell survival. Thus, DNA-8-MOP interstrand cross-links may be responsible for inhibition of DNA synthesis and cell killing.

8-MOP plus UV-A treatment of lymphocytes or lymphoblastoid cells in vitro resulted in approximately a doubling in the number of sister chromatid exchanges per metaphase. Further increases in 8-MOP plus UV-A were toxic. Thus, it is likely

that the doses of 8-MOP plus UV-A received by patients' lymphocytes are too low to permit routine detection of increased sister chromatid exchanges.

Mixed leukocyte reactivity of fresh human leukocytes was found to be inhibited in a dose dependent manner by 8-MOP plus UV-A in vitro. Stimulator and responder functions were both inhibited. This inhibition of immune reactivity may be exploited to provide immunosuppression.

Cultured cells from patients with Cockayne's Syndrome (CS) are hypersensitive to the growth-inhibiting effects of sunlight (UV-B radiation). Cells from one CS patient are hypersensitive to UV-B but have normal proliferative response to photosensitized 8-MOP. This implies that there is at least one human cellular recovery pathway that is different for UV-B and for photosensitized 8-MOP.

Significance to Biomedical Research and the Program of the Institute:

These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells. Further, photosensitized psoralen damage may, in part, be handled by human cellular recovery pathways different from that for sunlight-induced damage.

Proposed Course:

Lymphoblastoid cell lines from patients with cancer-prone genetic diseases are being examined for evidence of hypersensitivity to psoralen plus UV-A-induced killing and mutagenesis. This may indicate populations who are at increased risk of toxicity from photochemotherapy.

Publications:

Kraemer, K. H.: Assessment of human lymphoid cell damage induced by therapeutic levels of 8-methoxypsoralen and long wavelength ultraviolet radiation in vitro. In Castellani, A. (Ed.): The Use of Human Cells for the Assessment of Risk from Physical and Chemical Agents. London, Plenum (In Press)

Kraemer, K. H.: In vitro assay of the effects of psoralens plus ultraviolet radiation on human lymphoid cells. JNCI 69: 219-227, 1982.

Kraemer, K. H. and Waters, H. L.: Effects of psoralens plus ultraviolet radiation on human lymphoid cells in vitro. JNCI (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04517-07 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Repair In Human Cancer-Prone Genetic Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

K. H. Kraemer Research Scientist LMC NCI

COOPERATING UNITS *(if any)* W. C. Lambert, Dept. of Path., NJ Med. Sch., Newark, NJ; A. D. Andrews, Dept. of Derm., Columbia U., NY, NY; J. L. German, NY Blood Ctr, NY, NY; W. H. Clark, Dept. Derm., Hosp. U of Penn. Phila. PA; H. Slor, Tel Aviv Univ, Tel Aviv, Israel; G. Bynum, U of NM, Albequerque, NM.

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.1	3.1	2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human cancer-prone genetic diseases are being studied in order to identify groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting to discover the clinical consequences as well as the molecular basis of their cellular hypersensitivity. Patients with xeroderma pigmentosum (XP) and ataxia telangiectasia (AT), diseases with ultraviolet (UV) and X-ray hypersensitivity, respectively, and with the dysplastic nevus syndrome of hereditary cutaneous melanoma (DNS) are being studied. Detailed examinations of the clinical features of affected individuals are being made. A large retrospective XP literature study (more than 700 cases) and a prospective registry of XP patients is underway. Field studies in Israel detected a possible new form of XP with defective DNA repair without neoplasia. Clinical diagnostic features of DNS are being refined. Testicular abnormalities in AT are under study. Cultures of skin and blood from XP, AT and DNS family members were established. Effects on cell survival, mutagenesis, DNA synthesis and repair, histone synthesis and chromosome integrity after treatment with DNA damaging agents are being studied. DNS lymphoblastoid cell lines were found to be UV-hypermutable thus implicating DNS as a second hypermutable human disease (XP was the first recognized). We are using DNA mediated gene transfer to attempt to clone the genes responsible for UV hypersensitivity in XP cells. We have identified an XP cell line that is a highly proficient recipient of cloned genes. These studies may afford a means of detecting individuals with increased genetic risk of neoplasia and suggest modes of cancer prevention.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

M. H. Perera	Visiting Fellow	LMC NCI
M. Protic-Sabljic	Visiting Fellow	LMC NCI
J. Fagan	Sr. Staff Fellow	LMC NCI
M. H. Greene	Clinical Epidemiologist	EEB NCI
J. Scotto	Biometrician	BB NCI
R. Tarone	Mathematical Statistician	BB NCI
M. Bustin	Visiting Scientist	LMC NCI
B. Howard	Section Head	LMB NCI
R. Sherins	Senior Investigator	RR NICHD
R. Clark	Expert	RR NICHD

Objectives:

Human cancer-prone genetic diseases are being studied with a view toward identifying groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting (1) to correlate such sensitivity with clinical abnormalities, (2) to determine if there is genetic diversity within such groups, (3) to understand the molecular basis of their cellular hypersensitivity and (4) to develop tests to identify persons at increased risk of neoplasia.

Methods Employed:

Patients are examined with particular emphasis on cutaneous abnormalities, and cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory experimentation. Patients with xeroderma pigmentosum (XP), ataxia telangiectasia (AT), and dysplastic nevus syndrome of familial cutaneous malignant melanoma (DNS) have heen studied clinically. The English language medical literature on XP is being reviewed comprehensively, and information on individual patients abstracted and entered into a computer for analysis. Physicians treating patients with XP are encouraged to fill out a registry questionnaire about their patients. New clinical forms of XP are investigated in depth. Clinical diagnostic features of DNS are being refined. Gonadal abnormalities in males with AT are being evaluated. Histological sections of pigmented lesions in XP are being examined for microscopic abnormalities. Cultured XP, AT and DNS cells are being examined for the effects of DNA damaging agents (UV, X-ray, bleomycin) on cell survival, mutagenesis, DNA synthesis and repair, histone synthesis, and chromosome integrity. DNA mediated gene transfer experiments are being utilized to attempt to isolate the portion of DNA responsible for the UV hypersensitivity of the XP cells.

Major Findings:

XP is an autosomal recessive cancer-prone disease with clinical UV hypersensitivity, accompanied by cutaneous and neurological abnormalities. Cultured cells from XP patients have cellular UV sensitivity and defective DNA repair. We have compiled the most comprehensive review of the world literature to date on XP

including both clinical and laboratory observations. Data on more than 700 XP patients described in the literature have been entered into the computer. In this retrospective study, we have documented reduction of 50 years in the age of onset of skin neoplasms in XP in comparison to the U.S. population. There is a 1000-fold increase in all three major types of skin neoplasms: basal cell carcinomas, squamous cell carcinomas, and melanomas. The XP melanoma site distribution does not correspond to areas of greatest sun exposure implying that factors other than UV may be important in melanoma. Oral cavity neoplasms are increased, possibly due to UV exposure of the anterior tongue and/or to dietary carcinogens. We found lymphoblastoid cell lines from XP patients to be hypersensitive to killing by tryptophane pyrolysis products (carcinogens produced in charbroiled foods). Internal neoplasms, particularly sarcoma of brain, was found at increased frequency. A registry of XP patients is being established in collaboration with W. C. Lambert, A. D. Andrews, and J. L. German. On a collaborative study in Israel we identified kindreds with a form of XP that has not been previously recognized. Clinically, facial lesions predominate. One family member has reduced DNA repair with minimal clinical symptoms while a brother with equally reduced DNA repair has numerous neoplasms. This suggests that other, presently unmeasured, factors may be crucial to the development of neoplasms in XP. In our attempts to clone the genes responsible for UV hypersensitivity in XP cells, we have collaborated with the Laboratory of Molecular Biology and developed a protocol that gives high efficiency (about 10^{-3}) transfection of cloned selectable genes into XP cells. We found that SV40 transformed XP cells, but not primary fibroblasts, are suitable recipients of cloned genes. We developed a simple rapid thin layer chromatography assay for measuring xanthine phosphoribosyl activity in transferase (XPRT) activity transfected cells, and demonstrated stable transfection of XPRT activity into XP cells. We are presently modifying the transfection protocol for use with genomic (uncloned) DNA.

AT, an autosomal recessive cancer-prone disease with cutaneous, neurological, and immunological abnormalities, has X-ray sensitivity. We found unusual testicular defects in some AT males. We are studying the ability of cultured cells from AT patients and their parents to survive DNA damage induced by the chemotherapeutic agent, bleomycin. This agent also induced an abnormally large increase in chromosome breakage (but not in sister chromatid exchanges) in AT homozygous lymphoblastoid cells but not in heterozygous cells. DNA synthesis in AT homozygotes, but not heterozygotes, was found to be resistant to treatment by X-ray or bleomycin in comparison to the response of normal cells. The possible role of histones in the modulation of this response of DNA synthesis to cell damage is being investigated.

A newly recognized clinical disease, familial malignant melanoma with a characteristic precursor legion, the dysplastic nevus, is being examined in a collaboration with the Environmental Epidemiology Branch, NCI. This laboratory is contributing dermatological expertise to the clinical definition of the syndrome in a study of more than 400 family members. DNS family members with these distinctive nevi have several hundred-fold increased risk of developing cutaneous melanoma. Lymphoblastoid cell lines from selected patients are being examined for evidence of sensitivity to DNA agents as measured by cell survival and mutagenesis and examined for possible DNA repair defects. We found lymphoblastoid cell lines from two DNS patients to be hypermutable by UV. This indicates that DNS is a generalized disorder and is the second hypermutable human disease to be found.

Significance to Biomedical Research and the Program of the Institute:

These studies may identify persons with increased risk of cancer, may be useful in revealing the mechanism of cancer induction, and suggest modes of cancer prophylaxis. In addition, these diseases serve as models for studies of human environmental carcinogenesis.

Proposed Course:

This project will be continued along the lines indicated above.

Publications:

Elder, D. E., Greene, M. H., Guerry, D., Kraemer, K. H. and Clark, W. H.: The dysplastic nevus syndrome. Am. J. Dermatopathology 4: 455-460, 1982.

Greene, M. H., Goldin, L. R., Clark, W., Lourien, E., Kraemer, K. H., Tucker, M. A., Elder, D. E., Fraser, M. C. and Rowe, S.: Familial cutaneous malignant melanoma - an autosomal dominant trait possibly linked to the Rh locus. Proc. Natl. Acad. Sci. USA (In Press)

Kraemer, K. H.: Cellular hypersensitivity to physical and chemical agents in patients with ataxia telangiectasia: Disorders in cell growth and chromosomal integrity. Ann. Intern. Med. (In Press)

Kraemer, K. H.: Heritable diseases with cellular hypersensitivity. In Fitzpatrick, T. B., Eisen, A. Z., Austen, F., Freedberg, I. M. and Wolff, K. (Eds.): Dermatology in General Medicine, Update I. New York, McGraw-Hill, 1983, pp. 113-142.

Kraemer, K. H.: Use of human lymphoblastoid cell lines to determine cellular hypersensitivity to physical and chemical agents. In Castellani, A. (Ed.): The Use of Human Cells for the Assessment of Risk from Physical and Chemical Agents. London, Plenum (In Press)

Kraemer, K. H., Lee, M. M. and Scotto, J.: Early onset of skin and oral cavity neoplasms in xeroderma pigmentosum. Lancet 1: 56, 1982.

Protic-Sabljic, M., Whyte, D., Fagan, J. and Kraemer, K. H.: Transfection of xeroderma pigmentosum cells with cloned DNA. In Friedberg, E. and Bridges B. (Eds.): Cellular Responses to DNA Damage, UCLA Symposia on Molecular and Cellular Biology, New Series. New York, Alan R. Liss, Vol. II, (In Press)

Tucker, M. A., Greene, M. H., Clark, W. H., Kraemer, K. H., Fraser, M. C. and Elder, D. E.: Dysplastic nevi on the scalp of prepubertal children from melanoma-prone families. J. Pediatr. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04518-06 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Phosphoseryl-tRNAs and Hypomodified Aminoacyl-tRNAs in Mammalian Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Dolph Hatfield Research Biologist, LMC, NCI

COOPERATING UNITS *(if any)*

Northwestern University, Chicago, IL; University of New York at Stony Brook, Stony Brook, NY

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of two minor seryl-tRNAs from mammalian cells, each of which suppresses the nonsense codon UGA in protein synthesis and forms phosphoseryl-tRNA, is being investigated. Seryl-tRNA synthetase has been purified extensively and the kinase which phosphorylates these seryl-tRNAs is being studied.

In addition, it has been found that phenylalanyl-tRNA which lacks the Wye base and occurs in neuroblastoma tissue is used more extensively in protein synthesis than the corresponding phenylalanyl-tRNA which has the fully modified Wye base and occurs in normal tissue.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

J. F. Mushinski Senior Investigator LG NCI

Objectives:

One objective is to determine the cellular function of two minor seryl-tRNAs, both of which read the nonsense codon UGA in protein synthesis and form phosphoseryl-tRNA. Another objective is to determine the effect on protein synthesis of specific base modifications in isoacceptor aa-tRNAs and to determine whether misreading genetic code words by isoaccepter aa-tRNAs during protein synthesis may be involved in the expression of cancer.

Methods Employed:

Purification from mammary tissue of the kinase which phosphorylates the opal suppressor seryl-tRNAs from bovine liver (Hatfield et al. PNAS 79: 6215, 1982) is being undertaken by standard column chromatographic techniques. Rabbit liver seryl-tRNA synthetase has been purified by DEAE-cellulose and Blue Sepharose columns.

Large quantities of normal $tRNA^{Phe}$ from mouse liver and large quantities of $tRNA^{Phe}$ lacking the Wye base from neuroblastoma tissue (hypomodified $tRNA^{Phe}$) have been isolated and purified by Sepharose 4-B and reverse phase chromatography. Both isoacceptors were aminoacylated with ^{14}C and ^{3}H phenylalanine and the ability of each isoacceptor to donate phenylalanine to rabbit globin at specific sites was determined by cell free protein synthesis and subsequent tryptic peptide analysis. This project is a collaboration with Dr. J. F. Mushinski, Laboratory of Genetics, NCI.

Major Findings:

Seryl-tRNA synthetase has been purified extensively from rabbit liver and both the major serine tRNAs and the opal suppressor tRNAs serve as substrates for this enzyme. This finding suggests that a single synthetase is involved in the aminoacylation of each serine isoacceptor even though the primary structures of the major and suppressor serine tRNAs show less than 50% homology.

Hypomodified $tRNA^{Phe}$ was used more extensively in globin synthesis by about 15% over normal $tRNA^{Phe}$ regardless of whether UUU or UUC was translated with two exceptions: 1) one or more of the three Phe residues in tryptic peptide beta-T5 was incorporated preferentially by normal $tRNA^{Phe}$; and 2) the UUC encoded Phe residue in beta-T9 was used to a greater degree than any other Phe residue by hypomodified $tRNA^{Phe}$. These data suggest that preferential usage in protein synthesis of hypomodified $tRNA^{Phe}$ from neuroblastoma cells may facilitate the synthesis of certain proteins in tumor cells.

Significance to Biomedical Research and the Program of the Institute:

Among the major unresolved questions in biology is whether tRNA may play a role in cellular regulation and carcinogenesis. An approach to this problem is to determine the role of specific isoacceptors in protein synthesis and cell function and to determine if misreading genetic code words by isoacceptor aa-tRNAs in protein synthesis can induce cell transformation.

Proposed Course:

The proposed course is to pursue the cellular function of the opal suppressor seryl-tRNAs which form phosphoseryl-tRNA and to determine if phosphoserine is incorporated directly into protein.

Publications:

Hatfield, D., Diamond, A. and Dudock, B.: Opal suppressor serine tRNAs from bovine liver form phosphoseryl-tRNA. Proc. Natl. Acad. Sci. USA 79: 6215-6219, 1982.

Hatfield, D., Rice, M., Hession, C. and Melera, P.: Physarum polycephalum: Patterns of codon recognition. J. Bacteriol. 151: 1013-1021, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04525-11 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on Electrophoretic Techniques for Protein, RNA, and DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Andrew C. Peacock Chief, Protein Section, LMC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Genomic DNA from mouse and rat have been analyzed by a two-dimensional technique which permits greatly improved resolution of DNA segments defined on the basis of sites for restriction enzymes. After first digesting the DNA with one or more restriction enzymes, the generated fragments are separated by electrophoresis in a gel column of purified agarose which contains the second enzyme. The conditions of the first dimension run are chosen so that the second enzyme is immobilized and inactive during the run, while permitting the DNA fragments to migrate according to their length. After the first electrophoresis, the gel column is incubated under conditions to promote the activity of the second enzyme. Cleavage having been completed, the gel column is enbedded in a slab of agarose for a second electrophoretic run perpendicular to the first. Some sequences in the mouse and rat genome are sufficiently abundant to produce discrete spots, streaks, and secondary diagonals when the gels are stained; other sequences require the use of specific probes for identification. The secondary diagonals and streaks, generated by the second restriction enzyme, connect specific sequences that are more heterogeneously defined by the first enzyme.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Michael Seidman	Senior Staff Fellow	LMC, NCI
Susan Cole	Visiting Fellow	LMC, NCI

Objectives:

Current efforts are directed towards finding useful ways of examining whether, during carcinogenesis, there are changes in the genomic location of identifiable DNA sequences. For present purposes, the DNA sequences are defined on the basis of lengths between sites cleaved by selected restriction enzymes, and by ability to hybridize to specified probes. The immediate objective is to devise and evaluate electrophoretic methods that will give maximum information about the location of DNA sequences of low multiplicity.

Methods Employed:

Tissue cultures of rodent cells, assay of radioactivity, isolation and electrophoresis of RNA and DNA, preparative electrophoresis, hybridization, DNA restriction, and cloning.

Major Findings:

Continued development of the two-dimensional gel methodology has permitted the analysis of genomic DNA from mice and rats. The repeated family of murine sequences defined by EcoRI sites has been found to be contained within families defined by BglI and BamHI sites. Variability in the location of the Bam and Bgl sites was indicated by a secondary diagonal which passes through the spot generated by the most frequent members. Analogous results were obtained for the rat genome. The two-dimensional technique was used to examine the pattern of the repeats in the DNA from normal and transformed 3T3 cells. No differences were observed, but the high copy number of these sequences may be too great to detect one or two changes.

Experiments with specific probes for a moderately repeated sequence related to Harvey Sarcoma Virus DNA are in progress. These sequences serve as templates for an abundant "30S RNA." Early results indicate that this approach may be useful in characterizing the variety of genomic locations of this DNA, as well as showing sequence heterogeneity among the family members.

Significance to Biomedical Research and the Program of the Institute:

There is a widely-held (but disputed) belief that genomic rearrangements are important in the conversion of a cell from a normal to a malignant state. These studies are designed to provide suitable new technologies for investigating this problem, and to arrive at some firm conclusions as the validity of the hypothesis.

Proposed Course:

1) Improve two-dimensional technique to permit greater resolution of restriction fragments from mammalian genomes. 2) Develop digital scanning of gels to provide improved accuracy and sensitivity. 3) Seek additional probes for sequences susceptible to translocation. 4) Compare DNAs from normal and transformed cells to determine if translocation has occurred.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04555-09 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Assay Systems and Factors Involved in Malignant Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

T. Kakunaga Head, Cell Genetics Section LMC NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.2	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to clarify the mechanism of cell transformation by examining the genetic and physiological factors which affect cell transformation induced by chemicals. This project may also lead to development of an optimum system to study the mechanism of cell transformation as well as to obtaining an assay system for the assessment of human risk from environmental carcinogens. The structure-activity relationship of tumor promoters is being examined by using new promoters, aplysiatoxin (AP) and debromoaplysiatoxin (DBAP), with transformation of Balb 3T3A31-1-1 cells as an indicator of promoter potential. AP strongly enhances carcinogen-initiated transformation, whereas DBAP does not. The new factor which abolishes DBAP effects but not AP was found in serum which was used for transformation experiments, and was purified and identified as a protein of approximately 72,000 mol. wt. The lack of activity of DBAP in enhancement of transformation was ascribed to its high inactivation by the protein factor in serum. This transformation assay system correctly predicted the weak promoting potency of DBAP in vivo. Studies of the inhibitory effects of dexamethazone on cell transformation indicated the involvement of EGF-receptor down-modulation mechanisms in the cell transformation. A culture model system for co-carcinogenesis was developed using catechol, a mouse skin co-carcinogen, and Balb 3T3-A31-1-1 cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than Principal Investigator) engaged on this Project:

K. Shimomura Guest Researcher LMC, NCI

Objectives:

To clarify the mechanisms of malignant cell transformation by chemical and physical carcinogens, by defining and identifying the genetic and physiological factors affecting the transformation process, and to obtain an optimum system to study the mechanism of cell transformation as well as to obtain an assay system for the assessment of human risk from environmental carcinogens. Current interests are: 1) to determine the cellular and molecular mechanisms of tumor promotion by promoting agents using cell culture model systems, and 2) to develop a culture model system for co-carcinogenesis. Special attention is paid to the target stage of tumor promoters.

Methods Employed:

A quantitative assay system for malignant transformation by chemical carcinogens and its enhancement by tumor promoters has been developed in our laboratory using the Balb 3T3-A31-1-1 cell line. We determined stimulation of DNA synthesis in nuclei or glucose uptake across cell membranes by measuring the incorporation of [^{3}H]thymidine or [^{14}C]2-deoxyglucose, respectively. We employed a competitive binding assay for the determination of the number and affinity kinetics of receptors for promoters and epidermal growth factor on the cell surface membrane using [^{3}H]-phorbol dibutyrate and [^{125}I]-epidermal growth factor, respectively. Finally, we used separation on a thin layer chromatograph and measurement of [^{3}H]-arachidonic acid released into culture medium from the cells which were prelabeled with [^{3}H]-arachidonic acid.

Major Findings:

1) The effect of the removal of a bromine residue from aplysia toxin on its ability to promote transformation. Two members of a new chemical class of tumor promoters, aplysiatoxin (AP) and debromoaplysiatoxin (DBAP), were compared for their ability to enhance the transformation of Balb 3T3 cells pretreated with methylcholanthrene (MCA). DBAP differs from AP only by the lack of a bromine residue in its hydrophilic region. The hydrophilic region has been considered to be less important than the hydrophobic region for tumor promoting activity. AP strongly enhanced MCA-initiated cell transformation and DNA synthesis in GO-arrested cells, whereas DBAP did not. Potency of AP in enhancing transformation was 50-fold higher than that of 12-O tetradecanoyl phorbol-13-acetate (TPA) and similar to dihydroteleocidin B. However, AP and DBAP inhibited the binding of phorbol-12,13-dibutyrate (PDBu) and epidermal growth factor (EGF) to the cells equally. These results indicate that a minor change in chemical structure in the hydrophilic region of tumor promoters has a great influence on their potency to enhance transformation and that a discrepancy exists between the potencies of

promoters to enhance cell transformation and to inhibit the binding of PDBu and EGF. 2) The serum factor inactivating DBAP. AP and DBAP showed equal potencies to inhibit PDBu- and EGF-binding. However, DBAP did not enhance cell transformation or induce DNA synthesis. We realized that the buffer solution used for assaying PDBu- and EGF-binding does not contain serum whereas buffer solution used for cell transformation and DNA synthesis contains serum. Addition of bovine serum to the assay buffer completely abolished the inhibitory effects of DBAP on PDB uand EGF-binding, but did not affect AP or TPA activity. The factor in serum which abolishes DBAP effects was purified from bovine serum by ammonium sulfate fractionation, gel filtration and ion exchange chromatography. The factor was inactivated by digestion with proteolytic enzymes, and has an approximately 72,000 mol. wt. This serum factor is different from those reported by others, since this factor did not inhibit the effects of phorbol ester derivatives, whereas the others did. Recently, the promoting potency of DBAP was reported to be weak compared to AP in mouse skin carcinogenesis. These results indicate that the weak promoting activity of DBAP in vivo, which could be predicted from its effects on in vitro transformation, can be ascribed to inactivation by the factor present in serum. 3) Inhibition of cell transformation by dexamethazone (DX). Addition of DX into culture caused a marked reduction in the transformation frequency of the MCA-treated mouse Balb 3T3-A31-1-1 cells, in a dose-dependent manner. DX also greatly reduced the enhancement of cell transformation by a variety of tumor promoters. DX alone increased the number of EGF receptors on the plasma membrane. Pre-treatment with DX completely prevented the down-modulation of EGF-receptor induced by tumor promoters or EGF, but did not affect the promoter-induced reduction of PDBu-binding. Addition of EGF enhanced transformation frequency in MCA-treated cells. Combined with our previous results that the accumulated down-modulation of EGF receptors was most closely correlated with the transformation-enhancing potency of eleven promoter derivatives, these results suggest that enhancement of transformation is mediated through the biochemical events directly or indirectly associated with down-modulation of EGF-receptors. 4) Co-carcinogenesis model using in vitro cell transformation. "Co-carcinogen" identifies an agent which enhances tumor incidence when given to experimental animals simultaneously with carcinogens, but does not have the potency to induce tumors by itself. A co-carcinogen differs from a promoter in that a co-carcinogen is effective only when given simultaneously with a carcinogen, whereas promoters are effective only when given at some interval after carcinogen-treatment. When catechol, a mouse skin co-carcinogen, was given to the Balb 3T3-A31-1-1 cells simultaneously with a low dose of benzo[a]pyrene or beta-propiolactone, transformation was induced at high frequencies. Catechol alone, a low dose of carcinogen alone, or post-carcinogen treatment with catechol did not induce transformation. Thus, a model culture system to study co-carcinogenesis has been developed.

Significance to Biomedical Research and the Program of the Institute:

This project provides information on the biological identification and biochemical basis for the genetic and physiological factors affecting the induction of cell transformation by chemical carcinogens and irradiation. Such data will result in 1) the clarification of the mechanisms of cell transformation; 2) the development and improvement of the assay system for chemical and physical carcinogens, co-carcinogens and promoters; 3) the assessment of the role of environmental and

genetic factors in the incidence of human cancer; and 4) progress in the development of preventive measures of incidence of human cancer. Thus, this project directly aims at the aspect of the program of the Institute which seeks to find rapid meaningful assays for environmental carcinogens, to locate human populations with higher cancer risks, and ways to prevent cancer.

Proposed Course:

1) To continue to identify the biochemical effects of promoters which are directly involved in the enhancement of transformation. Special emphasis will be placed on the clarification of the requirement for the continuous presence of active promoters for enhancement of transformation. 2) To continue to investigate the molecular basis of the genetic differences in the susceptibility of A31-1 cell variants to chemically-induced transformation, with special attention to the expression of the transformed state. 3) To pursue the goals outlined in Objectives and to publish the results obtained.

Publications:

Atchison, M., Chu, S. C., Kakunaga, T. and Van Duuren, B. S.: Chemical cocarcinogenesis using a subclone derived from Balb/3T3 cells with catechol as cocarcinogen. JNCI 69: 503-508, 1982.

Cortesi, E., Saffiotti, U., Donavan, P. J., Rice, J. M. and Kakunaga, T.: Dose-response studies on neoplastic transformation of Balb/3T3 clone A31-1-1 cells by aflatoxin B_1, benzidine, benzo[a]pyrene, 3-methylcholanthrene and N-methyl-N'-nitrosoguanidine. Teratogen., Carcinogen., Mutagen. J. 3: 101-110, 1983.

Heidelberger, C., Freeman, A. E., Pienta, R. J., Sivak, A., Bertram, J. A., Casto, B. C., Dunkel, V. C., Francis, M. W., Kakunaga, T., Little, J. B. and Schechtman, L. M.: Cell transformation by chemical agents: A review and analysis of the literature. Mutat. Res. 114: 283-385, 1983.

Hirakawa, T., Kakunaga, T., Fujiki, H. and Sugimura, T.: A new promoter, dihydroteleocidine B, greatly enhances chemically-induced malignant transformation. Science 216: 527-529, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04578-07 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genes and Proteins Responsible for the Transformed Phenotype

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

T. Kakunaga Head, Cell Genetics Section LMC NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.1	3.9	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The nature of the genes and proteins responsible for the expression of the neoplastic phenotype of chemically transformed human cells has been investigated by using molecular cloning and other new techniques in molecular biology. A new polypeptide recognized in a chemically transformed human cell line has been identified as a mutant of beta-actin. The synthesis of the mutated beta-actin was correlated with the expression of the transformed phenotype in the variants of the transformed line and its hybrids with normal human fibroblasts. A mutation resulted in several defects in the function of beta-actin, such as increased instability, reduced incorporation into cytoskeletal elements, and decreased ability to polymerize in vitro. These defects in the beta-actin molecule were associated with the disruption and loss of the structural organization of the cytoskeleton, such as the actin cable network. The results suggest that a mutation in beta-actin leads the cells to express transformation by disrupting the cytoskeletal structure and its function. Two smooth muscle actin genes, stomach type and aorta type, were first isolated from human fibroblast DNA. They contain DNA sequences which completely match the amino acid sequences of the corresponding actin polypeptides. Comparison of the intron location in all actin genes in all species indicate that both deletion and insertion of the intron have been involved in the evolution of the actin gene family.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

N. Battula	Expert	LMC NCI
S. Taniguchi	Visiting Fellow	LMC NCI
S. Iijima	Visiting Fellow	LMC NCI
H. Ueyama	Visiting Fellow	LMC NCI

Objectives:

To identify the structure and function of genes and proteins responsible for the expression of the transformed phenotype. Current interests are: 1) to know the relationship between a mutation in actin genes and the expression of the transformed phenotype; 2) to determine the biological and molecular defects of the mutated beta-actin in vivo and in vitro; 3) to clone the normal and mutated beta-actin from a DNA library constructed from the DNAs of the chemically transformed human cell line, HuT-14, 4) to isolate and characterize human smooth muscle actin genes; 5) to construct a shuttle vector containing actin genes and examine the expression of actin in the transformed cells.

Methods Employed:

Biochemical techniques such as two-dimensional gel electrophoresis, in vitro translation, amino acid sequencing, ultracentrifugation, DNA-DNA and DNA-RNA hybridization, other techniques used for gene cloning and recombinant DNA work, standard tissue culture techniques of our laboratory, karyotype analysis using chromosome banding techniques, transplantation of cultured cells into nude mice, DNA transfection.

Major Findings:

1) Close correlation between the expression of the transformed state and the abnormality in actin molecules. The mutant beta-actin which was found in the chemically transformed human cell line has one amino acid substitution at position 245, where normal beta-actin has glycine and mutant beta-actin, aspartic acid. The rate of synthesis and half-life of the mutant beta-actin in the cells were identical to the normal beta-actin. However, the rate of incorporation into the cytoskeleton was significantly reduced compared to normal beta-actin. In the variant clone which showed increased tumorigenic properties and higher anchorage-independent cell growth, the rates of both synthesis and degradation were markedly increased. The electrical charge of mutant beta-actin molecule was altered by one negative net charge in the variant cell. This secondarily altered mutant beta-actin was synthesized in vitro under the direction of mRNA of the variant cell. The relationship between the expression of the transformed phenotype and the synthesis of the mutant beta-actin was also investigated in the hybrids and their segregants formed by fusing the transformed cells with normal parent cells. All hybrid cells which were confirmed to be 1: 1 hybrid cells expressed both the in vitro transformed phenotype and the mutant beta-actin.

One segregant obtained from the hybrid cell showed a normal-like phenotype in vitro and did not synthesize the mutant beta-actin. These results indicate that the mutation in the beta-actin molecule is related to the expression of the transformed phenotype. 2) Molecular and functional defects of the mutant beta-actin. The mutant beta-actin migrates at a significantly slower rate compared to normal beta-actin in SDS gel electrophoresis, as if the molecular weight of mutant beta-actin were 1-1.5 K dalton larger than that of normal beta-actin. The difference in the molecular weights of the two beta-actins is approximately only 58 dalton when calculated from their amino acid sequences. Thus the conformation of the mutant beta-actin seems different from the normal beta-actin even in the presence of detergent, sodium dodecyl sulfate (SDS). The procedures and conditions for isolating and purifying native cytoplasmic actins from the transformed human cells were extensively examined. The cytoplasmic actins of human cells were so unstable compared to muscle actins and amoeba actins that a very gentle and fast process was required for their isolation and purification and the yield was very low. A study of the kinetics of polymerization of the freshly purified normal beta- and gamma-actins and mutant beta-actins indicated that the mutant beta-actin has a reduced ability to polymerize in vitro compared to normal beta- and gamma-actins. The mutant beta-actin slightly reduced the polymerization of normal beta-actin, but not gamma-actin, when they were co-polymerized. The ion-dependency of the mutant beta-actin for self polymerization was much greater than that of the other two actins. These results, indicating that the mutant beta-actin is defective in at least self-polymerization, is consistent with in vivo observations that the incorporation of newly synthesized mutant beta-actins into the cytoskeleton is significantly reduced. 3) Cloning of human normal and mutated beta-actin genes. The genomic library was constructed by partial EcoRI digestion of DNA from a transformed human fibroblast line (HuT-14) and cloned into Charon-4A phage vector. This library was screened with the DNA fragment from the 3'-untranslated region of the human pseudo beta-actin DNA which we previously isolated as a probe. All phages that showed positive signals in the first autoradiograms of plaque hybridization were picked up and screened one more time. Four hundred phages that showed positive signals in the second screening were purified by repeated plating and plaque hybridization. Eight phages were picked up as the most likely candidates for beta-actins by their strong hybridizability to the DNA fragment, that is, DNA sequences coding for amino acid sequences near the N-terminal of beta-actin, and by determination of their approximate genomic size and restriction maps. Heteroduplex analysis of these eight cloned DNAs with pseudo beta-actin DNA and partial DNA sequencing are being conducted to identify these clones. 4) Isolation and characterization of two human smooth muscle actin genes. Alteration of gene expression is considered to be directly involved in oncogenic transformation. As one of the gene sources for studying the regulation of gene expression, families of actin genes were isolated from DNA library which was constructed using DNA of the human fibroblasts as described in (3). Screening of the DNA library with pcDd DNA, actin cDNA of Dictyostelium, gave six phages containing actin related DNA sequences. Two of them, termed Ha-315 and Ha-201, were found to contain smooth muscle actin genes by restriction mapping, hybridization with probes specific for the different parts of actin coding sequence obtained from the previously isolated human cardiac muscle actin gene, and finally nucleotide sequencing. Amino acid sequences translated from nucleotide sequences perfectly match with those of smooth muscle actins. Ha-315 was stomach type and Ha-201 was aorta type, since their translated amino terminal sequences are Glu-Glu-Glu-

Thr-Cys-Leu...- and Glu-Glu-Glu-Asp-Ser-Thr-Cys-Leu... respectively. The first five N-terminal amino acid sequences are the most variable region among actins and the only region in which stomach and aorta type differ. All the other 370 amino acid sequences are identical between them. The very interesting feature of the coding sequence is the presence of a cystein codon following an initiation methionin codon in both smooth muscle actin genes. Since both actin polypeptides do not have a cystein at their amino-terminal, this cystein must be removed after the translation of the corresponding mRNA, presumably by post-translational processing. A cystein codon at this site also exists in cardiac muscle actin and skeletal muscle actin mRNA; this codon was not found in non-muscle actin genes. The presence of a cystein codon at this position is characteristic of all muscle-type actin genes, at least in the vertebrate actin gene family. All exon-intron boundary sequences were in good agreement with the AG-GT rule. There was no termination codon in the coding sequence. The most interesting finding in the structure of smooth muscle actin genes is the new intron location at the position of amino acid 84/85, which had not been found in any actin gene families of any species. Two controversial concepts have been proposed to explain the evolutionary origin of the introns found in most of the eukaryotic structural genes; one concept is that introns are the ancient features of genes which have been lost during evolution, and the other is that introns are relatively recent insertions. Comparison of the intron location in all actin genes in all species examined indicates that both deletion and insertion of the intron have occurred during the evolution of the actin gene family. 5) Construction of a shuttle vector for studying the regulation of gene expression. As one of the systems for studying the regulation of gene expression, a shuttle vector containing actin genes which we have cloned from human cells was constructed. The shuttle vector (pdBPV) consists of the bovine papilloma virus DNA and pBR322 DNA. This vector replicates as an episome in mammalian cells, and morphologically transforms murine and bovine cells, which makes it easier to select the transformants and segregants. A reproducible transformation assay for the vector was established using C127 mouse recipient cells. A cardiac muscle actin gene consisting of the entire coding sequences and approximately 2000 - 3000 additional base pairs on 3' and 5' ends was excised from its parental clone, pBE 8.0, and transposed into pdBPV vector DNA. This new vector DNA is being introduced into mouse cells to examine actin gene expression.

Significance to Biomedical Research and the Program of the Institute:

The results from this project will provide information on the structure and function of the genes and their products controlling expression of the transformed state, and will also contribute to understanding the mechanism of carcinogenesis. Thus, this project is directly aimed at that aspect of the program of the Institute which seeks to find an essential difference in the biochemical nature between normal and malignant cells and a possible way to prevent and cure cancer.

Proposed Course:

1) To detect, isolate, and characterize the cellular genes and their products controlling the expression of transformed or normal phenotype of human cells by using tranfection and gene cloning techniques; 2) to determine the structure and function of human actin genes, the control mechanism of their expression, and the

role of actin in the maintenance of the transformed or normal phenotype of human cells; 3) to pursue the goals outlined in Objectives and to publish the results obtained.

Publications:

Hamada, H. and Kakunaga, T.: Expression of mutated actin gene associated with malignant transformation. In Cohn, W. D. (Ed.): Progress in Nucleic Acid Research and Molecular Biology. New York, Academic Press. (In Press)

Kakunaga, T.: Molecular mechanisms of chemical carcinogenesis. Cell Eng. 1: 108-118, 1982.

Kakunaga, T., Hamada, H., Leavitt, J., Lo, K.-Y. and Hirakawa, T.: Evidence both for mutational and non-mutational processing in chemically-induced cell transformation. In Cerruti, P. and Harris, C. (Eds.): Molecular Mechanisms of Chemical Carcinogenesis. New York, Alan R. Liss, 1982, pp. 517-529.

Kakunaga, T., Hashimoto, Y. and Sakamoto, T.: Molecular mechanisms of chemical carcinogenesis. Tokyo, Kodansha. (In Press)

Kakunaga, T., Leavitt, J., Hamada, H., and Hirakawa, T.: A point mutation in β-actin gene and neoplastic transformation. In: Vogel, H. J. and Weinstein, I. B. (Eds): Genes and Proteins in Oncogenesis, New York, Academic Press. (In Press)

Leavitt, J., Goldman, D., Merril, C. and Kakunaga, T.: Actin mutations and a human fibroblast model for carcinogenesis. Clin. Chem. 28: 850-860, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04782-13 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Hormones and Breast Tissue Interactions

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

M. R. Green Research Chemist, LMC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to contribute to the understanding of hormone action on metabolic processes in normal breast tissue and to understand the aberrant response to hormones in hyperplastic or neoplastic breast tissue. Phosphorylation-dephosphorylation reactions occur in response to a variety of hormonal stimuli. Using a staining method developed in this laboratory that detects certain phosphoproteins and sialoglycoproteins on gels and tissues, a phosphorylated glycoprotein was found in mouse colostral milk but not in mature milk. Mammary explants from mice prior to parturition were incubated with insulin, hydrocortisone, prolactin, and 32-P inorganic phosphate. In two-dimensional gels, the synthetically labeled tissue protein coincided with the unlabeled stained colostral phosphoprotein. Some qualitative and quantitative differences in colostral proteins obtained from normal and mammary tumor-bearing mice were observed. Analysis of milk has been the principal tool for determining products of human mammary gland synthesis because normal human breast tissue at various stages of development has not been available for labeling studies. Using the staining method, several modified proteins not detectable later in lactation were detected in human colostrum following separation by two-dimensional gel electrophoresis. Human breast tumors express some modified proteins not seen in mature milk, one or more of which might be secreted by the normal gland at an earlier stage of development.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

None

Objectives:

The objective is to gain a clearer understanding of the interaction of hormones with breast tissue. The topics of interest are interaction of hormones such as insulin, estrogens, hydrocortisone, and prolactin with breast tissue, and the metabolic effects of these interactions. Responses by breast tissue in culture to prolactin, carcinogens, and steroids in terms of nucleic acid synthesis and synthesis of macromolecules such as phosphoproteins, sialoglycoproteins, glycosaminoglycans, and proteins are under investigation. Modulation of these responses in rodent tissues following carcinogen treatment or in tissue bearing the mouse mammary tumor virus are investigated. Human milk and pathologic breast specimens from humans are also studied.

Methods Employed:

Methods employed include: culture of mammary tissue explants in vitro using defined medium, isolation and characterization of nucleic acids; separation on agarose-acrylamide gels and on gradients; radioactive tracer techniques; isolation and purification of proteins from milk and explants; measurement of enzymatic activities; polyacrylamide gel electrophoresis; histochemical analysis of gels and tissue sections; autoradiography and binding of hormones to macromolecules and immunochemical procedures.

Major Findings:

In the course of differentiation of the mammary gland from the immature virgin state to the fully mature lactating state, there are changes in the cell population and the kinds of proteins made. Secreted milk is composed primarily of proteins synthesized by the gland but also contains proteins made elsewhere. Electrophoretic analysis revealed a phosphorylated glycoprotein in colostral mouse milk that was not seen later in lactation. To determine whether this protein was synthesized by the gland, mammary explants from mice prior to parturition were incubated in synthetic medium with hormones and 32-Pi. Proteins in fractions of the tissue homogenate were separated by polyacrylamide gel electrophoresis on gels. In both one and two dimensional gels, the 32-Pi labeled tissue proteins coincided with the histochemically stained Ethyl Stains-all blue staining phosphoproteins present in colostral milk. Proteins from colostral milk and sera from normal and mammary tumor bearing mice were compared. Some proteins in milk of tumor bearing mice were seen to differ from those in milk of normal mice in quantity and charge. Unlike mammary gland tissue from animals, normal human gland in various stages of differentiation has not been available for study. To determine products of normal mammary gland synthesis, analysis of milk was utilized.

Colostral milk is more concentrated and contains proteins not seen later in lactation. Colostral milk 1, 2, and 4 days after parturition and mature milk were separated by one and two dimensional electrophoresis on polyacrylamide gels and examined for the presence of phosphoproteins and sialic acid containing proteins. The histochemical method was used because it is not possible to label human milk with isotopes in vivo. Several modified proteins not detectable in mature milk were detected in colostrum.

Significance to Biomedical Research and the Program of the Institute:

Modified milk proteins first detected on gels by means of a histochemical method developed in this laboratory have now been shown to be synthesized in response to hormones by mouse mammary explants in culture. The co-migration of unlabeled stainable phosphoproteins with a small amount of isotopically labeled tissue proteins on two-dimensional gels suggests that these proteins are the same. The staining method is useful as a pre-screen for the presence of phosphorus and/or sialic acid groups on proteins. Because these modifying groups are observed prior to purification of the protein, losses that may occur during isolation procedures may be monitored and prevented. By judicious use of chemical and enzymatic procedures, the nature of the chemical bond linking the substituent to the protein may be determined.

During the carcinogenic process various changes in protein composition of a tissue take place. Among these, enhanced phosphorylation of proteins as a result of retrovirus infection, and increased sialylation of proteins concomitant with enhanced metastatic potential of cells have been observed. Such changes in fluids and tissues from humans may be more easily monitored with the use of this staining method.

Proposed Course:

Post-translational modifications of proteins that take place in mammary gland during development and carcinogenesis will be studied. The response of rodent mammary gland explants to hormones will be studied by labeling with isotopes in vitro; modification of human proteins will be studied by special staining procedures.

Responses to hormones in the normal mammary gland of mice will be compared to responses in explants from mice bearing the mouse mammary tumor virus to evaluate changes that occur as a result of virus-host cell interaction. Synthesis of phosphoproteins in mammary gland explants from normal and MMTV-bearing mice will be studied to determine whether there are changes in enzymatic or receptor functions in the virus bearing versus the normal gland.

The nature of the modifying groups in the human colostral proteins will be assessed by use of enzymatic and chemical procedures followed by electrophoretic and staining analysis on gels. Antisera to milk or serum proteins will be used in an attempt to identify some of the proteins. Studies of modified proteins in milks at various stages of development in the human may reveal the source of certain proteins made by tumor tissue. The dye will be used as a pre-screen for

the presence of sialoglycoproteins in sections of tumor tissue. The presence of proteins known to contain high levels of sialic acid will then be tested by immunologic methods. Thus, it may be possible, in some patients, to find a marker suitable for monitoring the recurrence of metastatic disease by radioimmunoassay of the marker in serum.

Publications

Green, M. R.: Detection in milk of a 60,000 M_r mouse and a 68,000 M_r human phosphorylated glycoprotein by histochemical analysis on polyacrylamide gels. J. Histochem. Cytochem. 31: 709-716, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04785-13 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Repair Studies on Normal Human and Mouse, Tumor and Transformed Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

R.S. Day, III Research Physical Scientist, LMC, NCI

COOPERATING UNITS *(if any)*

D. Scudiero, Chemical Carcinogenesis Program, Basic Research Program, Litton Bionetics, Inc., Frederick, MD

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	3.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The study of human cells defective in repairing damaged DNA was extended, with the rationale that DNA-repair deficient cells are more susceptible to the adverse effects of carcinogens (cell killing, mutagenesis, sister chromatid exchange, and malignant transformation) than their repair-proficient counterparts. A group of human tumor (19) and SV40-transformed (7) strains deficient in the repair of O-six-methylguanine (O-6-MeG, a modified DNA base made by certain methylating agents) was identified earlier in this project. Such strains were called Mer (-). Like SV40 and Rous sarcoma virus, adenovirus was found to produce Mer (-) strains. An activity was present in 8 Mer(+) strains but not in 11 Mer(-) strains that demethylated O-6-MeG in DNA, thereby producing guanine and repairing this damaged base. After the reaction, the methyl group was found bound to a 22,000 M.W. protein, presumably the O-6-MeG-DNA methyltransferase. Using methyl group transfer as a stoichiometric measure, Mer(+) strains were judged to contain on the average 60,000 methyltransferase molecules per cell. A group of 5 Mer(+) cell strains, found sensitive to cell killing by MNNG was termed Mer(+) Rem(-). This group was found able to repair approximately one-third as much O-six-methylguanine as Mer(+) Rem(+) cell strains. To provide a basis for somatic cell genetic studies of DNA repair, hybrids between cells having the various DNA repair phenotypes were produced after preparing cells with selectable markers by transfection with plasmids. Primary fibroblasts and established cell lines from various strains of mice show differences in their ability to respond to agents that produce O-6-MeG. Finally, studies with hydroxyurea indicate that human tumor cells and normal human fibroblasts depend upon ribonucleotide reductase(s) to different extents when repairing UV-damaged adenovirus.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

D.B. Yarosh	Staff Fellow	LMC, NCI
T. Yagi	Visiting Fellow	LMC, NCI

Objectives:

To learn more about DNA repair mechanisms in human and other mammalian cells and about their role in carcinogenesis. In particular, to determine the nature of the DNA repair defects both in human tumor cells and in cells from persons who are genetically predisposed to cancer. In addition, to use human cell strains with characterized defects to study the mechanisms of action of carcinogens, or suspect carcinogens, chemotherapeutic agents, and other chemicals, in altering either DNA or the repair of damaged DNA.

Methods Employed:

1. Plaque assay: An adenovirus-host cell reactivation assay, developed previously in this project, was used to quantify the deleterious effects of various chemical and physical treatments on the ability of the virus to initiate and sustain infection. The method involves establishing monolayer cell cultures which are infected with treated or non-treated adenovirus. The infected cells are then incubated 12-14 days with feeding by means of periodic overlaying with a nutrient agar. Non-treated virions or those treated ones which have been "reactivated" by cellular repair mechanisms form countable plaques of dead, lysed cells.

2. Cellular extracts and partially purified fractions were assayed for O^6-methylguanine-DNA methyltransferase repair activity using as substrate either DNA methylated by [^{3}H-methyl]-methylnitrosourea or a synthetic double-stranded polymer (kindly supplied by Dr. S. Mitra, Oak Ridge National Laboratory) containing [^{3}H-guanine]-O^6-methylguanine. Reaction mixtures were incubated at 37°C, then heated in acid to remove purines, which were separated by high pressure liquid chromatography and quantified by liquid scintillation counting. A reduction of the O^6-methylguanine to guanine ratio or a conversion of O^6-methylguanine to guanine was interpreted to mean that repair of O^6-methylguanine had occurred.

3. Discontinuous SDS polyacrylamide gel electrophoresis was used to measure the size of O^6-methylguanine-DNA methyltransferase. After cellular extracts had been incubated with a substrate (prepared by treating DNA with [^{3}H-methyl]-MNU), and the labeled methyl group of O^6-methylguanine was transferred to the O^6-methylguanine-DNA methyltransferase, the proteins were precipitated, resuspended in buffer, boiled with SDS, and subjected to electrophoresis.

4. DNA repair synthesis was measured by incorporation of ^{3}H-thymidine into TCA-insoluble-double stranded DNA either according to the method of Scudiero et al. (BND-cellulose assay) or according to the method of Pettijohn and Hanawalt (CsCl/BrdUrd assay).

5. The survival of cells treated with ultraviolet light or chemicals was assayed by growth of the cells into colonies of at least 50 cells.

6. Plasmid production and purification together with transfer to E. coli and human cells, and assays for their presence followed published protocols.

7. Sister chromatid exchanges were assayed essentially by the method of Latt as modified by Perry and Wolfe. MNNG-treated and non-treated cultured cells are grown for two rounds of DNA synthesis in the presence of BrdUrd, a manipulation that labels (with BrdUrd) one chromatid of each chromosome in both DNA strands and the other chromatid in one DNA strand. After dye photosensitized alteration of the DNA that contains BrdUrd, DNA without BrdUrd is more heavily stainable by Giemsa, a fact that affords differential visualization of the two sister chromatids of each chromosome. Any somatic recombination that occurred between sister chromatids is visualized as a reciprocal exchange of heavily for lightly Giemsa-stained material.

Major Findings:

A major part of this year's research concerned human tumor cell strains defective in the repair of alkylation damage in DNA. One such group of 19 such cell strains, called Mer^-, which was identified earlier in this project on the basis of relative inability to support the growth of MNNG (N-methyl-N'-nitro-N-nitroso-guanidine) treated human adenovirus 5, is also characterized by inability to repair O^6-methylguanine produced in DNA by MNNG. Mer^- tumor cells are extremely sensitive to MNNG-produced reduction in colony forming ability and to the MNNG-production of sister chromatid exchanges. Further, in collaboration with Dr. Dominic Scudiero, FCRF, NCI, we have characterized human tumor cell strains in terms of their post-MNNG colony forming ability. The range of survival given by 13 normal human fibroblast strains defines the Rem^+ phenotype. Strains whose post-MNNG survival indicates greater sensitivity than the normal range are Rem^-. While sorting strains according to their Mer and Rem phenotypes, we identified a group of five strains which are Mer^+ Rem^-, i.e., repair O^6-methylguanine and MNNG-treated adenoviruses but are sensitive to MNNG produced inactivation of colony forming ability. With respect to MNNG-treated Mer^+ Rem^+ strains, MNNG-treated Mer^+ Rem^- strains are observed to 1) have a greater sensitivity to sister-chromatid exchanges, 2) have less ability to support the growth of MNNG-treated adenoviruses, 3) are more slow in restoring supercoiled structure to their nucleoids, 4) show higher levels of DNA repair synthesis, 5) show less ability to remove O^6-methylguanine from their DNA after a further low-level treatment of the cells with an [^{3}H-methyl]-MNNG "tester dose" (see below). Thus it appears that, for as yet undetermined reasons, the Mer^+ Rem^- strains behave as if they have one-third as much O^6-methylguanine-DNA methyltransferase as Mer^+ Rem^+ strains do.

1. The Mer^- phenotype occurs in transformed, not normal human cell strains. We have now studied 40 non-transformed human fibroblast strains and find all to be Mer^+. Of 93 human tumor strains studied 19 are Mer^-; 74 are Mer^+. Viral transformation of Mer^+ human cells produces cell strains with the Mer^- phenotype in the case of SV40 (7 of 11 SV40 transformed human fibroblast strains were Mer^-), Rous sarcoma virus, and adenovirus. Epstein-Barr virus is also implicated as causing such a conversion.

2. When MNNG-damaged adenoviruses were used to infect Mer$^-$ cell strains, 2.2+0.4 O^6-methylguanine residues were present per virion at the dose that introduced one lethal hit per virion in the viral population. This may mean that an O^6-methylguanine in one viral DNA strand is lethal whereas, if in the other DNA strand, it is not.

3. Extracts of Mer$^-$ and Mer$^+$ cells were assayed for ability to alter O^6-methylguanine. Extracts from eight Mer$^+$ cell strains contained an activity which appeared to remove the methyl group from O^6-methylguanine. As determined first by Lindahl and coworkers, the same is true for E. coli; others have shown similar results with rat, mouse, and human tissue. The activities from one of the Mer$^+$ strains and from human placenta were partially purified by DNA-cellulose chromatography. By contrast, none of the extracts of eight Mer$^-$ cell strains repaired O^6-methylguanine, an indication that the fundamental difference between Mer$^+$ and Mer$^-$ strains is likely a difference in repair protein levels. A similar activity was detected in extracts of NIH3T3 cells.

By allowing extracts to react with a substrate containing [^{3}H-methyl]-O^6methylguanine, the labeled methyl group was transferred to a methyl acceptor protein - presumably the O^6-methylguanine-DNA methyltransferase - as in the case of E. coli. After SDS - polyacrylamide gel electrophoresis, the molecular weight of the protein in Mer$^+$ tumor strains, was determined to be 22,000. This was also true for proteins prepared from certain Mer$^+$ Rem$^+$ and Mer$^+$ Rem$^-$ strains as well as human placenta, and NIH3T3 mouse cells.

4. Mer$^+$ strains, treated with MNNG to deplete O^6-MeG-DNA methyltransferase activity, were incubated for 0, 1, or 2 days. During this time the activity was restored to levels about 1.5-fold greater than that present originally. Blocking either protein synthesis or RNA synthesis during this period blocked the restoration of methyltransferase activity, indicating that the normal restoration process requires mRNA and protein synthesis.

5. To prepare cell strains for studies of the somatic cell genetics of the Mer and Rem phenotypes, cell strains having the Mer$^+$ Rem$^+$, Mer$^+$ Rem$^-$ and Mer$^-$ Rem$^-$ phenotypes were transfected with either of two plasmids. One confers resistance to G418, a neomycin derivative; the other to mycophenolic acid. Resistant transfectants were selected. Hybrids between various strains were produced by fusing a strain resistant to G418 with a strain resistant to mycophenolic acid and selecting cells resistant to both. No Mer$^+$ strains have been detected among hybrids of one Mer$^-$ strain with another, but in a case of fusing a Mer$^-$ Rem$^-$ strain with a Mer$^+$ Rem$^-$ strain, a Mer$^+$ Rem$^+$ strain was obtained. Work continues in this area, and the use of transfection technology is being used to transfer the Mer$^+$ gene into Mer$^-$ cells as a first step to cloning the Mer$^+$ gene as well.

6. Cells having the Mer$^+$ Rem$^+$, Mer$^+$ Rem$^-$, and Mer$^-$ Rem$^-$ phenotypes were assayed for remaining colony forming ability after treatment with MNNG (22 strains), MMS (22 strains), ENNG (13 strains), BCNU (20 strains) and HECNU (13 strains). The rationale for such a study was that if Mer$^-$ Rem$^-$ cells are sensitive to killing by MNNG because O^6-MeG (or a DNA alteration that undergoes a similar repair process) is lethal, then they should show different behavior after treatment with an agent that 1) produces less O^6-MeG (MMS); 2) produces O^6-ethylguanine,

not O^6-methylguanine (ENNG); 3) is an MNNG-like chemotherapeutic agent whose non-alkylating decomposition product is reported to have biological activity (BCNU) or whose non-alkylating decomposition product should have less biological activity (HECNU).

The results are consistent with the conclusions that MMS produces much cell killing due to damage other than O^6-methylguanine; that ENNG produces lethal lesions, presumably O^6-ethylguanine, that are not as repairable by the O^6-methylguanine-DNA methyltransferase as is O^6-methylguanine; that the non-alkylating decomposition product of BCNU (likely chloroethyl-isocyanate) is indeed active biologically and that the analogous decomposition product of HECNU is less active biologically. Another conclusion drawn is that if BCNU is effective in killing the tumors sensitive to it because those tumors are largely composed of Mer^- Rem^- cells, then HECNU should be a better chemotherapeutic agent because it gives a larger differential kill of Mer^- Rem^- cells and Mer^+ Rem ($^+$ or $^-$) cells.

7. Cell strains prepared from mice were assayed both for their post-MNNG survival and post-MNNG sister chromatid exchange frequency. Primary strains or established lines prepared from NIH3T3 cells were quite resistant in these assays, as were primary strains prepared from C57BL mice. However a C57BL established line was very much more sensitive in both assays, showing that cell culture sometimes can produce phenotypic alterations, perhaps in a strain-specific way.

8. Studies with DNA repair inhibitors using the adenovirus 5 host cell reactivation system were continued. Ultraviolet radiation causes a quantifiable, dose-dependent, decrease in the ability of the virus to grow in cells. The inclusion of repair inhibitors such as caffeine, hydroxyurea, or aphidicolin (an inhibitor of alpha-type DNA polymerases) causes ultraviolet-irradiated, but not non-treated virus, to form fewer plaques. This effect is likely due to inhibition of repair processes that act upon the irradiated virus. There are several new findings. 1) When the four deoxyribonucleosides are supplied to cells infected by UV-irradiated virus, the inhibition by hydroxyurea of repair of these viruses is reversed. This is strong evidence that hydroxyurea does indeed block repair by blocking ribonucleotide reductase. 2) While hydroxyurea at 1 mM is as effective as 10 mM in blocking repair of UV-irradiated virus in tumor cells, the maximum repair blockage seen is less than that due to a 10mM treatment of each of three normal human fibroblast strains. In the one human fibroblast strain we have studied closely, 1 mM hydroxyurea has little effect on inhibition of repair, and 30 mM has a greater effect than 10 mM, indicating that the mechanisms by which hydroxyurea supresses repair in human fibroblasts and tumor cells may be different. 3) Even though 1.25-2.5 mM hydroxyurea treatment of virus-infected cells produces maximal supression of the UV damaged virus, the addition of aphidicolin along with the hydroxyurea further depresses repair of the virus, showing that the two agents likely act by inhibition of different parts of the repair pathway(s).

Significance to Biomedical Research and the Program of the Institute:

The results of this project suggest the possibility that a fraction, one-fifth, of all human tumors is composed of repair-defective cells. Certain bifunctional alkylating agents (BCNU, CCNU) are known to be effective against some individual

human tumors and to be relatively ineffective against others, also indicating the possibility that the molecular basis for the success of alkylation chemotherapy may be specific to a tumor or a group of tumors. Our studies of the mechanisms by which repair occurs and upon the inhibition of such repair are designed, in part, to understand ways by which tumors might be more successfully treated and ways by which tumors might arise. Physical, chemical, and viral carcinogens are all known to alter the structural integrity of the cellular genetic apparatus. An evaluation of the role of DNA repair and/or related mechanisms in conferring resistance or susceptibility to mutagenesis and carcinogenesis is an important facet in any overall program having as its goal the understanding of the molecular pathways which, when perturbed, give rise to carcinogenesis in humans. It is the long-range goal of this project to determine whether or not the elucidation of repair mechanisms is important to the understanding of carcinogenesis. It is expected that an understanding of human repair mechanisms, in general, will benefit many areas of biomedical research.

Proposed Course:

1) To continue surveying human strains for Mer^- strains using the MNNG-treated adenovirus plaque assay and the O^6-methylguanine DNA methyltransferase activity assay; 2) to clone the O^6-methyltransferase gene by linking it to a selectable gene and transferring it into Mer^- cells. The source of the O^6-MeG DNA methyltransferase gene will be genomic DNA from a Mer^+ cell and a human fibroblast mRNA library cloned in phage 2; 3) to purify the O^6-methylguanine DNA methyltransferase from human tissue, including placenta and liver; and 4) to study the DNA repair defect in Mer^+Rem^- cells by investigating their survival after treatment with closely related carcinogens, and by measuring their repair of damaged DNA bases.

Publications

Day, R. S., III and Ziolkowski, C. H. J.: Differential effects of hydroxyurea on the survival of UV- and MNNG-treated adenovirus 5. Mutat. Res. 94: 257-262, 1982.

Day, R. S., III, and Ziolkowski, C. H. J.: Human adenoviruses as indicators or inhibition of DNA repair. In Collins, A. C. S. and Johnson, R. (Eds): Inhibition of DNA Repair. Marcel Dekker (in press).

Day, R. S., III and Ziolkowski, C. H. J.: Induced reversion using human adenovirus. In Kilbey et al (Eds.): Handbook of Mutagenecity Test Procedures. Amsterdam, Elsevier/North-Holland Biomedical Press, Vol. 2 (in press).

Yarosh, D. B., Foote, R. S. , Mitra S. and Day, R. S., III: Repair of O^6-methylguanine in DNA by demethylation is lacking in Mer^- human tumor cell strains. Carcinogenesis 4: 199-205, 1983.

Yarosh, D. B., Mattern, M. R., Scudiero, D. A. and Day, R. S., III: The Mer phenotype: Human tumor cell strains defective in repair of alkylation damage. In Castellani, A. (Ed.): The Assessment of Cancer Risk from Physical and Chemical Agents. New York, Academic Press (in press).

Yarosh, D. B., Rice, M., Ziolkowski, C. H. J., Day, R. S., III, Scudiero, D. A., Foote, R. S. and Mitra, S.: O^6-methylguanine methyltransferase in human tumor cells. In Friedberg, E. C. and Bridges, B. A. (Eds.): Cellular Repair of DNA Damage. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05001-06 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of DNA Damage, Repair and Mutational Process in Chemical Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Takeo Kakunaga Head, Cell Genetics Section, LMC, NCI

COOPERATING UNITS *(if any)*

Northrop Environmental Sciences, Inc., Research Triangle Park, NC

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The possible involvement of mutation in the transformation process is being examined by two approaches. First, the pattern of stable alteration of the structure of cellular genes induced by treatment with chemical carcinogens was investigated by comparing the DNA sequences of four actin genes isolated from a human fibroblast line transformed by 4-nitro-quinoline-1-oxide (4NQO) with those of normal genes. The results suggest that the most frequent alterations of DNA sequence, either induced by 4NQO treatment or arising spontaneously, and base substitution, one point mutation and, less frequently, one-base insertion or deletion. Secondly, the possibility that chemically-induced cell transformation results in genetic instability which increases the probability of neoplastic progression, was tested by comparing the mutation rates of normal diploid human fibroblasts and a chemically transformed human fibroblast line. When two genetic loci, i.e., the hypoxanthine-guanine phosphoribosyl transferase locus, a X-linked recessive locus, and the Na^+-K^+ ATPase locus, an autosomal codominant locus, were used, the mutation rates of the normal and transformed cells did not differ significantly. Thus, it was concluded that increased mutation rates do not necessarily occur following chemical transformation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than Principal Investigator) engaged on this Project:

Hisao Ueyama Visiting Fellow LMC, NCI

Objectives:

To study cellular and molecular mechanisms of cell transformation by chemical carcinogens, with special attention to the role of DNA damage, its repair and mutational events in this process. Current interests are; 1) to determine the pattern of stable structural changes in cellular genes induced by treatment of the cultured cells with chemical carcinogens, by determining and comparing with those of corresponding normal genes, the DNA sequences of four actin genes isolated from a human fibroblast line transformed by 4-nitro-quinoline-1-oxide (4NQO) and; 2) to examine the possibility that chemically-induced transformation results in genetic instability which increases the probability of neoplastic progression.

Methods Employed:

Cell culture, DNA cloning techniques, DNA sequencing, amino acid sequencing, restriction mapping, molecular hybridization, gel electrophoresis, autoradiographs, Southern blot, nick-translation, column chromatography and somatic mutation assay.

Major Findings:

1) The pattern of stable alteration of gene structures induced by chemical carcinogens. The primary structures of four actin genes which were isolated from a human fibroblast line transformed by a single treatment with 4-nitro-quinoline-1-oxide (4NQO) were compared to normal actin genes. First, in the aorta type smooth muscle actin gene isolated from the transformed cells, one point mutation (GC to TA, transversion mutation) was found, which corresponded to substitution of glycine by valine. The smooth muscle actin gene is not expressed in the transformed cells and thus has not been exposed to the selection for survival of the genes after 4NQO-treatment. No other missense mutation was found in the coding sequences. Secondly, the stomach type smooth muscle actin gene, which is also dormant in the parent transformed cells, did not show any missense mutation in its coding sequences. Third, the pseudo beta-actin gene, which has not been expressed for many generations, thus containing numerous structural changes accumulated in germ cells, has many mutations, predominantly with single base substitution point mutation. Single base insertion and deletion mutations were frequently found. Large insertions or deletions were not found. Fourth, one mutation, corresponding to a single base substitution point mutation, was found at the level of the amino acid sequences of beta-actin. Expression of beta-actin is essential for survival of cells. Neither gene rearrangement nor polymorphism were detected in the four actin genes examined. These results suggest that the most frequent type of alteration of DNA sequence, induced by 4NQO-treatment and spontaneous, is a single base substitution point mutation and, less frequently, a single base insertion or deletion. 2) Comparison of mutation rates between

normal and transformed cells. The possibility that chemically-induced cell transformation results in genetic instability, which increases the probability of neoplastic progression, was tested by determining and comparing the mutation rate of normal diploid human skin fibroblasts (KD) and a chemically induced, transformed line (HuT-11A) derived from KD cells. The two genetic loci used in this study were the hypoxanthine-guanine phosphoribosyl transferase locus, an X-linked recessive locus, and the Na^+K^+adenosine triphosphatase (ATPase) locus, an autosomal codominant locus. Hypoxanthine-guanine phosphoribosyl transferase mutants were selected by resistance to a 10-ug/ml dose of 6-thioguanine, and Na^+-K^+ ATPase mutants were selected by resistance to 10^{-7} M ouabain. The growth conditions used permitted cloning efficiencies of 70 to 85% and population-doubling times of 16 to 17 hours with both normal and neoplastic human cells. Mutation rates were determined by Luria-Delbruck fluctuation analysis. The hypoxanthine-guanine phosphoribosyl transferase mutation rates of KD (1.6 to 2.1 X 10^{-6}/cell/generation) and Hut-11A (1.0 to 1.8 X 10^{-6}/cell/generation) cells were not different and compared favorably with previously reported rates. The Na^+-K^+ ATPase mutation rates of KD (3.8 to 8.5 X 10^{-7}/cell/generation) and HuT-11A (6-13 X 10^{-7}/ cell/generation) cells were also similar. The observed Na^+-K^+ ATPase mutation rates are 5- to 25-fold higher than reported previously. Use of improved growth conditions allowed for increased recovery of ouabain-resistant mutants. In conclusion, increased mutation rates do not appear to be a necessary factor in carcinogen-induced transformation of human cells.

Significance to Biomedical Research and the Program of the Institute:

The results from this project will provide information about the mechanism of cell transformation and, therefore, clues to develop methods to prevent and cure cancer.

Proposed Course:

Activation of pre- or pro-oncogenes will be attempted in vitro and in vivo, using chemical carcinogens and mutagens and the DNA transfection method. Elucidation of the kinds of structural alterations of the oncogenes which lead to their activation. The goals outlined in Objectives will be pursued.

Publications:

Elmore, E., Kakunaga, T. and Barrett, J. C.: Comparison of spontaneous mutation rates of normal and chemically transformed human skin fibroblasts. Cancer Res. 43: 1650-1655, 1983.

Hamada, H., Petrino, M. G. and Kakunaga, T.: Molecular structure and evolutionary origin of human cardiac muscle actin gene. Proc. Natl. Acad. Sci. USA 79: 5901-5905, 1982.

Kakunaga, T.: Involvement of a point mutation in the neoplastic transformation of human fibroblasts. Fed. Proc. (In Press)

Lo, Ko-Yu and Kakunaga, T.: Similarities in formation and removal of DNA adducts in Benzo[a]pyrene-treated BALB/3T3 variant cells with different induced transformation frequencies. Cancer Res. 42: 2644-2650, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05086-05 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Monoclonal Antibodies to Human Carcinogen Metabolizing Enzymes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. S. Park Senior Staff Fellow, LMC, NCI

COOPERATING UNITS *(if any)*

F. P. Guengerich, Vanderbilt University School of Medicine, Nashville, Tennessee

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Benzo(a)pyrene (BP) may be metabolized to nontoxic products or to the carcinogen, BP-7,8 diol-9, 10-epoxide. The direction of BP metabolism depends on the presence of specific forms of enzymes in individuals exposed to chemical carcinogens. Monoclonal antibodies (MAbs) are specific probes for particular antigenic determinants and are useful tools for identification of particular isoenzymes. We prepared MAbs to cytochrome P-450 and epoxide hydrolase (EH), which are two key components of the system responsible for the metabolism of BP. The MAbs bind to human liver cytochrome P-450 but do not immunoprecipitate or inhibit enzymatic activity; the last observation suggests that the MAbs were raised against an antigenic determinant whose binding does not interfere with the functioning of the catalytic site. The MAbs against EH bind and precipitate only the form which was used as antigen but do not interact with other forms.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

H. V. Gelboin	Chief	LMC, NCI
H. Miller	Biological Lab Technician	LMC, NCI

Objectives:

Benzo(a)pyrene is metabolized by mixed function oxidases to carcinogenic products and nontoxic water soluble compounds. Many cytochrome P-450 forms have been reported but specific forms appear to predominate following treatment with specific inducers. Specific carcinogen-metabolizing enzymes might predominate in individuals who smoke heavily, or who are exposed to carcinogenic environmental chemicals. Microsomal preparations of placenta from women who smoke heavily possess high levels of mixed function oxidases, as do human monocytes, lymphocytes, and lung cell lines induced with benzo(a)anthracene. The objective of this work is to prepare monoclonal antibodies (MAbs) to enzymes involved in BP metabolism, and to use these MAbs as a tool to study enzyme multiplicity and identify the predominant isozymes induced by carcinogens. A long range goal is to identify patterns of cytochromes P-450 that are responsible for differences in carcinogen susceptibility.

Methods Employed:

Balb/c female mice were immunized with cytochrome P-450 derived from human liver and placental microsomes, and with human liver microsomal epoxide hydrolase. The primed spleen cells were isolated and fused with myeloma cells using polyethylene glycol. The hybrid cells were grown in a selective medium (HAT) and screened by radioimmunoassay for hybridomas producing MAbs to their respective immunogens. The effect of MAbs on enzyme activities were measured by fluorometric and HPLC assays. Ouchterlony double immunodiffusion analyses were performed to assay for precipitin reactions.

Major Findings:

Four IgG1 MAbs were obtained to human liver cytochrome P-450. Mouse serum to the cytochrome P-450 precipitatd the antigen but the MAbs did not. Direct inhibition of aryl hydrocarbon hydroxylase activity was not observed but activity could be removed by precipitating the MAb-enzyme complex from solution with protein-A bound to Sepharose 4B. We also observed that the hybridomas gradually lost their capacity to produce MAbs. Since it is not possible to obtain an induced form of human liver cytochrome P-450, cytochrome P-450 from different individuals were used for immunization of mice. Preparation of MAbs with new hybrids is in progress. Four MAbs were obtained against EH. These MAbs bound and precipitated the form of EH that was used as antigen, but not other forms.

Significance to Biomedical Research and the Program of the Institute:

Cytochrome P-450 and epoxide hydrolase are two key enzymes leading to the formation of the carcinogenic metabolite, BP-7,8-diol-9,10-epoxide. Both of these enzymes are present in multiple forms. Preparation of MAbs to specific forms of these enzymes is essential for the study of the activation and regulation of BP metabolism. Provided with a series of MAbs to enzymes in BP metabolism, not only can we better understand chemical carcinogenesis, but we will be able to analyze the relationship between enzyme content and differences in individual susceptibility to cancer. We can thus develop techniques for screening populations to identify individuals with high risk for cancer. The MAbs to carcinogen-metabolizing enzymes would also be useful for the study of chemotherapeutic agents which require activation during cancer treatments.

Proposed Course:

1) Additional MAbs to several of the cytochromes P-450 and EH isoenzymes will be prepared. 2) Competitive radioimmunoassays with the MAbs are being developed. 3) The cross-reactivity of MAbs to human enzymes with enzymes from different species will be assessed.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05109-04 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Changes in Chromatin Structure During Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Michael Bustin Visiting Scientist, LMC, NCI

COOPERATING UNITS *(if any)*

Department of Human Genetics, Tel Aviv University, Israel

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Antibodies to DNA modified by benzo(a)pyrene diol epoxide-1 (BPDE-1) were used to study the influence of chromatin structure on the binding of BPDE-1. The results indicate that the packaging of DNA into its nucleosomal conformation affects the binding of BPDE-1 to DNA. The linker region between core particles is 3-fold more susceptible to modification than the DNA in the core particle conformation. Chromatin regions which are highly enriched in transcribed sequences show an increased susceptibility to carcinogen binding. A gene coding for a 3-methylcholanthrene induced P-450 enzyme has been cloned in pBR322. Various parts of the gene have been subcloned yielding 12 overlapping gene regions.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Leo Einck	Staff Fellow	LMC, NCI
John Fagan	Senior Staff Fellow	LMC, NCI
Michael Seidman	Senior Staff Fellow	LMC, NCI

Objectives:

To study the mechanism of the interaction of chemical carcinogens with the genome. To elucidate changes in the chromatin structure of particular genes associated with carcinogenesis.

Background and Research Strategy:

The interaction of a carcinogen with the genome of a target cell seems to be a critical event leading to transformation. While it is known that certain chemical carcinogens interact with various constituents of the genome, it is not clear how the structure of chromatin and chromosomes influence the binding of the carcinogen to its target. The program involves three different, albeit interrelated, experimental approaches to study the relation between chromatin structure and carcinogen binding.

The first approach involves the use of the SV40 minichromosome as a well-defined model for the eukaryotic chromosome. The major question under consideration is whether all reactive nucleotides are uniformly accessible to the carcinogen. This major question can be subdivided into the following more specific questions: 1) Is there a "hot spot" for carcinogen binding? 2) Is there variability between types of sequences, i.e., coding vs. regulatory, transcribed vs. nontranscribed? 3) Is there variability between coding and noncoding strand accessibility? 4) Is there a difference between nucleosomal or spacer DNA?

The SV40 chromosome is an ideal system to study these questions since it is a well-defined system whose DNA sequence has been established. Immunological approaches are used to detect modified sequences.

The second approach involves the use of polytene chromosomes. These giant chromosomes are a convenient system to study various structure-function relations in the genome. Specific genes can be visualized by conventional light microscopy and the chromosomes can be experimentally manipulated so as to induce and visualize transcriptional activity in defined genetic loci.

While the SV40 system allows investigation at the DNA sequence level, the polytene chromosome system allows investigation of similar questions, but on a more macroscopic level. The following questions will be investigated: 1) Are there "hot spots" for carcinogen binding? 2) Does the carcinogen bind to a few selected genes? 3) Does a change in the macromolecular structure of a genetic locus affect

the binding? 4) Is there a difference in the accessibility of the carcinogen between transcribed, condensed and uncondensed regions of the chromosome? Immunological approaches are used to visualize the location of the carcinogen on chromosomes. The third approach involves studies on the gene structure of a P-450 gene which is induced in rats after treatment with 3-methylcholanthrene. Possible changes in the chromatin structure of this gene resulting from the administration of the carcingen to rats will be examined.

Methods Employed:

SV40 propagation, restriction analysis of DNA, polytene chromosomes, antibody preparation, immunoblotting, immunofluorescence, chromatin isolation, gene cloning, radioactive labelling of nucleic acid, chromatin digestion and analysis.

Major Findings:

The first two phases of this project have been completed (see Z01O5109-03 LMC). The findings can be summarized as follows: 1) The packaging of DNA into a nucleosome structure affects the binding of the carcinogen to the genome. The DNA found in the linker region between core particles is three-fold more susceptible to modification by benzo(a)pyrene than the DNA in the core particle conformation. 2) In SV40 there is no particular DNA region which is specifically modified with this carcinogen. 3) The origin of replication of SV40 is in a non-nucleosomal conformation. Yet, it is less susceptible to modification than the average nonnucleosomal linker DNA between two-core particles. Thus, certain features present in the origin of replication protect the DNA from carcinogen interaction. Most probably these are specific proteins associated with that DNA region. 4) In polytene chromosomes there are specific loci which display enhanced binding of the carcinogen. These regions are associated with high transcriptional activity.

The third approach, namely analysis of the chromatin structure of a P-450 gene is now in progress. A particular P-450 gene has been previously selected from a rat library (Report no. Z01CP05196-02 LMC) and cloned. This gene has now also been cloned in pBR322. In addition, various regions of the gene have also been subcloned in pBR322. The gene has been subdivided into 12 overlapping subclones. Using these subclones, we found that the intervening sequences of the gene contain at least two distinct classes of repetitive DNA. Furthermore, the gene codes for two different messages. The chromatin structure of various regions of the gene in control and in carcinogen treated mice is studied using these clones.

Significance to Biomedical Research and the Program of the Institute:

Understanding the processes involved in the interaction of chemical carcinogens with the genome of a target cell may help elucidate various aspects of the mechanism of carcinogenesis. The approaches used may be generally applicable to studies on the damage of cells induced by carcinogens and its subsequent repair.

Proposed Course:

Studies aimed at accomplishing the goals of this project will be continued using the experimental approaches described above. In the next year, the research will center on elucidation of the chromatin structure of the P-450 gene described.

Publications:

Seidman, M., Mizusawa, H., Slor, H. and Bustin, M.: Immunological detection of carcinogen modified DNA fragments after in vivo modification of cellular and viral chromatin. Cancer Res. 43: 743, 1983.

Seidman, M., Slor, H. and Bustin, M.: The binding of a carcinogen to the nucleosomal and non-nucleosomal regions of SV40 chromosome in vivo. J. Biol. Chem. 258: 5215-5220, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05125-03 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Preparation of Monoclonal Antibodies to Rat Liver Cytochromes P-450

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. S. Park Senior Staff Fellow, LMC, NCI

COOPERATING UNITS *(if any)* F. P. Guengerich, Vanderbilt Univ. School of Med., Nashville, TN; J. B. Schenkman, Univ. of Connecticut, Farmington, CT; J. B. Stegeman, Woods Hole Oceanographic Institute, Woods Hole, MA., E. S. Vesell, PA State Univ., Hershey, PA., S. S. Thorgeirsson, NCI, J. R. Gillette, NHLBI

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cytochrome P-450 plays a key role in the metabolism, activation, and detoxification of drugs, endogenous steroids, xenobiotics, and environmental carcinogens. Cytochromes P-450 were purified from the livers of rats treated with phenobarbital (PB-P-450), 3-methylcholanthrene (MC-P-450), -naphthoflavone (BNF-P-450), and pregnenolone 16- -carbonitril (PCN-P-450); monoclonal antibody-producing hybridomas were prepared to these enzymes. Among twelve hybrid clones producing MAbs active toward PB-P-450, seven were IgG1 and five IgM. Three classes of MAbs were produced: One class bound but neither precipitated the PB-P-450 nor inhibited its aryl hydrocarbon hydroxylase (AHH) activity; a second class bound and immunoprecipitated, but did not inhibit enzyme activity; a third class comprising a single clone, MAb 2-66-3, bound, immunoprecipitated and completely inhibited the AHH of PB-P-450. MAb 2-66-3 did not inhibit the AHH activities of MC-P-450, BNF-P-450, or PCN-P-450. The MAb 2-66-3 inhibited the AHH, ethoxycoumarin deethylase, and benzphetamine demethylase of liver microsomes from PB-treated rats, but did not inhibit these activities in microsomes from control, BNF, or MC-treated rats. The MAb 2-66-3 showed high cross-reactivity in binding, immunoprecipitation, and inhibition of enzyme activity of PB-induced cytochrome P-450 from rabbit liver. Monoclonal antibodies to different cytochromes P-450 will be extraordinarily useful for a variety of studies, including phenotyping, genetic analysis, and purification of cytochromes P-450.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

H. V. Gelboin Chief LMC, NCI

Objectives:

Benzo(a)pyrene (BP) is activated by aryl hydrocarbon hydroxylase (AHH) and epoxide hydrolase, both of which exist in multiple forms. Metabolism of BP by these enzymes leads to a number of products: to the formation of nontoxic, water soluble, excretable conjugates, and/or to the formation of active carcinogenic forms. Exposure of individuals to environmental chemical carcinogens influences metabolism through induction of specific isoenzymes. We previously observed that monoclonal antibodies (MAbs) to 3-methylcholanthrene-induced rat liver microsomal cytochrome P-450 (MC-P-450) were cross-reactive with placental microsomes of women who smoked heavily. We used these MAbs to identify and characterize human carcinogen-metabolizing enzymes. This report describes similar experiments with MAbs to phenobarbital-treated rat liver microsomal cytochrome P-450 (PB-P-450).

Methods Employed:

Balb/c female mice were immunized with purified cytochrome P-450 of rats which were treated with phenobarbital or other inducers. The primed spleen cells were isolated and fused with myeloma cells, using polyethylene glycol. The hybrid cells were grown in a selective medium (HAT) and screened by radioimmunoassay to identify hybridomas producing MAbs to PB-P-450. The effect of MAbs on enzymatic activity was measured both fluorometrically and with HPLC. Ouchterlony double immunodiffusion analysis was also carried out for immunoprecipitin reactions.

Major Findings:

1) Twelve hybridomas producing MAbs were obtained: seven were IgG1 and five were IgM. 2) Based on the interaction with PB-P-450, these MAbs can be classified in three groups: binding but not precipitating; binding, precipitating but not inhibiting; binding, precipitating, and inhibiting. MAb 2-66-3 bound, precipitated, and completely inhibited the AHH activity of PB-P-450 but did not inhibit the AHH activities of cytochrome P-450 of rats treated with 3-methylcholanthrene (MC-P-450), -naphtoflavone (BNF-P-450) or pregnenolone-16- -carbonitril (PCN-P-450). 3) The MAb 2-66-3 inhibited the AHH, ethoxycoumarin deethylase, and benzphetamine demethylase of liver microsomes from PB-treated rats by 22-38%. However, this MAb did not inhibit these activities in microsomes from control, BNF, or MC-treated rats, and had no effect on ethylmorphine demethylase. 4) MAb 2-66-3 also inhibited the formation of BP metabolites by PB-P-450 by more than 85%. 5) The MAb 2-66-3 showed high cross reactivity in binding, immunoprecipitation, and inhibition of enzyme activity of PB-P-450 from rabbit liver.

Significance to Biomedical Research and the Program of the Institute:

The various cytochromes P-450 display stereoselectivity for both substrate and product formation. Therefore, the balance of BP metabolism between detoxification and carcinogen formation is dependent on the types and amounts of isoenzymes present. Each isozyme of cytochrome P-450 possesses specific antigenic determinants and the MAbs prepared to PB-P-450 are specific to PB-P-450, whereas previously prepared MAbs to MC-P-450 are active toward both MC-P-450 and BNF-P-450. Therefore, antigenically related cytochromes P-450 can be induced by different chemicals. MAbs originally prepared to rat or rabbit enzymes that also react with human cytochrome P-450 would be useful in phenotyping isozyme patterns of individuals and perhaps relating these to individual differences in carcinogen sensitivity. The monoclonal antibody technique is also a powerful new tool for numerous studies on the genetics of these crucial enzymes of carcinogen metabolism.

Proposed Course:

To gain more insight into the catalytic mechanisms of cytochromes P-450, we will examine the influence of different MAbs on the spectrum of metabolites obtained from the action of cytochromes P-450 on various substrates (e.g. 2 aminoacetylfluorene, imipramine, propranolol). We will prepare additional MAbs using a variety of P-450 preparations from rats and other animals. These additional probes for different cytochromes P-450 will make feasible a more extensive characterization of the isoenzymes in various sources, including human tissues.

Publications:

Fagan, J. B., Park, S. S., Pastewka, J. V., Gelboin, H. V. and Guengerich, F. P.: DNA cloning and monoclonal antibodies to cytochrome P-450: regulation and function. In Kato, R. and Sato, R. (Eds.): 5th International Symposium on Microsomes and Drug Oxidations. Tokyo (Wiley-Interscience, New York), Japan Scientific Soc. Press, 1982, pp. 597-604.

Fagan, J. B., Pastewka, J. V., Park, S. S., Guengerich, F. P., and Gelboin, H. V.: Identification and quantitation of a 2.0 kilobase mRNA coding for 3-methylcholanthrene-induced cytochrome P-450 using cloned cytochrome P-450 cDNA. Biochemistry 21: 6574-6580, 1982.

Fujino, T., Park, S. S., West., D. and Gelboin, H. V.: Phenotyping of cytochromes P-450 in human tissues with monoclonal antibodies. Proc. Natl. Acad. Sci. USA 79: 3682-3686, 1982.

Park, S. S., Cha, S. C., Miller, H., Persson, A. V., Coon, M. J., and Gelboin, H. V.: Monoclonal antibodies to rabbit liver cytochrome P-450 LM_2 and cytochrome P-450 LM_4. Mol. Pharmacol. 21: 248-258, 1982.

Park, S. S., Fagan, J. B., Gelboin, H. V., and Guengerich, F. P.: Monoclonal antibodies and the cloning of cytochrome P-450 genes. In Korean Scientists and Engineers Assoc. Summer Symposium. Seoul, Korea, 1982, pp. 265-271.

Park, S. S., Fujino, T., West, D., Guengerich, F. P. and Gelboin, H. V.: Monoclonal antibodies inhibiting enzyme activity of cytochrome P-450 from 3-methylcholanthrene treated rats. Cancer Res. 42: 1798-1808, 1982.

Thorgeirsson, S. S., Sanderson, N., Park, S. S. and Gelboin, H. V.: Inhibition of 2-acetylaminofluorene oxidation by monoclonal antibodies specific to 3-methylcholanthrene induced rat liver cytochrome P-450. Carcinogenesis (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05195-03 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetics and Regulation of Cytochrome P-450 Biosynthesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

John B. Fagan Senior Staff Fellow, LMC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cytochrome P-450 is a central component of the aryl hyrdocarbon hydroxylase (AHH) system. Regulation of AHH enzymatic activity has been carefully studied in vivo, as well as in cell culture using Buffalo rat liver (BRL) cells and other cell types. The accessibility of the BRL cell culture system to well-controlled experimental manipulation makes it ideal for studying the regulation of biosynthesis of the cytochromes P-450 and of other AHH components. There is no direct information on the regulation of synthesis, processing and turnover of AHH system polypeptides, nor is there direct information on the regulation of rates of transcription, processing and degradation of the mRNAs for AHH system enzymes. Our objective is to use the BRL cell culture system to study regulation of the AHH system at these two levels.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project: .

H. V. Gelboin Chief LMC, NCI

Objectives:

1) The primary objective is to use the BRL cell culture system to study regulation of the cytochromes P-450 at the levels of a) synthesis, processing and turnover of P-450 peptides and b) transcription, processing and turnover of P-450 mRNAs.

2) Select clones of BRL cells that are highly inducible for AHH and are of diploid or near diploid karyotype. These cells will be used for all subsequent studies.

3) Characterize the rates of P-450 synthesis in control and polycyclic aromatic hydrocarbon (PAH)-induced BRL cells.

4) Characterize the stability and half-life of P-450 polypeptides in control and PAH-induced BRL cells.

5) Aryl hydrocarbon hydroxylase activity in BRL cells can be induced by a number of different treatments, including culture with PAHs, cycloheximide, cyclic AMP, or butyrate. To understand and compare the underlying mechanism(s) of induction, we will characterize the effects of these treatments on P-450 polypeptide synthesis, processing, stability, and turnover.

6) Compare the extent and time courses of induction of P-450 peptide synthesis with previously reported data on the effects of inducers on AHH enzymatic activity to examine the relationship between these two events. The P-450(s) responsible for AHH activity in BRL cells will be further characterized by determining whether P-450 specific antibodies, which have been studied in other systems, exert inhibitory effects on enzyme activity.

7) Identify different P-450s in the BRL cells using polyclonal and monoclonal antibodies to purified P-450s; compare results with those obtained in other systems.

8) Study processing of newly synthesized P-450 peptides by proteolysis, glycosylation and heme insertion.

9) Objectives 3 through 8 will also be carried out for the regulation of other enzymatic components of the AHH system including epoxide hydratase (EH).

10) Study the regulation of synthesis, processing and degradation of mRNAs for AHH system proteins under various induction conditions. This work will require the use of cloned cDNAs complimentary to the P-450s and other AHH enzymes (see

annual report Z01CP05196-03 LMC). Amounts, sizes and rates of transcription, degradation, and processing of P-450 mRNAs will be examined and compared quantitatively.

Methods Employed:

Standard cell culture, karyotyping, and cloning procedures are used with BRL cells. Rates of peptide synthesis, stability, turnover, and degradation are measured by labeling cells in culture with ^{35}S-methionine (either steady-state or pulse-chase labeling). This is followed by immunoprecipitation of solubilized, ^{35}S-labeled peptides with various monoclonal antibodies described in annual report Z01CP05125-03 LMC. The labeled peptides and their corresponding immunoprecipitates will be analyzed by SDS-polyacrylamide gel electrophoresis. Regulation of synthesis, processing and degradation of AHH system mRNAs will be studied using the procedures outlined in annual report Z01CP05196-03 LMC. BRL variants, lacking various components of the AHH system, will be isolated using two different procedures. Variants which are unable to metabolize PAHs will be identified by fluorescence-activated cell-sorting of cells cultured in the presence of a PAH such as benzo(a)pyrene (BP), whose fluorescence spectrum changes when it is metabolized. AHH-negative variants will also be selected by growth in the presence of a PAH which is relatively nontoxic until it is metabolized via the AHH system.

Major Findings:

1) Culture of BRL cells in the presence of BA for 12-18 hours causes a 4- to 8-fold increase in steady-state labeled peptides which are immunoprecipitated with IgG specific for highly purified MC-type P-450. 2) The rate of synthesis of peptides immunoprecipitable with MC-P-450-specific IgG is linear for about 60 minutes, and this rate is increased in cells induced with BA. 3) Two immunoprecipitable peptides of about 55 and 57 kilodaltons are induced by BA. 4) Tunicamycin, an inhibitor of peptide glycosylation, blocks the accumulation of ^{35}S-labeled peptides immunoprecipitated with MC-P-450 IgG. 5) Messenger RNA has been isolated from control and benzanthracrene-treated BRL cells, and characterized by in vitro translation and immunoprecipitation, and by Northern hybridization.

Significance to Biomedical Research and to the Program of the Institute:

The AHH system is central to the activation and detoxification of carcinogens, to the detoxification of xenobiotics and to drug metabolism. A wealth of evidence indicates that the AHH system is very carefully regulated in vivo. However, the molecular mechanisms of this regulation have not yet been elucidated. The significance of this regulation, especially the induction of specific P-450 species, in determining the response of individuals to specific carcinogens, drugs, and xenobiotics has not been assessed. Accomplishment of the objectives described above should provide new insights into the molecular mechanisms of regulation of the biosynthesis of the enzymatic components of the AHH system. Furthermore, comparisons of the abilities of a series of cell lines lacking various components of the AHH system to convert PAHs to carcinogenic and cytotoxic forms will be useful in evaluating the roles of those components of the

AHH system in rendering PAHs carcinogenic and/or cytotoxic. Accomplishment of these objectives will result in a better understanding of the balance between activation and detoxification of carcinogens, the influence of this system on drug action and other questions in carcinogenesis, toxicology and pharmacology. It also should be noted that the properties of the BRL system make it an extremely useful model system for studying the regulation of eukaryotic gene expression. Thus, this work should not only contribute in a practical way to the areas of carcinogenesis, toxicology and pharmacology, but will also contribute to the basic understanding of eukaryotic gene regulation.

Proposed Course:

Objective 2 has been attained and work is in progress and some preliminary goals have been attained for objectives 3 through 8. In this upcoming research period, these objectives, as well as the other objectives, will be pursued further.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05196-03 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cloning, Structure, and Regulation of the Genes for the Cytochromes P-450

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. B. Fagan Senior Staff Fellow, LMC

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	1.8	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have adopted recombinant DNA and related techniques to study the molecular biology of the cytochromes P-450 (P-450s), a family of enzymes central to the activation and detoxification of carcinogens and to the metabolism of xenobiotics and drugs. Our goal is to understand the structure and regulation of P-450 genes, to elucidate the molecular basis for the multiplicity of the P-450s and for the structural-functional relationships among them, and to determine the extent to which individual susceptibility of humans to cancer depends on the regulation of expression of specific P-450s. This year we have determined the detailed structure of a 3-methylcholanthrene-induced P-450 (MC-P-450) gene whose isolation we reported last year. We have also cloned and characterized cDNAs corresponding in sequence to the two most abundant forms of MC-P-450 mRNA in liver. We have determined the sizes of these two P-450 mRNAs and the peptides for which they code, and have quantitated them in control and induced tissues. We have also used these MC-P-450 cDNAs as hybridization probes to isolate the corresponding new P-450 genes from a library of the rat genome.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

H. V. Gelboin	Chief	LMC, NCI
S. C. Chalberg	Guest Researcher	LMC, NCI
J. Pastewka	Chemist	LMC, NCI

Objectives:

1) The primary, long range objective of this project is to elucidate the structure and regulation of expression of the genes for the cytochromes P-450.

2) Construct, identify and characterize clones carrying cDNA complementary to the mRNAs for the cytochromes P-450 induced by methylcholanthrene, phenobarbital and other inducers.

3) Isolate and characterize the native genes for inducible cytochromes P-450 from recombinant bacteriophage libraries of the rat and other eukaryotic genomes.

4) Study the regulation of P-450 gene expression at the mRNA level and at the gene level. Specific objectives: a) Quantitate intracellular levels of P-450 mRNAs under various induction conditions. b) Determine the extents to which P-450 mRNA transcription, processing and degration contribute to the intracellular levels of P-450 mRNAs by characterizing these individual processes and by studying the responses of these processes to P-450 inducers.

5) Analyze the structure of the P-450 genes and compare both the regulatory and structural sequences of the genes for different P-450's. Specific objectives: a) electron microscopic analysis of heteroduplexes between P-450 mRNA and cloned P-450 genes will be used to determine the location and size of intervening sequences and other gross structural characteristics of the P-450 genes; b) cloned P-450 genes will be used as hybridization probes against Southern blots of other cloned P-450 genes in order to identify regions of homology within the genes of this family; c) the organization of the P-450 gene family within the rat genome and the copy number of these genes will be determined by Southern hybridization and other mapping techniques; d) regulatory regions and other interesting regions of the P-450 genes will be analyzed by DNA sequencing; and e) the functional properties of regulatory sequences will be studied by cloning these sequences into eukaryotic expression vectors. The latter and derivatives which have been subjected to site specific mutagenesis will then be introduced into cells in culture by DNA mediated gene transfer, and the regulatory properties of these sequences assessed in cell culture.

6) Correlate regulatory data from objective 4 with structural data from objective 5 for the purpose of identifying relationships between gene structure and regulation.

7) Use the P-450 cDNA and genomic clones to assess a) the multiplicity of P-450s expressed in different tissues; b) the relatedness of P-450s expressed in different tissues; and c) the relatedness of P-450 genes in different eukaryotes. Both the regulatory and structural sequences will be assessed.

Methods Employed:

Standard methods (see references 2 and 3 for details) for RNA isolation, fractionation and characterization have been modified, where necessary, for use with rat liver. These include RNA extraction and isolation with guanidine-HCl, RNA size-fractionation by methylmercuric hydroxide agarose gel electrophoresis and by sucrose gradient centrifugation, in vitro translation of mRNA, immunoprecipitation of translation products with P-450 specific IgG, and SDS polyacrylamide gel electrophoretic analysis of translation products and immunoprecipitates. Levels and rates of RNA synthesis are studied by Northern blot hybridization and solution hybridization to P-450 cDNAs. DNA complementary to P-450 mRNA is synthesized and cloned in the bacterial plasmids by modifications of previously reported methods (see references). Clones of the complete, native P-450 genes are isolated from libraries of rat genomic DNA ligated into phage lambda and screened by standard plaque hybridization techniques. Cloned cDNAs and genes are prepared in large amounts by standard microbiological and biochemical procedures. Cloned cDNAs and genes are characterized and studied by a wide range of techniques including electron microscopic analysis of RNA-DNA heteroduplexes, hybridization selected translation and immunoprecipitation, colony and plaque hybridization, restriction endonuclease mapping, DNA sequencing, Southern hybridization, Northern hybridization, solution hybridization, and in vitro transcription. Regulatory sequences are characterized by subcloning into eucaryotic expression vectors, introducing these recombinants into cells in culture by the calcium phosphate precipitation technique for DNA mediated gene transfer, and analysis of the response of such cells to P-450 inducers. Site specific mutagenesis is used to alter regulatory sequences cloned into these expression vectors to identify specific nucleotides essential to the activity of these sequences.

Major Findings:

1) We have characterized in greater detail the structure of the MC-P-450 gene whose isolation we reported last year. Our main findings are: a) We have constructed a series of over 12 subclones of this gene, each of which contains a different domain of the P-450 gene or of adjacent sequences. b) Using these subclones we have discovered repetitive DNA in the intervening sequences nearest the 5' and the 3' ends of the gene. The intervening sequences in these two locations do not cross-hybridize and are therefore different from each other in sequence. c) The large intervening sequence near the 5' end of the gene has been found to contain sequences that code for a second mRNA. By Northern hybridization we find that this second mRNA is relatively abundant in rat liver and is not induced by P-450 inducers, although these inducers may suppress the level of this mRNA slightly. By hybridization selection and translation we have found that this mRNA encodes a peptide of about 65 kilodaltons that is not recognized by MC-P-450 specific antibodies. It appears that the coding sequences of this second mRNA do not overlap the P-450 mRNA coding sequences, since recombinant plasmids that are known to contain only P-450 coding sequences and not intervening

sequences do not hybridize to this second mRNA. Preliminary evidence indicates that sequences coding for other portions of this second mRNA are to be found outside of, but in the vicinity of, the P-450 gene.

2) This year we have also constructed a cDNA library in the plasmid pBR322 that represents all of the mRNAs present in the liver of MC-treated rats. From this library we have isolated and characterized several recombinant plasmids containing cDNAs corresponding in sequence to two mRNAs for two different MC-induced cytochromes P-450. Thus, including the one cloned MC-P-450 cDNA reported last year, we now have recombinant plasmids containing cDNAs complementary to a total of three distinct MC-P-450s. To date our findings with the two new recombinant plasmids are these: a) These recombinants correspond to the mRNAs encoding the two major forms of MC-induced cytochrome P-450. b) These mRNAs are 2000 and 2700 bases long. c) These mRNAs are induced many thousand fold since they are undetectable in control liver but are highly abundant in liver from MC-treated rats. d) These major MC-P-450 mRNAs encode proteins that are about 54 and 58 kilodaltons. Both are recognized strongly by polyclonal MC-P-450 specific antibodies. The interaction of these peptides with monoclonal MC-P-450 antibodies is now under investigation.

3) During this year, we have used the two new MC-P-450 cDNA recombinant plasmids as hybridization probes for isolating the corresponding native MC-P-450 genes from a library of the rat genome. a) One of these two genes is presently isolated and is being characterized by restriction enzyme mapping, electron microscopic analysis of DNA-RNA heteroduplexes and other techniques. b) The gene corresponding to the other cloned cDNA is now being isolated.

4) We have quantitated the levels of translatable mRNA encoding several P-450s using in vitro translation of mRNA and immunoprecipitation of translation products with polyclonal and monoclonal antibodies specific for a variety of P-450's including those induced by MC, phenobarbital (PB), and pregnenolone-16-carbonitrile (PCN). The kinetics of induction of translatable mRNA for these P-450s have been determined and the differences between male and female rats have been explored.

Significance to Biomedical Research and the Program of the Institute:

The P-450s, as part of the mixed-function oxidase system, are central to the activation and detoxification of carcinogens, to the detoxification of xenobiotics and to drug metabolism. There is a wealth of evidence indicating that these enzymes are highly inducible and that they exist in multiple forms. The source of the diversity of P-450s and the mechanisms of their regulation have not yet been elucidated at the molecular level. The significance of the diversity and regulation of the P-450s in determining the response of individuals to specific carcinogens, drugs and xenobiotics has not been assessed. Accomplishment of the objectives described above should result in new insights into the molecular basis for the diversity of P-450s and into the molecular mechanisms of regulation of P-450 gene expression. This will result in a better understanding of the balance between activation and detoxification of carcinogens, of the

influence of this system on drug action and of other questions in carcinogenesis, toxicology and pharmacology. Our work has already begun to address the question of the molecular basis for the multiplicity of P-450s by showing that different MC-P-450s are encoded by different mRNAs (see refs. 2 and 3). In the year to come we will move our attention from the mRNAs to consider the question of the molecular basis of the multiplicity of P-450s at the level of the genes themselves. This will be done by comparing the structures and sequences of the three MC-P-450 genes that are now available in this laboratory. Besides being of practical interest in the fields of cancer biology and toxicology, the P-450 system is also interesting on a more fundamental level as a system for studying the regulation of expression of a family of genes. Thus, the work with this system should contribute to the basic understanding of eukaryotic gene structure and regulation. Our recent discovery that the coding sequences of one gene are present in the intervening sequences of another gene is an example of such a contribution. This is the first instance in which coding sequences for one mRNA have been found in the intervening sequence of another gene.

Proposed Course:

In the coming year we will:

1) Investigate further the structure of the P-450 gene whose isolation was reported last year. The focus of this work will be to a) elucidate the detailed structure of the region of this gene that contains coding sequences for a second gene by sequencing that region of the gene already identified by Southern and Northern hybridizations; b) delineate the 5' end of the transcribed region of the P-450 gene by primer extension and other techniques; and c) sequence the stretch of DNA upstream of the 5'end of the P-450 gene as the first step in locating possible regulatory sequences associated with this gene.

2) Complete the isolation and characterization of the other two P-450 genes mentioned above. The initial characterization will include r-loop analysis and restriction mapping.

3) Analyze the structure of these genes in detail. This will include sequence analysis of selected regions of each gene and analysis of gene organization and copy number by hybridization of subcloned fragments of these genes to Southern transfers of genomic DNA and Northern transfers of RNA isolated from control and MC-treated rats.

4) Compare the three P-450 genes available to us by Southern transfer and hybridization. Among other things, this will provide evidence concerning the existence and extent of homology among these genes and provide an indication of the relatedness of these genes.

5) Characterize the kinetics of induction of the three MC-P-450 mRNAs for which we now have cloned cDNAs that can be used as hybridization probes. RNAs will be isolated from rat liver and from cells in culture at specific times after administration of inducers. The level of each MC-P-450 mRNA in these preparations will be quantitated by Northern and by dot-blot hybridization. Similar procedures will be used to compare the levels of these mRNAs in liver from males and females.

6) Determine the rate of initiation of P-450 gene transcription in liver from control and MC-treated rats by measuring ^{32}P incorporation into nascent mRNA transcripts completed in vitro in isolated nuclei. Incorporation of ^{32}P into specific P-450 transcripts will be measured by hybridization of ^{32}P labeled RNA to cloned P-450 gene sequences immobilized on nitrocellulose.

7) In order to functionally identify and to characterize the properties of regulatory (esp. promotor) regions associated with the three P-450 genes that we have isolated we will: a) identify possible regulatory regions of these genes based on sequence information and on their location relative to structural regions of the P-450 genes; b) introduce these putative regulatory regions into the pSVO-CAT vector of Gorman, Moffat and Howard in such a way that these regions can control chloramphenachol acetyl transferase (CAT) gene expression; and c) introduce these recombinants into cells in culture by the calcium phosphate precipitate technique of DNA mediated gene transfer and test the ability of these putative regulatory sequences to modulate the expression of CAT. During the upcoming year we will construct these recombinants and carry out preliminary expression experiments. In depth regulatory studies with these recombinants, including site-specific mutagenesis experiments to delineate detailed correlations between regulatory activity and specific bases within the putative P-450 regulatory regions, will be carried out in the subsequent year.

Publications:

Fagan, J. B., Park, S. S., Guengerich, F. and Gelboin, H. V.: DNA cloning and monoclonal antibodies: Analysis of cytochrome P-450 regulation. In Sato, R. and Kato, R. (Eds.): Microsomes, Drug Oxidation and Drug Toxicity. Tokyo, Japan Sci. Soc. Press, 1982, pp. 597-604.

Fagan, J. B., Pastewka, J. V., Guengerich, F. P. and Gelboin, H. V.: Multiple cytochromes P-450 are translated from multiple mRNAs. Biochemistry 22: 1927-1934, 1983.

Fagan, J. B., Pastewka, J. V., Park, S. S., Guengerich, F. and Gelboin, H. V.: Identification and quantitation of a 2.0 kilobase mRNA coding for 3-methylcholanthrene-induced cytochrome P-450 using cloned cytochrome P-450 cDNA. Biochemistry 21: 6574-680, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05204-03 LMC

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Sequence Alterations in vivo Following DNA Modification by Carcinogens

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Michael Seidman Senior Staff Fellow, LMC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Bacterial plasmids have been used to study repair and mutagenesis of benzo-pyrene diol epoxide (BPDE)-damaged DNA in E. coli. In nontargeted experiments the plasmids were randomly modified by BPDE and introduced into E. coli strains which differed in their capacity for repair and mutagenesis. By measuring the survival of bacteria containing plasmids and mutagenesis of a plasmid gene, it was possible to identify host cell functions for error-free repair and for mutagenesis. It was found that repair functions can be distinguished temporally from mutagenic activities after induction of the SOS response. In "targeted" experiments, a specific fragment of the plasmid from a nonessential marker gene was modified with BPDE and ligated back into the plasmid. The survival curves of these constructs were virtually identical to those of the randomly modified plasmids, suggesting that the principal determinant for survival of BPDE-damaged DNA is the simple presence of the carcinogen rather than secondary mutational events in essential functions. Mutants were found in the targeted regions but not in another nontargeted gene, indicating that mutagenesis is targeted. A collection of these mutants has been sequenced and transitions, transversions, and frame shift mutations identified.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Sekhar Chakrabarti	Visiting Fellow	LMC, NCI
Hiroshi Mizusawa	Visiting Associate	LMC, NCI

Objectives:

To study repair and mutagenesis of carcinogen modified DNA, to distinguish direct from indirect effects and to identify the cellular processes involved.

Methods Employed:

Bacterial plasmids were constructed which have marker genes (chloramphenicol resistance, galactose kinase, tetracycline resistance) as well as functions required for replication and ampicillin resistance. In the targeted experiments, fragments from either the tetracycline gene, in one case, or galactokinase in another, were covalently modified with BPDE and then ligated back into the remainder of the unmodified plasmid. The randomly modified and target-modified plasmids were used to transform E. coli strains which are defective in specific repair and/or mutagenesis gene functions. The number of bacteria which survived on ampicillin medium were counted and the number of mutants at the appropriate locus were determined. These data were plotted as a function of the amount of BPDE on the plasmid. Selected mutants were sequenced.

Major Findings:

1) Mutagenesis in this system is targeted. Mutations arose in the targeted regions, not in other nondamaged regions of the plasmids. The mutations generated by BPDE damage of DNA include transitions, transversions, and frame shifts. 2) After SOS induction (by UV light) of recipient bacteria, functions for survival (error-free repair) and mutagenesis were induced above constitutive levels. The uvrA dependent activity rose and fell in the first 30 minutes after induction, while the mutagenesis activity was fully induced only after 60 minutes after induction. Thus, the two functions could be distinguished temporally. 3) Survival of BPDE-modified plasmids is largely a function of the presence of the carcinogen and not due to secondary effects such as mutations in essential functions.

Significance to Biomedical Research and the Program of the Institute:

The study provides a direct estimate of the consequences of covalent modification of DNA by chemical carcinogenesis. The cellular processes involved in carcinogen-induced mutagenesis are being detailed.

Proposed Course:

This project will not be continued.

Publications

None

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DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05205-03 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Naturally Occurring Inhibitors of Different Forms of Cytochrome P-450

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

H. V. Gelboin Chief, LMC, NCI

COOPERATING UNITS *(If any)*

D. Gottlieb, University of Sao Paulo, Brazil; P. Dewick, University of Nottingham, England

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mixed-function oxidases containing different forms of cytochrome P-450 are the key enzymes that metabolize a wide variety of drugs, chemicals and carcinogens. The focus of this project is the utilization of specific inhibitors and inducers of aryl hydrocarbon hydroxylase (AHH) to probe the multiplicity, diversity, and different catalytic properties of the cytochromes P-450. 7,8-benzoflavone and L-maackiain acetate are inhibitors that are effective against different forms of AHH. We are using these inhibitors to probe the active catalytic sites of the multiple forms of cytochrome P-450. Also, other compounds, i.e., medicarpin and maackiain, are being studied and their structural and inhibitory effects on cytochrome P-450 are being determined using microsomes and purified cytochromes P-450.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

T. Fujino	Expert	LMC, NCI
F. K. Friedman	Senior Staff Fellow	LMC, NCI

Objectives:

To investigate naturally occurring inhibitors which are present in our environment and to characterize their effects on cytochromes P-450 from animal and human tissues.

Methods Employed:

1) Naturally occurring flavonoid compounds, some of which are purified from plants and some of which are chemically synthesized, are used; 2) microsomal preparations are made from liver, lung and kidney of nontreated, PB- and MC-treated rats, and from available human tissues; and 3) AHH, 7-ethoxycoumarin deethylase and benzphetamine hydroxylase are determined for each of these preparations in the absence and presence of inhibitors. Analysis of benzo(a)-pyrene metabolism is performed with the aid of high pressure liquid chromatography.

Major Findings:

7,8-Benzoflavone, L-maackiain acetate, medicarpin, and maackiain are inhibitors that affect different forms of AHH. 7,8-benzoflavone inhibits the AHH from MC-treated rat tissues, but has little or no inhibitory effect or stimulates the AHH of untreated or PB-treated rat tissues. L-maackiain acetate greatly inhibits the AHH from noninduced and PB-induced rat tissues, but has no inhibitory effect or stimulates the AHH from MC-treated rat tissues. 7,8-benzoflavone and L-maackiain acetate have a similar effect on the AHH of human tissues. 7,8-benzoflavone stimulates the AHH of normal human liver tissue and strongly inhibits the AHH of placental microsomes from women who smoke. In contrast, L-maackiain acetate strongly inhibits the AHH of normal human liver and has little effect on the AHH of human placenta.

Significance to Biomedical Research and the Program of the Institute:

L-Maackiain acetate, 7,8-benzoflavone, and other flavones may be useful inhibitors of specific forms of AHH in human as well as animal tissues. The unusual specificity of L-maackiain acetate as an inhibitor renders it a valuable tool for probing the structure and function of cytochrome P-450, assessing the factors that influence polyaromatic hydrocarbon metabolism, and clarifying the relationship between drug and carcinogen activation and detoxification. Some of the compounds under study may be powerful natural inhibitors or modulators of chemical carcinogen-induced cancer.

Proposed Course:

To examine the effects of specific inhibitors on tumorigenesis by various classes of carcinogens. To investigate other classes and related flavonoids as modulators of carcinogenesis. To investigate the interaction of these flavones and related compounds with various forms of cytochrome P-450. We are in the process of obtaining additional amounts of these and other inhibitors to continue our studies.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05208-03 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Typing of Cytochrome P-450 in Human Tissues Using Monoclonal Antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Tadahiko Fujino Expert, LMC, NCI

COOPERATING UNITS *(if any)* H. L. Gurtoo, Roswell Park Memorial Institute, Buffalo, N.Y.; K. Gottlieb, University of Colorado, Denver, CO.; D. Manchester, University of Colorado, Denver, CO., C. Kapitulnik, Hebrew University, Jerusalem, Israel; H. Slor, University of Tel Aviv, Tel Aviv, Israel

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The diversity of the cytochromes P-450 in human tissues, i.e., placenta, monocytes and lymphocytes was investigated using monoclonal antibodies (MAbs) to 3-methylcholanthrene (MC) and phenobarbital (PB)-induced rat liver cytochrome P-450. MAbs to rat liver MC-induced cytochrome P-450 not only inhibited the aryl hydrocarbon hydroxylase (AHH) of MC-induced rat liver microsomes but also inhibited AHH of human placenta and lymphocytes. The 7-ethoxycoumarin deethylase activity of human placenta and lymphocytes was also inhibited. The degree of enzyme inhibition by monoclonal antibodies was different for different individuals and tissues. The content of antigenically unique types of cytochrome P-450 responsible for different drug and carcinogen reactions can therefore be measured in different individuals by the extent of their inhibition by monoclonal antibodies.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Sang S. Park	Senior Staff Fellow	LMC, NCI
Harry V. Gelboin	Chief	LMC, NCI

Objectives:

Differences in the profile of cytochromes P-450 present in different tissues may account for differences in metabolism that result in either the carcinogenic activation, or the detoxification of drugs, mutagens, and potential carcinogens. We are using cytochrome P-450-specific monoclonal antibodies (MAbs) to investigate the diversity and multiplicity of these enzymes by: 1) development of a phenotypic description of the number and quantity of cytochromes P-450 in tissues and individuals; 2) determination of their role in the detoxification or activation of specific xenobiotics; 3) determination of their role in individual variation in drug and carcinogen responsiveness; and 4) examination of the complex interactions and influence of hereditary and environmental factors through an investigation with twin placentas.

Methods of Employed:

Monoclonal antibodies were obtained from hybridomas made by the fusion of myeloma cells and spleen cells derived from BALB/c mice that had been immunized with MC-induced rat liver cytochrome P-450. Human monocytes and lymphocytes were isolated from peripheral blood and treated with benzanthracene. Human placental microsomes were prepared from single birth and twin birth placentas from women who are smokers and nonsmokers. Aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (ECD) activities were measured by a fluorometric assay after incubation with monoclonal antibodies. The effects of monoclonal antibodies on the metabolism of benzo(a)pyrene by cytochrome P-450 were also studied by high pressure liquid chromatography (HPLC).

Major Findings:

1) Monoclonal antibodies to MC-induced rat liver cytochrome P-450 not only inhibited the AHH activity of MC-induced rat liver microsomes, but also inhibited the AHH activity of human placental microsomes, lymphocytes and some human liver microsomes. 2) Monoclonal antibodies detected common antigenic sites in human placenta and lymphocytes. 3) The AHH activity of human placenta was inhibited 90%, while the ECD activity was inhibited from 0-70% in different individuals and varied widely. 4) The placentas from both dizygotic and dichorionic, monozygotic twins show extraordinarily high intrapair concordance for both the absolute amounts of AHH and ECD and their extent of inhibition by MAb, compared to unrelated individuals. 5) AHH and ECD in human monocytes and liver are catalyzed by cytochromes P-450 that are different from the MAb-sensitive cytochrome P-450 in placenta and lymphocytes.

Significance to Biochemical Research and Program of the Institute:

Monoclonal antibodies provide us with the ability to analyze the complicated phenomenon of polycyclic hydrocarbon metabolism from a new perspective. Use of monoclonal antibodies to cytochrome P-450 should identify those P-450 species involved in carcinogen activation and clarify the metabolic pathways responsible for chemical carcinogenesis. Knowing the enzymatic steps leading to the activation of the carcinogen will be useful for the detection and prevention of chemical carcinogenesis.

Proposed Course:

Additional MAbs will be explored as a tool for the study of human cytochromes P-450. These might recognize the different antigenic determinants of the multiple forms of cytochrome P-450. MAbs will help define catalytic specificities of the various cytochromes P-450 by studying their inhibitory effect on metabolism of substrates other than benzo(a)pyrene and 7-ethoxycoumarin. MAbs may be utilized to phenotype cytochromes P-450 in tissues, organs and individuals. Such information is useful in determining the relationship between cytochrome P-450, drug activation, and susceptibility to chemical carcinogenesis.

Publications:

Fujino, T., Park, S. S., West, D. and Gelboin, H. V.: Phenotyping of cytochromes P-450 in human tissues with monoclonal antibodies. Proc. Natl. Acad. Sci. USA 179: 3682-3686, 1982.

Park, S. S., Fujino, T., West, D., Guengerich, F. P. and Gelboin, H. V.: Monoclonal antibody specificity to cytochrome P-450 from methylcholanthrene treated rats. Cancer Res. 42: 1798-1808, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05236-02 LMC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
5-Methyl Cytosine-Containing Sequences in the Chicken Genome

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Francine C. Eden Expert, LMC, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Genome rearrangement can be directly responsible for oncogenic transformation, and its structural requirements need to be investigated. A specific site of genomic rearrangement has now been correlated with undermethylation in the chicken genome. Recombination involves a short, tandemly repeating region contained within a longer repeated sequence element and the recombination event generates a polar deletion. DNA sequencing of cloned DNA segments provides a complete description of this undermethylated recombination site. Another way methylation might affect oncogenic transformation is through repression of incoming (virally carried) oncogenes; cytosine methylation apparently inhibits transcription. The specific requirements for de novo methylation of DNA sequences are unknown. A "natural" gene transfer system, the introduction of unmethylated mitochondrial DNA into the nuclear genome, is being used to investigate sequence requirements for de novo methylation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Veronica A. O'Neill Visiting Fellow LMC NCI

Objectives:

To relate cytosine methylation in DNA to two cellular processes important to oncogenic transformation: 1) genome rearrangement and 2) inhibition of the expression of newly introduced genes. The general strategy will be to: 1) Determine whether an undermethylated region within a family of chicken repeated DNA sequences is associated with recombination events and 2) clone DNA sequences that have moved from an unmethylated to a methylated genome and determine the sequence requirements for de novo methylation and its concomitant inhibition of transcription.

Methods Employed:

5-methyl cytosine in DNA is detected using methylation-sensitive restriction endonucleases. Methylation patterns within specific DNA sequences are determined by hybridizing ^{32}P labelled cloned DNA segments to Southern blots of genomic DNA fragments separated in agarose gels. DNA is cloned in PBR322 or in bacteriophage lambda and is sequenced by the method of Maxam and Gilbert.

Major Findings:

The undermethylated segment that occurs within a family of long repeated DNA sequences in the chicken genome has now been implicated directly in the process of genomic recombination. The family of repeats in which the undermethylated region occurs exhibits considerable structural diversity in the genome. Thirteen members of this family have been cloned individually in bacteriophage lambda and compared to determine the nature of this variability. About half of the family members have a large deletion. Sequencing and restriction endonuclease digestion reveals the presence of a short, tandemly repeated sequence at one boundary of this deletion. This tandem repeat corresponds to the previously detected undermethylated region in the genome. The deletion it generates is polar and removes the tandem repeats and a much larger segment of 3' flanking DNA. The boundary of the deletion has been mapped at 50 bp segments and may be precise to the nucleotide. Through further subcloning and sequencing of these deletion boundaries in different family members, it will be possible to write out a specific DNA sequence that both participates in recombination and is maintained in an unmethylated state in the genome. This will establish the first firm connection between cytosine methylation and recombination in the eucaryotic genome.

The study of the acquisition of a new methylation pattern by DNA sequences as they are introduced into the nuclear genome is now underway. It has been reported recently that mitochondrial sequences are occasionally integrated into the nucleus, and in this transfer they are moved from an unmethylated to a methylated

cellular compartment. This provides an opportunity to study how new methylation patterns are acquired. We have cloned a fragment of mitochondrial DNA that is also represented in nuclear DNA. This fragment will be used to study de novo methylation by comparing mitochondrial and nuclear counterparts of the same sequence using methylation-sensitive restriction endonucleases and by sequencing newly methylated DNA sequences. This will reveal the sequence requirements for establishment of a new methylation pattern.

Significance to Biomedical Research and the Program of the Institute:

Our present understanding of the molecular basis of cancer indicates that the altered location of genes and/or alteration of their expression plays a key role. Thus it is important to understand what influences genome rearrangement and gene expression. We are studying the methylation of cytosine in DNA, which is directly related to both these important processes.

Proposed Course:

During the next year the unmethylated deletion boundary regions will be subcloned and sequenced. The DNA sequence involved in recombination will be recloned into a new vector transmissible to heterologous eukaryotic cells. The participation of this sequence in recombination will be studied further and subsequently the influence of sequence alterations introduced in vitro will be tested.

The sequences that undergo de novo methylation will be identified and the sequence requirements for establishment of a methylation pattern will be determined.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05240-02 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Rearrangement of a Plasmid Sequence in Mammalian Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Michael Seidman Senior Staff Fellow, LMC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A shuttle vector plasmid has been constructed which can be used to study gene stability, rearrangement, and recombination in mammalian cells. The plasmid contains sequences derived from a bacterial plasmid, from SV40 virus, and a marker gene, galactokinase, which can be scored in the appropriate bacterial host. This construct replicates in mammalian cells and bacteria. An experimental protocol was designed in which mammalian cells were infected with the plasmid, replication permitted and then the plasmid DNA extracted from the cells. After purification and elimination of residual infectious DNA, the plasmid was introduced into a bacterial host which permitted the detection of the presence or absence of a functional galactokinase gene. With this assay it was possible to assess quantitatively the stability of the plasmid in the mammalian cells. It was found that approximately 1% of the progeny plasmids had lost a functional marker gene. The defective plasmids contained point mutations, deletions, and insertions of cell DNA. Many of the mutational events occurred early in infection and were found with different methods of DNA infection.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Abdur Razzaque	Visiting Fellow	LMC, NCI
Sekhar Chakrabarti	Visiting Fellow	LMC, NCI

Objectives:

To study DNA sequence rearrangement in mammalian cells and to identify DNA sequences and gene functions involved in DNA rearrangement and recombination.

Methods Employed:

A shuttle vector was constructed which consists of sequences from the bacterial plasmid pBR322 which confer ampicillin resistance and a bacterial origin of replication, the early genes and origin of replication from SV40 virus which permit replication in permissive mammalian cells, and a marker gene, galactokinase, which can be scored in the appropriate E. coli strain. After DNA infection and replication in the mammalian cells, the plasmid DNA is extracted from the cells and purified. It is then treated with a restriction enzyme which cleaves only the infectious DNA so that only the plasmid molecules which replicated in the mammalian cells are introduced into the bacterial strain, which is galactokinase negative. A comparison of the total bacterial colonies and those which are galactokinase negative is made and a mutation frequency for the galactokinase gene in mammalian cells calculated. By using constructs with direct repeated sequences flanking the marker, it is possible to study homologous recombination by screening for plasmids with the precise deletion of the intervening sequence.

Major Finding:

There was a significant level of spontaneous mutagenesis of the plasmid. Many of the mutant plasmids arose early in the infection although it is also clear that mutations may arise at any time during the replication period. The defective plasmids contained point mutations, deletions, and insertions of cell DNA, some insertions as large as 7-8 kb.

Significance to Biomedical Research and the Program of the Institute:

DNA transfection techniques are widely used in current molecular and cell biology programs including studies designed to detect and characterize oncogenes. Our results indicate that spontaneous mutagenesis of the DNA used in these experiments does occur at high frequency and must be considered when interpreting data from transfection experiments.

Proposed Course:

Construction of vectors with direct repeats of specific DNA sequences flanking the marker allows the study of homologous recombination in animal cells using the shuttle vector technology. We have performed such experiments, and find that molecules arising from homologous recombination events can be detected and characterized. We plan to study this process with different repeated sequences and to follow the effects of treatment of the host cells with DNA damaging agents (carcinogens, UV lights) on the frequency and precision of the recombination.

Publications

Razzaque, A., Mizusawa, H. and Seidman, M.: Rearrangement and mutagenesis of a shuttle vector plasmid after passage in mammalian cells. Proc. Natl. Acad. Sci. USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05241-02 LMC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Left-Handed Helical DNA in Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Hiroshi Hamada Visiting Associate, LMC, NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies have shown that purine-pyrimidine alternating sequences, such as (C-G)n·(C-G)n and (T-G)n·(C-A)n, can form a left-handed helical conformation called Z-DNA. We previously found indications for the existence of such potential Z-DNA-forming sequences in natural genomes by hybridization. Here, we have comfirmed our previous observation by direct DNA sequencing. The human DNA library was screened by hybridization with poly d(T-G)·poly d(C-A). Among many positive clones, five were randomly isolated and characterized in detail. DNA sequencing revealed that four clones contain a precisely alternating (T-G)n (n = 20,15,10,10), while the remaining one has a (T-G)15, with four bases out of alternation. The sequence data also revealed the characteristics of (T-G)n sequence with respect to its structure and organization in the human genome. The same strategy was applied directly to show the presence of (C-G)n sequence in the genome. However, such a sequence has not been isolated from the mouse DNA library, probably because the hybridization procedure does not recognize a (C-G)n sequence. These results have confirmed the general and abundant occurrence of (T-G)n, a potential Z-DNA-forming sequence. Attempts are now underway to establish an assay system to detect Z-DNA in biological samples, and to test the hypothesis that Z-DNA may play a crucial role in the regulation of gene expression and in carcinogenesis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than Principal Investigator) engaged on this project.

Takeo Kakunaga Head, Cell Genetics Section LMC, NCI

Objectives:

To know the general occurrence of Z-DNA sequences in nature and to study their biological implication, such as a role in the regulation of gene expression, in cell differentiation, and in carcinogenesis.

Methods Employed:

The human DNA library was screened by the hybridization with ^{32}P-labeled poly d(T-G)·(poly d(G-C). The hybrizing DNA regions in isolated clones were subjected to DNA sequencing.

Major Findings:

1) Confirmation of the abundant occurrence of a dT-dG alternating sequence in natural genomes. Screening of a human DNA library with ^{32}P-labeled poly d(T-G)·poly d(C-A) indicated that about 20% of recombinant phages hybridized to the probe. This value (20%) is in good agreement with the copy number of (T-G)n sequence in the human genome we estimated previously. Five positive clones were randomly isolated and characterized. Nucleotide sequencing of the hybridized DNA region in each clone showed the presence of a precisely-alternating (T-G)n (n=20,15,10,10) in four clones, and a (T-G)15 with four bases out of alternation in the remaining one clone.

2) The organization of a (T-G)n sequence in the genome. Structural analysis of five clones suggested the following characteristics of a (T-G)n sequence: a) n = 10 - 30; b) randomly dispersed in the genome, approximately one copy of (T-G)n in every 100 Kb of human DNA; c) sequence flanking a (T-G)n has virtually no homology, indicating that (T-G)n itself is a repeated DNA element; d) minor population of (T-G)n is associated with Alu-family in the human genome.

3) Survey of (G-C)n sequence in the mouse genome. 5-10% of recombinant phages of a mouse DNA library hybridized to ^{32}P-labeled poly d(G-C). Nine clones were randomly isolated and characterized. DNA sequencing of the hybridized regions, however, revealed that all clones contain a (G-A)n sequence, but not a (G-C)n sequence. Thus, the conventional hybridization procedure failed to detect (G-C)n sequence in the genome.

Significance to Biomedical Research and the Program of the Institute:

Evidence has been accumulating that suggests Z-DNA exists in vivo. Although the biological significance of Z-DNA is an open question, it could be involved in essential step(s) of cell function. Especially, considering the importance of

the tertiary structure of DNA in the regulation of gene expression, it is very likely that Z-DNA may modulate gene expression at the transcriptional level. If this is the case, factors that affect B -- Z transition of a potential Z-DNA sequence would play a crucial role in gene expression. Interestingly, a number of mutagens have been known to affect the B -- Z transition. Our approaches, thus, will allow us to study the quite novel mechanism of gene regulation and to understand the cause of abnormal gene expression in tumor cells.

Proposed Course:

1) To establish an assay system to detect Z-DNA in a biological system, and 2) to know whether or not and how Z-DNA modulates gene expression.

Publications:

Hamada, H., Petrino, M. G. and Kakunaga, T.: A novel repeated element with Z-DNA-forming potential is widely found in evolutionarily diverse eukaryotic genomes. Proc. Natl. Acad. Sci. USA 79: 6465-6469, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05242-02 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Monoclonal Antibody Mapping of Cytochromes P-450 in Different Species and Tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Tadahiko Fujino Expert, LMC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Multiplicity of microsomal cytochromes P-450 was studied with the aid of monoclonal antibodies (MAbs) to 3-methylcholanthrene induced rat liver cytochrome P-450. These MAbs specifically bind and inhibit the enzymatic activity of the purified cytochrome P-450. The contribution to enzyme activity of a particular MAb-sensitive P-450 in a mixture of different types of cytochrome P-450 can be determined from the effect of MAb on enzyme activity. We are using such enzyme-inhibition assays to develop a detailed atlas of the cytochromes P-450 for different drug and carcinogen metabolism and their distribution in different strains, species and tissues. Such information will aid us in further understanding the diversity of cytochrome P-450 and its role in tissue, strain and species susceptibility to carcinogenesis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Harry V. Gelboin	Chief	LMC, NCI
Sang S. Park	Senior Staff Fellow	LMC, NCI

Objectives:

In order to understand the detailed genetics and the role of cytochrome P-450 in carcinogen and drug metabolism, a phenotypic description of the number and quantity of P-450s in various species and tissues is necessary and is examined in this project.

Methods Employed:

Monoclonal antibodies were prepared by the general methods of Kohler and Milstein. Microsomes from different species and tissues, i.e., rat, mouse, hamster were collected. The genetically defined mouse strains C57BL/6 and DBA/2 were included. Aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase were measured by a fluorometric assay.

Major Findings:

1) Monoclonal antibodies to 3-methylcholanthrene (MC)-induced rat liver microsomes inhibited both the AHH and 7-ethoxycoumarin deethylase of Sprague Dawley rat liver microsomes by 75-80%. 2) In mice, the monoclonal antibodies clearly distinguish a large difference between the MC-induced enzyme in the livers of C57BL/6 and DBA/2 mice. AHH activity of liver from C57BL/6 mice was inhibited 87%, while that from DBA/2 mice was not inhibited. 3) The hepatic 7-ethoxycoumarin deethylase of C57BL/6 mice was inhibited 43% and did not parallel the degree of inhibition of AHH, while that of DBA/2 mice was not inhibited. 4) The pulmonary AHH activities of both C57BL/6 and DBA/2 mice behaved differently from hepatic AHH activities; both were inhibited 80%. 5) Pulmonary 7-ethoxycoumarin deethylase activity was inhibited less than the liver activity in both C57BL/6 and DBA/2 mice. 6) AHH activity in livers of guinea pigs and hamsters were inhibited, but the corresponding ECD activity was unaffected.

Significance to Biochemical Research and the Program of the Institute:

An atlas of cytochromes P-450 classified by monoclonal antibodies may lead to a better understanding of the multiplicity of the cytochromes P-450, their genetic control, and their relationship to drug and carcinogen metabolism, and to individual differences in rates of drug metabolism and carcinogen sensitivity.

Proposed Course:

Tissue microsomes from different species have been collected. Some crosses of genetically defined mouse strains will be investigated in order to clarify the involvement of genetic factors in the regulation of these systems. Monoclonal antibodies will be used to discriminate between different forms of cytochrome P-450 on the basis of their effects on enzymatic activities. We will make an atlas of the cytochromes P-450 using a library of monoclonal antibodies to differentiate the different forms.

Publications:

Fujino, T., Park, S. S., West, D. and Gelboin, H. V.: Phenotyping of cytochromes P-450 in human tissues with monoclonal antibodies. Proc. Natl. Acad. Sci. USA 79: 3682-3686, 1982.

Park, S. S., Fujino, T., West, D., Guengerich, F. P. and Gelboin, H. V.: Monoclonal antibodies that inhibit enzyme activity of 3-methylcholanthrene-induced cytochrome P-450. Cancer Res. 42: 1798-1808, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05317-01 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Opal Suppressor tRNA Genes in Chicken, Human, and Rabbit Genomes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Dolph Hatfield, Research Biologist and Francine Eden, Expert LMC, NCI

COOPERATING UNITS *(If any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein and Cell Genetics Sections

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Protein phosphorylation is important in many cellular processes including oncogenic transformation. Opal suppressor tRNAs may mediate specialized aspects of protein phosphorylation through read-through in response to termination codons and simultaneous insertion of phosphoserine. We are investigating the structure and function of opal suppressor genes by molecular cloning. A chicken opal suppressor gene has been isolated and sequenced. It is encoded in a single genomic copy and has an unusual internal 5' promoter region. In vitro transcription experiments indicate that it has an active but weak promoter. Gene copy number and promoter strength together account for the presence of a low intracellular level of this tRNA. The human and rabbit genomes contain DNA sequences that hybridize to the chicken gene, which is being used as a probe to isolate the corresponding human and rabbit genes from DNA libraries. `Evolutionarily conserved regions integral to gene expression will be identifed through gene comparison.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Veronica O'Neill Visiting Fellow LMC, NCI

Objectives:

To understand the structure and function of opal suppressor tRNA genes in higher vertebrates and their role in the overall process of protein phosphorylation.

General Strategy:

1) Isolate and characterize opal suppressor tRNA genes from chicken, human, and rabbit DNA libraries; 2) sequence the genes and their flanking DNA segments; 3) investigate the structure of the genomic regions that contain these genes with respect to transcription and evolutionary conservation; 4) study the control of transcription using in vitro and in vivo transcription systems; and 5) use in vivo transcription systems to study processing and localization of the tRNA product.

Methods Employed:

The minor seryl-tRNAs are purified by DEAE-cellulose, BD-cellulose, and RPC-5 column chromatography and by two-dimensional polyacrylamide gel electrophoresis. Purified tRNAs are characterized by specific aminoacylation activity, codon recognition in a ribosome binding assay, and RPC-5 chromatography.

Purified tRNAs are labelled for use as probes by dephosphorylation with bacterial alkaline phosphatase and addition of ^{32}P from α-^{32}P-ATP using T4 polynucleotide kinase.

Genes corresponding to the tRNA probes are identified by hybridization to genomic blots of total DNA digested with restriction endonucleases. Genes are isolated from DNA libraries in bacteriophage lambda by plaque hybridization and the gene-containing segments are subcloned in the plasmid PBR322 for further analysis and DNA sequencing by the method of Maxam and Gilbert.

DNA segments flanking the tRNA genes are subcloned in PBR322 after construction of the necessary restriction map. Subcloned segments are in turn used as probes against genomic DNA of the same or different species or are used in in vitro transcription experiments.

A partially purified extract from KB cells and α-^{32}P-GTP were used in in vitro transcription experiments. Products were analyzed using polyacrylamide gels containing 7M urea. In vivo transcription, processing, and localization will be studied by injection of cloned genes into Xenopus oocytes. (We thank Jose Castano and Michael Zasloff for help with transcription).

Major Findings:

An opal suppressor tRNA purified from chicken liver has been successfully used as a ^{32}P-labelled probe to identify restriction fragments of chicken genomic DNA containing the corresponding gene and to isolate this gene from a library of chicken DNA cloned in bacteriophage lambda. Standard hybridization conditions were adequate for both genomic blotting and library screening even though the maximum length of the homology region is 87 bp and the gene is present as a single copy in the genome.

The cloned chicken opal suppressor gene has been localized in the recombinant phage containing it by restriction analysis and subcloned in the plasmid PBR322. After further restriction analysis, a 540 bp segment containing the gene and flanking DNA segments was sequenced by the method of Maxam and Gilbert. The gene is encoded in an 87 bp segment without introns. The CCA terminus of the mature tRNA is not encoded. The gene encodes a tRNA which would read the termination codon UGA, establishing that the genome actually encodes an opal suppressor tRNA, rather than assembling it at the RNA level through modification or splicing. Information bearing on transcriptional control emerged from the DNA sequence of this gene. It contains an unusual internal 5' promoter region which differs both by base substitution and insertion. The 3' portion of the internal promoter and the termination signal are both normal.

Analysis of the recombinant phage carrying this gene and of total genomic DNA revealed that it is not part of a cluster of tRNA genes and that it is a single copy gene. The segments flanking the gene were analyzed by restriction analysis and have been subcloned in PBR322 for use as probes to further characterize the larger genomic segment containing this gene in chicken and other genomes.

The cloned chicken gene has been used as a probe to identify corresponding DNA sequences in the human and rabbit genomes. These genes are currently being isolated by screening of their respective DNA libraries.

Preliminary in vitro transcription experiments indicate that the cloned chicken gene has an active but weak promoter. The transcription initiation point and processing intermediates will be identified by fingerprinting the primary and processed transcripts after purification by polyacrylamide gel electrophoresis.

Significance to Biomedical Research and the Program of the Institute:

The function of suppressor tRNAs that contain phosphoserine seems totally unique and probably lies outside all of our present concepts of tRNA utilization. Its full understanding is more likely to open a new area of eucaryotic molecular biology than to contribute to knowledge of tRNA genes per se. It is especially important to understand this special means of protein phosphorylation in light of new information relating protein phosphorylation directly to carcinogenesis.

Proposed Course:

1) Determine whether the chicken genome contains opal suppressor genes through more exhaustive library screening. This is important to understanding how the intracellular level of suppressor tRNAs in the cell is regulated. 2) Isolate and

sequence opal suppressor genes from human and rabbit DNA libraries. 3) Compare the sequences of genes and flanking regions in chicken, human, and rabbit genomes to identify regions important to the control of gene expression. (DNA sequences outside the internal tRNA promoters also influence transcription). 4) To determine where transcription of these tRNAs initiates, how processing proceeds, and where the final tRNA product is localized in the cell (nucleus vs. cytoplasm). This is needed because utilization of a tRNA can be influenced not only by gene copy number and promoter strength but also by processing and transport to the cytoplasm.

Publications:

Hatfield, D., Dudock, B. and Eden, F.: Characterization of nucleotide sequence of a chicken gene encoding an opal suppressor tRNA and its flanking DNA segments. Proc. Natl. Acad. Sci. USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05318-01 LMC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunopurification and Characterization of Cytochrome P-450

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Fred K. Friedman Senior Staff Fellow, LMC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mixed-function oxidase system includes cytochromes P-450 that metabolize a variety of drugs and carcinogens. The multiple forms of this enzyme display broad, overlapping substrate specificity. The type and amount of each form varies among species and individuals. The focus of this project is the identification, characterization, and elucidation of structure-function relationships of these isoenzymes. Monoclonal antibodies (MAbs) to specific cytochromes P-450 are an essential tool in these studies. In particular, a Sepharose-based immunoadsorbent has been prepared with a MAb to the major cytochrome P-450 induced in the livers of rats treated with 3-methylcholanthrene. The resin strongly adsorbs from microsomes some cytochrome P-450 which appears as two polypeptides of MW 50,000 and 52,000. The MAb interaction with the former species is relatively acid labile. The immunoadsorbent also binds with comparable affinity a lesser amount of a polypeptide of MW 48,000 from liver microsomes of untreated and phenobarbital-treated rats. These results demonstrate the utility of the MAb approach to analysis and preparation of various cytochromes P-450.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Richard Robinson	Biologist	LMC, NCI
Sang S. Park	Senior Staff Fellow	LMC, NCI
Harry V. Gelboin	Chief	LMC, NCI

Objectives:

To identify, purify, and characterize the multiple forms of cytochrome P-450. Monoclonal antibodies (MAbs) are utilized as highly specific reagents for recognition of individual cytochromes P-450.

Methods Employed:

MAbs were prepared to several liver microsomal cytochromes P-450 from rats treated with inducing agents. Methodologies were developed primarily with MAb 1-7-1 to the 3-methylcholanthrene-induced enzyme. The MAb was covalently linked to Sepharose to yield an immunoadsorbent. This resin was combined with rat liver microsomes, and the proteins bound to the column characterized spectrally, and upon elution from the resin, electrophoretically on SDS polyacrylamide gels.

Major Findings:

Spectral analysis demonstrates that a fraction of the total cytochrome P-450 in microsomes from treated rats binds tightly to the immunoadsorbent. Acid conditions (pH 3) are necessary to elute bound protein, which appear as two bands of MW 50,000 and 52,000 on SDS gels. The MW 50,000 band was partially eluted in milder acid (pH 5), indicating that the strength of MAb interaction differs between these two species. Much less protein is bound when microsomes from control or phenobarbital-induced rats are applied to the immunoadsorbent. However, each yields one band on electrophoresis that is of higher mobility (MW 48,000) than either band from the MC-induced microsomes.

Significance to Biomedical Research and the Program of the Institute:

Purification of individual cytochrome P-450 by a simplified procedure using specific MAbs offers a new approach to studying their multiplicity. The relationship of type and amount of cytochrome P-450 to drug and carcinogen metabolism can then be assessed.

Proposed Course:

Purification will proceed from an analytical to a preparative scale. The individual forms of cytochromes P-450 will then be analyzed for enzymatic activity and subjected to various physical and chemical structural studies.

Such detailed characterization should aid in gaining further insight into the role of cytochrome P-450 multiplicity in the metabolism of various drugs and carcinogens.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05320-01 LMC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Oncogene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
T. Kakefuda Medical Officer, LMC, NCI

COOPERATING UNITS *(if any)*
Y. Ito LMM, NIAID NIH; Y. Hamagishi LMM, NIAID NIH and M. Kimura, National Institute of Industrial Health, Japan

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Recombinant plasmids for the expression of polyoma virus middle tumor antigen (MT) were constructed in order to obtain a large quantity of the antigens and to study their properties. The plasmids contain an ampicillin resistance gene, lambda phage PL promoter, CII, and a portion of the O-gene that was fused with MTcDNA. The expression of the hybrid gene (OMT) was controlled in temperature shift experiments. The host E. coli (CI857) strain carries a temperature sensitive mutation in the CI gene that controls PL promoter. The OMT produced in the E. coli was immunoprecipitated specifically by MT monoclonal antibody. The OMT was poorly auto- or trans-phosphorylated by comparison to authentic MT. Antibody specific for MT was produced by injection of OMT into rabbits. The experimental system established may be applied to produce oncogenic proteins of other viral and cellular origins. The mechanisms controlling oncogene expression are studied hy cloning and transfection of the hybrid fused DNA consisting of the 5' control regions of the human metallothionein (Met) gene and oncogenes cloned from viruses and normal or tumor cell lines. These studies will give insight to the molecular mechanisms of gene expression associated with the oncogenic processes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

K. Kohno Visiting Fellow LMC, NCI

Objectives:

1) To observe the molecular mechanism of cellular malignant transformation by active expression of oncogenes of viral and cellular origins; 2) to produce oncogene products in large quantities for their characterization and the production of monospecific antibodies; and 3) to establish a model system for the study of the control mechanisms of gene expression associated with malignant transformation.

Methods Employed:

1) A plasmid containing lambda phage strong promoter PL, CII and O-gene was constructed (pKld). 2) A plasmid containing modified polyoma virus DNA which encodes only MT was obtained from R. Kamen and subcloned in pBR322. 3) The MT gene was inserted in the pKld plasmid and transformed into a CI857 lysogen of E. coli which contains temperature sensitive mutation in CI gene. 4) Transformants were selected by culturing the cells in medium containing ampicillin. 5) The temperature shift from 32°C to 42°C activates PL promoter and initiates OMT production. 6) The OMT produced was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by transblotting onto a nitrocellulose filter. The filter was incubated with the rabbit anti-peptide serum specific for MT, washed, incubated with the second antibody (goat anti-rabbit IgG) conjugated with horse radish peroxidase, and treated with 4-chloro-1-naphthol-H_2O_2 solution. 7) A band containing MT was further purified by HPLC using a C-3 reverse phase or TSK column. The gel extracts were injected into rabbits for antibody production. 8) Peptide mapping was carried out by labeling of OMT with ^{35}S-methionine, banding on SDS-PAGE, followed by autoradiographic analysis. Positive bands were crushed and were loaded onto SDS-gels containing various amounts of V8-enzyme. The gels were treated with PPO, and processed for autoradiography.

Major Findings:

A strain of E. coli lysogenized by CI857 was transfected with the expression vector containing the PL promoter and the OMT gene. Soon after shifting the incubation temperature from 32°C to 42°C, it produced a large quantity of the fused protein. The protein was identified by SDS-PAGE and immunoperoxidase staining after transblotting onto a nitrocellulose filter membrane. From DNA sequencing, it was deduced that the 23rd amino acid from NH_2 terminal of O-gene had been fused with the 21st amino acid of MT in the pMT1-8 clone. We isolated 30 other clones that have different sizes and joining sites because the antigenic site is assumed to be located at amino acid residue 311 to 319 near the COOH terminal. One of the clones, having 114 amino acids of COOH terminal side, was used for injection into rabbits to produce MT specific antibody. A partial cleavage pattern produced by staphylococcus V8 protease treatment of OMT from the MT1-8 clone was compared with and was indistinguishable from that of authentic MT

isolated from productively infected 3T6 cells. It is known that only a subpopulation of MT is associated with MT kinase activity. Post-translational modification of MT is likely required either to activate its kinase activity or to activate it as a substrate for phosphorylase. As expected, OMT did not demonstrate kinase activity. However, the purified protein will serve as a desirable model for studies of post-translational modification and activation of biological function. Exogeneous addition of cellular enzymes and microinjection of the OMT will be tested in future studies.

Significance to Biomedical Research and the Program of the Institute:

The mechanism of cellular transformation has not yet been elucidated, but comparison of modes of action of oncogenes of viral and cellular origins provides a clue that transformation is mediated by activation of an oncogene. The interactions of oncogene products with cellular components are important to transformation but are still unclear. It has been difficult to evaluate the roles of individual oncogene products because the purification of large quantities of oncogene products is difficult. Recombinant DNA techniques have made possible the production of specific proteins encoded by specific genes which are usually not expressed at high levels in cells. We constructed an expression vector that allowed us to produce OMT in a large quantity. The OMT produced in E. coli lacks secondary modification and, therefore, is not active either in terms of auto- or trans-phosphorylation or protein kinase activity. This will serve as an ideal substrate for post-translational modification and possible activation under appropriate experimental conditions. The pure protein can be used for production of monospecific antibodies. Artificial MT-like proteins and corresponding antibodies may be produced by gene manipulation. Application of such antibodies for immunotherapeutic purposes may open a new methodological approach for cancer treatment.

Proposed Course:

1) The expression vector system developed in the present study will continue to be used for production of other oncogene products. The proteins are purified and used for studies of post-translational modification and activation. 2) The genomic clones of human metallothionein genes are divided into three major components: the 5' control regions, the coding sequences and the 3' uncoded region. The control region is linked to polyoma or SV40 early region from which their own 5' control region was removed. The hybrid DNA is cloned and transfected into human cells. The expression of the early gene by heavy metals (Cd, Zn) is assayed by expression of the early gene and the cell morphology. Deletion, insertion and point mutations of the 5' control region will be constructed and studied for their expression mechanisms.

Publications:

Kakefuda, T.: Human carcinogenesis studied by DNA transfection. Oncologia 4: 143, 1983.

Kakefuda, T.: Mechanistic studies of mutation and cancer by chemical carcinogens. In Yamamoto, M. (Ed.): Challenge to Incurable Diseases. Tokyo, Univ. Tokyo Press, (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05339-01 LMC

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Radioimmunoassay of Cytochromes P-450 Using Monoclonal Antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
B. J. Song Visiting Fellow, LMC, NCI

COOPERATING UNITS *(if any)*
none

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Multiplicity of microsomal cytochrome P-450 is being studied by utilizing monoclonal antibodies (MAbs) to 3-methylcholanthrene (3MC) and phenobarbital (PB) induced rat liver cytochrome P-450. These MAbs directly bind both the corresponding microsomes and purified cytochrome P-450. A semiquantitative, direct radioimmunoassay (RIA) has been developed to measure cytochrome P-450 in the microsomes from various tissues in animals that are untreated, or treated with 3MC or PB. The amounts of cytochrome P-450 in different tissues and species are also being examined by competitive RIA. This analysis provides an approach to the study of cytochrome P-450 multiplicity that is complementary to enzymatic and structural studies. A detailed atlas of the cytochromes P-450 present in different tissues, species, and strains will aid in understanding the diversity of the cytochromes P-450 and their role in individual susceptibility to carcinogenesis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

T. Fujino	Expert	LMC, NCI
S. S. Park	Senior Staff Fellow	LMC, NCI
F. K. Friedman	Senior Staff Fellow	LMC, NCI
H. V. Gelboin	Chief	LMC, NCI

Objectives:

In order to understand the detailed genetics and the role of cytochromes P-450 in carcinogen and drug metabolism, a phenotypic description of their exact number and quantity in various species and tissues is necessary. Monoclonal antibodies (MAbs) to 3-methylcholanthrene (3MC) or phenobarbital (PB)-induced rat liver cytochrome P-450 are used as highly specific probes in the development of radioimmunoassays (RIAs) for different cytochromes P-450.

Methods Employed:

Monoclonal antibodies were prepared by the general method of Kohler and Milstein. The microsomes were prepared from different tissues from species which were untreated, or treated with either 3MC or PB. Rat, hamster, guinea pig, and the genetically defined mouse strains C57BL/6 and DBA/2 were studied. The cytochrome P-450 content of the microsomes from these tissues were examined by either a direct or competitive RIA using [^{35}S] labeled MAb. The RIA data were compared with enzymatic activities in the absence and presence of monoclonal antibody. Aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (ECD) were measured by a fluorometric assay; the hydroxylations of benzphetamine and ethylmorphine, which are more specific indicators of the presence of PB-induced cytochrome P-450, were assayed by the colorimetric method of Nash.

Major Findings:

1) Monoclonal antibodies are a useful tool in the identification of specific cytochromes P-450 in crude microsome preparations, as evidenced by solid-phase RIA. In rat liver, there is considerable elevation (greater than 50-fold) in the level of MC-induced cytochrome P-450 in MC-treated rats, relative to the level in control and PB-treated rats. Similar differences were also observed for livers from guinea pig and mouse C57BL/6 mice, but not in those from hamster and DBA/2 mice. 2) The species-dependent differences in the amount of MC-induced cytochrome P-450 is consistent with the corresponding data on AHH activity. 3) In 3MC-induced rats, tissue-dependent differences were observed: liver has a more than 30-fold higher level of cytochrome P-450 than lung or kidney. 4) Comparable tissue-dependent differences were also observed in measurements of AHH activity. 5) The apparent tissue-dependent difference in the amount of MC-induced cytochrome P-450 was evident with mouse strain C57BL/6, but not with the relatively nonresponsive DBA/2 strain.

Significance to Biochemical Research and the Program of the Institute:

Development of RIAs and an atlas of cytochromes P-450 classified by MAbs will result in a better understanding of cytochrome P-450 multiplicity and genetic control. It will also help to determine the role of particular cytochromes P-450 in drug and carcinogen metabolism, and in assessment of individual differences in cytochrome P-450 content.

Proposed Course:

Our RIA procedures will be refined to develop a quantitative, sensitive, and reproducible method for detection of cytochromes P-450 that are recognized by specific MAbs. Tissue microsomes from different species have been collected. Some crosses of genetically defined mouse strains will be investigated in order to clarify the involvement of genetic factors in the regulation of cytochrome P-450 systems. The RIA will also be applied to human tissues to examine inter-individual differences; for comparison, the influence of MAbs on enzymatic activity will be determined.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF MOLECULAR ONCOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1982 to September 30, 1983

The Laboratory of Molecular Oncology conducts research on the molecular elements responsible for the development and expression of malignant phenotypes in humans and animals. The Laboratory applies skills in molecular biology, recombinant DNA technology and hybridoma-monoclonal antibody production in a comprehensive program to identify and isolate cellular transforming genes and to characterize products expressed by these genes. This is accomplished by bringing together expertise in the diverse disciplines of eukaryotic and prokaryotic virology, molecular biology and genetics. The Carcinogenesis Regulation Section studies the relationship between oncogenic viral gene expression and the conversion of cells from normal to transformed phenotype. Specific regions of molecularly cloned acute transforming retroviruses genomes are tested for transforming activity and molecular mechanisms of interaction with the cell are elucidated. The Cellular Transformation Section investigates the malignant transformation of cells by avian sarcoma viruses, including the function of the virus-coded protein directly responsible for transformation, the primary physiological effects of the functioning protein, the sequence of metabolic changes resulting in the altered metabolic profile characteristic of malignant cells, and the metabolic changes necessary for the maintenance of the malignant state. The Microbiology Section investigates the mechanism of cell transformation using biological, biochemical and immunological techniques. The transforming potential in mammalian cells of specific viral and cellular DNA sequences amplified by cloning in appropriate prokaryotic vectors is determined under selective conditions using characterized markers. Transformation studies are augmented using monoclonal antibodies prepared against various viral and cell-coded proteins. The Molecular Control and Genetics Section conducts studies to control gene expression in the prokaryote, E. coli and its phage lambda. The molecular basis of gene regulation is determined at the level of transcription initiation, transcription termination, RNA translation, and RNA processing. Mutants are isolated in control sites and in control genes and the effects of these mutations on RNA and protein synthesis are studied in vivo. The Tumor Biochemistry Section investigates the genetic elements responsible for cellular transformation. Specific regions of viral and cellular DNA thought to be involved in the expression of cellular transforming phenotypes are isolated by recombinant DNA techniques. Structural properties of the cloned fragments are determined and their transforming activities characterized in biological assays. The characterization and identification of these elements provides a means for examining normal mammalian DNA for the occurrence of molecular elements with similar properties.

The major portion of the present and future emphasis of this Laboratory concerns the identification and analysis of DNA sequences and gene products involved in neoplastic transformation. We have pursued these studies in several major areas. First we have analyzed the structural and biological properties of viral onc genes present in both avian and mammalian acute transforming retroviruses. We have identified, isolated and characterized normal cellular homologues of these viral onc genes from their species of origin as well as from the human genome. Through

a combined effort of collaborators from two Sections within the Laboratory of Molecular Oncology a procaryotic vector has been developed which allows the expression of high levels of onc genes in E. coli. This has provided a means for both studying the chemistry of the protein and for preparing appropriate immunological reagents for studying the expression of the oncogene product in transformed cells and tumor cell lines. These studies could only have been accomplished by having this unique combination of investigators skilled in prokaryotic genetics and in oncogene transformation systems of higher eukaryotes.

The Tumor Biochemistry and Microbiology Sections have collaborated and analyzed the cloned transforming virus of Moloney sarcoma virus (MSV) containing the transforming sequence v-mos and its cloned normal cellular homolog c-mos from both mouse and human. We have been interested in identifying the sequences required for mos transformation and in understanding the mechanism by which the normal cellular homologs can be activated to express transforming potential. We have attempted to develop techniques for efficient detection and isolation of transforming sequences present in human tumors and tumor derived cell lines. In both cases we utilized sensitive and newly developed DNA transfection procedures in conjunction with recombinant DNA techniques to isolate dominant transforming genes. We have also developed a novel eukaryotic-prokaryotic retroviral shuttle vector which allows sequences inserted into this vector to be propagated in E. coli as well as following transfection into eukaryotic cells, to be rescued as a retrovirus. This virus can subsequently be used to infect cells capable of amplifying retroviral intermediates as DNA copies in sufficient quantity so that the DNA can be directly transfected back into E. coli. The potential of this vector is many fold. First, it can be used to generate cDNA copies from genomic DNA by rapidly transferring cloned genomic sequences processed as a retrovirus intermediate in eukaryotic systems back into E. coli. Secondly, the system appears to have transducing potential. It may also provide a means for expressing genes in eukaryotic cells at high levels.

The provirus of the MSV contains both the MSV-specific transforming sequence v-mos as well as sequences which enhance or activate RNA synthesis. These latter sequences are found in the MSV long terminal repeat (LTR) which has been shown to be necessary for the efficient expression of the transformation potential of v-mos and its murine cellular homolog c-mos. The LTR acts to enhance v-mos transformation when linked either 5' or 3' relative to v-mos. An analysis of polyadenylated RNA indicates RNA synthesis either initiates or terminates in the LTR depending on its location relative to mos. The enhancing properties of the proviral LTR have been shown to reside within the 72 bp repeat sequence present in the unique 3' region of the LTR. We have also identified a cis-acting sequence within the 5' normal mouse DNA flanking sequence upstream from the murine cellular mos locus that inhibits activation of transformation by a 3' LTR. Removal of this normal mouse sequence permits activation of the transforming potential of c-mos. This locus has been pinpointed to within 200 nucleotides and resides 1800 bases upstream from the beginning of the open reading frame of the cellular mos locus. This is the first characterization of a negative acting cis control element. We have identified DNA sequences upstream from the mos gene in molecularly cloned human and mouse cellular loci and have identified a region 400 bases in front of the conserved mos open reading frame in human and mouse which is highly homologous. These studies were performed in order to identify the origin of the mos gene specific mRNA. Since expression from this locus has never been detected in vivo, we have attempted to identify the mos specific domain by identifying repetitive elements surrounding the mos gene.

Often these elements have been shown to bracket structural gene domains. The two conserved regions in mouse and human (i.e., upstream sequence of 200 nucleotides and the open reading frame) may represent a transcription locus. This is testable with some of the in vitro constructed mos-LTR recombinants that we have generated in this lab and will be the subject of studies in the forthcoming year.

We have determined the retroviral DNA sequences upstream from the v-mos gene in two molecularly cloned MSV isolates in order to determine the origin of the viral subgenomic mos mRNA. A number of sequence alterations have been identified which we believe to be responsible for the production of the mos gene mRNA and we are currently making recombinants between the two MSV isolates in order to test this hypothesis. Collectively, these studies will provide us with a thorough understanding of the regulation of expression of this transforming gene in the virus. Our analyses indicate that the viral mos gene in cellular transformation is effected with low levels of expression (i.e., approximately 1 to 10 copies of RNA per cell). This is consistent with the amount of protein believed to be expressed in transformed cells (i.e., approximately one thousand molecules per cell). This identifies mos as one of the most potent transforming genes yet to be described.

In a model for transformation whereby a retrovirus integrates adjacent to a normal cell gene with transforming potential it is important to know whether the methylated state of this DNA precludes activation of transformation. We have shown that methylation of v-mos results in a marked reduction of transformation efficiency; however, in experiments where both sequences are cotransfected far less reduction is observed when the LTR is methylated instead of the viral mos sequence. When both v-mos and LTR sequences are methylated, the reduction in transforming frequencies are greater than the reduction observed when the entire MSV provirus is methylated. However, we have demonstrated that supermethylation of DNA with two different methylases does not yield an additive effect. It appears from these analyses that methylation of a few sites in DNA is sufficient to inhibit transformation. Methylation of additional sites does not further reduce transforming activity and suggests that regulation of expression by methylation be effected by only a few sites in a genetic locus and there does not appear to be site specificity in this regulation.

A human DNA fragment containing a region homologous to the v-mos transforming gene of MSV was sequenced and compared to the sequence of the mouse cellular homologue of v-mos. The human gene contained an open reading frame of 346 codons that converged with an initiation codon (ATG) found in the mouse cellular mos locus. The polypeptides predicted from the open reading frame were extensively homologous as well. Attempts to activate the human mos gene with LTRs and hybrid recombinants containing human mos and the first 50 codons from the v-mos of MSV were unsuccessful. We developed a technique for isolating unique hybrid DNA recombinants between human mos and the viral mos region of Moloney sarcoma virus in E. coli. We have characterized these recombinants by both identifying the region in mos where the hybrid molecules have been formed and testing their biological transforming activity in order to identify the region of the mos gene that is essential for focus formation. These analyses have provided the first application of recombinant DNA to make hybrid protein molecules and more importantly they have allowed us to identify important domains in the mos gene that are essential for transforming activity. It is now possible by conventional recombinant DNA techniques to specifically identify the amino acids contributing to the structure of a transforming protein.

We have developed a screening method in nude mice for detecting dominant transforming genes. Five such genes have been identified by this technique. One gene has been partially cloned from a human pancreatic carcinoma cell line which has been shown to be a member of the family of human sequences related to the Kirsten ras oncogene. A second dominant transforming gene has been isolated from a human tertocarcinoma cell line. This oncogene also is a member of the human ras oncogene family but is the n-ras oncogene. Several other genes are in the process of being cloned. One is clearly not a member of the ras family and is derived from a chemically transformed human tumor cell line. This transfection assay appears to be as sensitive as the focus forming assay and has the additional feature of allowing the assay to be expanded to test for many more DNA samples. We also are attempting to develop human cells as recipients for transforming genes in DNA transfection assays since the use of the single recipient cell line (NIH 3T3) raises questions regarding the range of oncogenes detectable and the role of genes isolated in rodent cells. We are utilizing continuous human cell lines which express the normal phenotype, are nontumorigenic in athymic nude mice and which are transfectable at reasonable efficiencies. Certain hybrids of human fibroblasts and tumorigenic HeLa cells fulfill these criteria and are currently being tested for their ability to be transformed by a collection of cloned retroviral oncogenes. We are also examining diploid human and rodent primary cells for their ability to be transfected and transformed and we are attempting to determine what multiple factors are required to render these cells tumorigenic following DNA transfection. These approaches should allow us to detect a wider range of human oncogenic sequences in a system more closely analogous to the one in which tumors are naturally induced in animals and man.

The overall objectives of the Carcinogenesis Regulation Section are to delineate the relationship between virus gene expression and conversion of cells from normal to malignant state. These studies included termination of the molecular anatomy of known tumor viruses and the mechanism by which subviral structures act in concert with cellular factors to regulate oncogenesis. To investigate the process by which viral oncogenes, as well as their cellular homologs, induce the activation of the metabolic processes and participate in malignant transformation. To delineate at the molecular level the mechanism by which oncogenes act in concert with cellular factors to induce oncogenesis. To introduce functionally modified oncogenes to specific target cells in an effort to analyze and alter the function of their normal counterparts. The technique of molecular cloning, DNA sequence analysis, and site mutagenesis have been used to implicate specific nucleotides in the transformation process.

The nucleotide sequence of the integrated proviral genome of avian myelocytomatosis virus (MC29) coding for gag-myc protein has been determined. By comparison of this nucleotide sequence with the helper virus as well as the c-myc region, it was possible to localize the junction points between helper viral and v-myc sequences. These studies demonstrate that (i) the large terminal repeat sequence of MC29 is very similar to that of Rous sarcoma virus; (ii) the viral genome has suffered extensive deletions in the gag, pol, and env genes; (iii) the gag region can code for p19, p10, and part of p27; (iv) the recombination between viral and cellular sequences occurred in the coding region of p27 such that the open reading frame extends for an additional stretch of 1,266 base pairs, resulting in a gag-myc hybrid protein; (v) the open reading frame terminated within the v-myc region 300 bases upstream of v-myc-helper viral junction; and (vi) the v-myc helper-viral junction at the 3' end occurred in the middle of env gene, rendering it defective.

Myelocytomatosis virus MC29 is a defective avian retrovirus with a hybrid transforming gene (Δgag-myc) consisting of a 1,358-bp sequence from the retroviral gag gene and a 1,568-bp sequence (v-myc) shared with a cellular locus, termed c-myc. We have subjected to sequence analysis 2,735 bp of the cloned c-myc gene, which includes the v-myc-related region of 1,568 bp, an intervening sequence of 971 bp, and unique flanking sequences of 45 bp and 195 bp at the 5' and 3' ends, respectively. Analysis of the genetic information and alignment of the c-myc sequence with the known sequence of MC29 indicates that: (i) the two myc sequences share the same reading frame, including the translational termination signal; (ii) there are nine nucleotide changes between c-myc and v-myc that correspond to seven amino acid changes; (iii) the 971-bp intervening sequence of c-myc can be defined as an intron by consensus splice signals; (iv) the unique 5' sequence of c-myc could either extend its reading frame beyond the homology with v-myc or could be an intron because its junction with the myc region of the locus is a canonical 3' splice-acceptor site; (v) the v-myc contains 10 nucleotides at its 5' end not shared with the c-myc analyzed here and also not with known gag genes, probably derived from an upstream exon; and (vi) the c-myc locus can generate a mRNA whose termination signals have been identified to be located 83 bp and 119 bp from the point of divergence between the v-myc and c-myc. We conclude that the gene of the c-myc locus of the chicken and the onc gene of MC29 share homologous myc regions and differ in unique 5' coding regions, and we speculate, on this basis, that their protein products may have different functions. The hybrid onc gene of MC29 must have been generated from the c-myc gene by deletion of the 5' cellular coding sequence, followed by substitution with the 5' region of the viral gag gene.

We have determined the complete nucleotide sequence of human cellular c-myc, which is homologous to the transforming gene, v-myc, of myelocytomatosis virus MC29. Analysis of the genetic information and alignment with the known sequence of chicken c-myc and v-myc indicates: (i) an intervening sequence can be identified by consensus splice signals. The unique 5' sequence of c-myc and its junction with the v-myc region may be a canonical 3' splice acceptor. (ii) The c-myc locus can generate a mRNA whose termination signals are downstream from the translational termination signal. (iii) The three myc genes share the same reading frame, including translational termination signals. (iv) The homology is conserved only in the coding region. (v) Most changes at the nucleotide level result in no change in the amino acid. (vi) There are two distinct domains--the 5' unique domain, which is different from the viral, and the 3' coding domain, which contains amino acids coded by the two exons whose sequences have been determined here. In the latter domain, the amino acid variation between v-myc and chicken c-myc is less than 2%, whereas that between the chicken v-myc and the human is 27%, with the variation concentrated in the region that flanks the splicing points.

The genome of the avian myeloblastosis virus (AMV) has undergone a sequence substitution in which a portion of the region normally coding for the env protein has been replaced by cellular sequences. We have determined the complete nucleotide sequence of this region. Examination of the AMV oncogenic sequence revealed an open reading frame starting with the initiation codon ATG and terminating with the triplet TAG within the acquired cellular sequences and terminating with the triplet TAG at a point thirty-three nucleotides into helper viral sequences to the right of the helper-viral-cellular junction. The stretch of 795 nucleotides would code for a protein of 265 amino acids with a molecular weight of 30,000 daltons. The eleven amino acids at the carboxy terminus of such a protein

would be derived from the env gene of helper virus. Antibodies were prepared against synthetic peptides derived from the predicted amino acid sequences. One such antibody precipitated two magnesium proteins of apparent nucleotide weight of 30,000 daltons and 51,000 daltons.

A plasmid, pJL6, was constructed that contains a unique Cla I site twelve codons beyond the bacteriophage λ cII gene initiation codon. This site allowed us to fuse the carboxy-terminal portion of the cII gene. Transcription of the hybrid gene is controlled from the phage λp_L promoter. When this promoter is derepressed, E. coli cells harboring the chimeric plasmid produce a level of cII-myc fusion protein greater than 5% of total cellular protein. Antibodies raised by this protein form an immunoprecipitate with the MC29 gag-myc gene product, P110gag-myc.

The Cellular Transformation Section investigates the malignant transformation of cells by avian sarcoma viruses, including identification of virus-coded proteins directly responsible for transformation, determining the cellular location and direct function of these proteins, the primary physiological effects of the functioning protein, the sequence of metabolic changes resulting in the altered metabolic possible characteristic of malignant cells, and the metabolic changes necessary for the maintenance of the malignant state.

Current investigations include the identification of transforming proteins coded by avian myeloblastosis and MC29 viruses. Neither protein was found to have protein kinase nor to be glycosylated, although the MC29 myc protein is phosphorylated. Although no structural similarity is evident in these proteins, both are found predominantly in the nuclei of infected cells, suggesting a direct role for these tumor-inducing proteins in altering transcription. A normal cellular homologue of the viral myc coding sequence is involved in certain human murine and avian lymphomas, and cellular proteins similar to the transforming region of the viral protein are being investigated.

In cells transformed by Rous sarcoma virus, attempts have been made to relate characteristic changes with the activity of the viral transforming protein. Also, the interrelationships of ion transport, glucose transport, the enzymes involved in ion transport and glucose metabolism, and the production and utilization of ATP have been examined in an attempt to explain the metabolic changes observed after cells become malignant.

The Molecular Control and Genetics Section is continuing its studies of gene expression. New developments in the control of gene expresion have recently been published by this laboratory. These studies cover the following areas:

1. Positive activation of transcription initiation by RNA polymerase and an auxilliary factor, λ cII protein.

2. Transcription termination control on gene expression where the amount of termination or read through at a particular terminator controls and determines expression rates of downstream genes.

2. Negative control of expression from a controlling site distal to the gene. This is called retroregulation. Retroregulation is a new form of gene control discovered by this section in collaboration with Dr. Gabriel Guarneros of Mexico City.

4. The effect of transcription termination on mRNA stability.

These studies have enabled us to better understand how genes are controlled and expressed. We have been using this knowledge to express genes from higher organisms (man included) in bacterial systems. This provides many benefits for the study of these genes and their products. Additionally, an understanding of the basis of gene control in E. coli has allowed us to better observe and analyze gene expression in higher organisms.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04846-11 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Analysis of Viral Infection and Its Control at the Cellular Level

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

B. I. Gerwin Chemist, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.67	1.0	0.67

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In the course of studying a non-conditional polymerase mutant of B-tropic MuLV we have established the existence of pauses during endogenous reverse transcription and suggested a consensus sequence which may determine pausing by reverse transcriptase. We have located the mutation in the pol gene within a 400-bp region and are currently determining the DNA sequence of this region in the mutant and wild-type viruses. In addition, we have determined the DNA sequence upstream from the transforming gene in two molecularly cloned sarcoma viruses which either do or do not efficiently splice a subgenomic mRNA for the transforming gene. We have shown that the expected splice acceptor has been deleted in the MSV which does not efficiently splice subgenomic mRNA. In addition, sequence alterations have been detected which would account for known alterations in the gag gene product by one of these MSV isolates.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

J. Levin Chemist LMG, NICHD

Objectives:

The goal of this project is to understand at the molecular level, the life cycle of RNA tumor viruses, i.e., the expression of these viruses in cells infected both nonproductively and productively, as well as to understand the cellular control mechanisms applicable to these viruses. The topics of present interest are:

(A) Analysis of the products of in vitro DNA synthesis by a mutant polymerase molecule. Further characterization of the mutant viral genome which codes for this protein.

(B) Determination of the DNA sequences controlling the mRNA specific for the transforming mos protein in Moloney MSV.

Methods Employed:

(A) Enzymes are purified from disrupted virions and cells using affinity and ion-exchange chromatography and velocity gradients. Products of DNA synthesis by mutant and wild-type enzymes are compared by gel electrophoresis, DNA blotting techniques and filter hybridization. Intermediates are isolated and characterized by annealing to specific probes and by restriction enzyme digestion. Kinetics of enzymatic reactions are analyzed.

(B) Restriction endonuclease cleavage patterns of DNA of interest are determined. DNA base sequence is determined by the Maxam-Gilbert technique.

Major Findings:

(A) We have shown that several intermediate DNA species arise from enzyme pausing during endogenous reverse transcription in MuLVs. Using the expected sequences of the intermediates and the known sequence of the AKR genome, we have located consensus sequences which are significantly correlated with pause sites.

In addition, through techniques of molecular cloning, we have isolated the defect of the polymerase mutant to a 400 bp region of the pol gene.

(B) The sarcoma virus which splices inefficiently contains a 48 bp deletion just 3' of the mos gene which contains the most likely splice acceptor for the subgenomic env gene message. This acceptor sequence is present in the sarcoma virus which splices efficiently. This finding indicates that (i) the env gene splice acceptor from Moloney leukemia virus is utilized in the predominant splicing pathway for generating subgenomic mos message, and (ii) other acceptor sequences may be utilized albeit inefficiently to generate a mos message in ml murine sarcoma virus.

In addition, sequencing of HT-1 and ml-MSV has located several interesting changes from the parental Moloney leukemia virus which are located in the region corresponding to the genes for p30, p10, and pol. In ml-MSV, a single base change near the carboxyl terminus of p30 changes a histidine to a tyrosine. This change introduces an iodination site which accounts for the new p30 peptides of ml described in earlier work. After 72 bp of p10 a single deletion of 3.05 kb occurs from map position 2108 to 5159 of the the Moloney leukemia virus sequence after which no other major changes from the leukemia sequences occur until the 48 bp deletion described above and the mos junction.

In HT-1 MSV, which produces no detectable gag or pol protein, several major changes from the leukemia virus sequence occur in the p30-pol region. At 1830 bp, where a stretch of 6 As occurred in Moloney leukemia sequence, an additional 16 As have been added. A 2.3 kb deletion occurs between 2150 and 4518 bp of the leukemia virus where a segment is deleted between a pair of 7 bp direct repeats. From this point to the mos junction, the sequence is that of the leukemia virus except for a region at approximately 5200 bp where several Gs are converted to As resulting in a A,T rich stretch. Similarly at approximately 5275 bp a polyA stretch of 20 bp occurs interrupted by only two Ts. This stretch results from changes of 4 Gs and 1 T to As and the insertion of 4 As and a T. It is not clear at this time whether these changes result in changes in gene expression.

Significance to Biomedical Research and the Program of the Institute:

(A) Further characterization of this polymerase mutant will increase our understanding of the mechanisms of RNA tumor virus replication. Identification of a sensitive portion of this reaction may reveal a phase of the viral life cycle which would provide a target for chemotherapy.

(B) Information on genetic signals for control of mRNA production and processing are of major importance to understanding and manipulation of regulated growth of mammalian cells.

Proposed Course:

(A) The 400 bp fragment containing the mutation will be sequenced in the mutant and a wild-type virus.

(B) The region containing the 48 bp deletion will be exchanged between cloned ml-MSV (inefficient splicing) and HT-1 MSV (efficient splicing). This exchange should be all that is required to provide efficient splicing to ml-MSV if the splice acceptor deletion is the only cause of the splicing failure. In addition the two mutants will be sequenced in the region just 3' of the 5' LTR in order to determine signals allowing gag gene expression (ml-MSV) or lack of expression (HT-1 MSV).

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04876-11 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Oncogenic Virus Influence on the Biochemical Events of Host Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Paul Ebert Research Chemist, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Succinylacetone (SA) is a specific irreversible inhibitor of delta-aminolevulinic acid dehydrase (ALAD), the second enzyme of the heme pathway. ALAD from broken L1210 cells was inhibited completely by 1 micro mole SA. The compound was considerably less active against the enzyme in whole cells, but stopped cell growth at concentrations which only partially inhibited the enzyme. The rapid uptake of hematoporphyrin (HP) could be further enhanced by pretreatment of L1210 cells with SA. SA inhibited L1210 cell growth by a mechanism independent of the inhibition of heme biosynthesis. HP uptake in the nonmalignant line, NIH3T3, was lower than in malignant L1210 cells and this low rate could not be increased by incubation of the NIH3T3 cells with SA. Treatment of L1210 cells with SA for three days decreased the endogenous respiration by 50% and inhibited cell growth. An enhancer fragment from the long terminal repeat region derived from the Moloney sarcoma virus and an Alu sequence from a human source were cloned into the vector pi AN-7.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

G. F. Vande Woude	Chief	LMO, NCI
Michael Tainsky	Staff Fellow	LMO, NCI

Objectives:

To determine how succinylacetone (SA) affects the metabolism of cancer cells and inhibition growth. SA, a new potent inhibitor of heme biosynthesis, inhibits growth of L1210 cells by some unknown mechanism not related to inhibitor of heme synthesis. To determine where in the electron transport chain SA is causing a block and if this block is the prime cause of the growth inhibition observed. To construct a plasmid containing an enhancer fragment to be used to observe if certain unexpressed genes can be activated in its presence.

Methods Employed:

Malignant L1210 cells and nonmalignant NIH3T3 cells grown in culture were incubated with hematoporphyrin and the uptake of this compound was determined by a fluorimetric method and compared to the cellular protein concentration. Endogenous respiration (oxygen uptake) of L1210 cells incubated with and without SA was measured in a polarimeter. Alu and enhancer DNA sequences were cloned into a mN-7 vector using standard molecular biology techniques. Various bacterial clones receiving ligated fragments were screened for the presence of Alu sequences with P^{32}-Alu probes. Phage containing a human library (Alu segments) and another strain containing MSV-LTR (enhancer sequences) were used to screen bacterial clones for the presence of Alu and enhancer sequences in the plasmids.

1. Augmented hematoporphyrin (HP) uptake by succinylacetone in L1210 cells. Four-6-dioxoheptanoic acid (succinylacetone, SA) is a specific irreversible inhibitor of δ-aminolevulinic acid dehydrase (ALAD), the second enzyme of the heme biosynthesis pathway. ALAD from broken leukemia L1210 cells was inhibited 100% by μM SA. SA was considerably less inhibitory against ALAD of whole cells suggesting that this watersoluble drug passes through the membrane with difficulty. L1210 cells rapidly take up HP from the medium. Hematoporphyrin is of interest because of its propensity for malignant tissue. This rapid uptake can be further enhanced by pretreatment of the cells with SA for several days. SA-treated cells reached the saturation concentration of cellular HP with lower HP concentrations in the medium than did untreated cells. SA inhibited the growth of L1210 cells by some mechanism independent of the inhibition of heme synthesis, since the addition of serum (which contains heme) to cultures of cells containing SA did not reverse the growth inhibition due to SA. HP uptake in the nonmalignant line, NIH3T3, was lower than in malignant L1210 cells, and this low uptake rate could not be increased by incubation of the 3T3 cells with SA.

2. Effect of succinylacetone on respiration of L1210 cells. L1210 leukemia cells have an active respiration rate of about 16 nanoatoms oxygen per 10^7 cells per minute. The respiration is of mitochondrial origin as demonstrated by its inhibition by inhibitors of mitochondrial electron transport such as rotenone, antimycin, azide, and cyanide. Moreover, the inhibition observed with oligomycin was released by uncouplers of oxidative phosphorylation, an observation that strongly suggests the presence of mitochondrial respiration. Oxygen uptake by the L1210 cells was completely inhibited by 500 μM TFNB, a transition metal chelator. Addition of menadione enhanced the respiration six-fold and overcame the inhibition by TFNB. It is thought that the site of action of the transition metal chelator is between the ubiquinone pool and the non-heme iron of the mammalian respiratory chain. Cells grown in the presence of SA exhibited diminished respiration of approximately 50% of the untreated control values. Addition of SA to the cell suspension during the polarigraphic assay of oxygen consumption had a very minimal effect. This diminished respiration could be overcome by menadione which acts in the region of the primary dehydrogenases and effectively bypasses the electron carriers of the chain by reacting directly with molecular oxygen.

3. Cloning of Alu and enhancer sequences into the vector πAN-7. Alu sequences from πBlur and enhancer sequences from pM1SP DNA were ligated with the vector πAN-7 and inserted into the bacteria P3W3110. Colonies were screened for the presence of Alu sequences by hybridization with Alu probes. Several bacterial colonies containing linear plasmids of about 1.3 kb or greater were further examined for the presence of Alu segments by Southern blotting against Alu probes. Two clones were found to contain both the Alu and enhancer sequences by phage recombination studies.

Significance to the Biomedical Research and the Program of the Institute:

SA has shown antitumor activity against several malignant cell systems in vitro and in vivo, but it appears to have growth inhibitory activity which is not associated with its demonstrated potent activity against the heme biosynthetic pathway. Investigation of the mechanism of action of SA may lead to a better understanding of the growth properties of malignant cells and to the design of possible analogs of SA which may pass through the cell wall more efficiently. Tumor tissue selectively takes up HP and certain other porphyrins including heme. This property has been utilized for the detection of malignant neoplasms and for the selective destruction of tumor tissue by phototherapy. The biochemistry of this process is poorly understood at present. A better understanding of this process could lead to increased use of this recently developed antitumor technology.

Proposed Course:

We propose to identify a new specific protein(s) which appears after transformation of cells by avian tumor viruses, and which are dependent upon new transcription. The goal of this project is to analyze the sequence of events leading to changes in transcription occurring during virus-induced transformation.

Publications:

Ebert, P. S., Frykholm, B. C., Hess, R. A., and Tschudy, D. P.: Characteristics of hematin uptake in malignant, embryonic and normal cells. Cancer Biochem. and Biophys. 6: 157-165, 1983.

Ebert, P. S., Hess, R. A., and Tschudy, D. P.: Augmentation of hematoporphyrin uptake and inhibition of L1210 leukemia cell growth in vitro by succinylacetone. JNCI (In Press).

Ebert, P. S., Smith, P., Bonner, R., Hess, R. A., Costa, J., and Tschudy, D. P.: Effect of defined wavelength and succinylacetone on the photoinactivation of leukemia L1210 cells in vitro by hematoporphyrin. Photobiochem. and Photobiophys. (In Press).

Tschudy, D. P., Ebert, P. S., Hess, R. A., Frykholm, B. C., and Atsmon, A.: Antitumor activity of succinylacetone against Walker 256 carcinosarcoma, Novikoff hepatoma and L1210 leukemia in vitro and in vivo. Oncology 40: 148-154, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04882-09 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Events in Tumor Virus Replication and Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

G. F. Vande Woude Chief, Tumor Biochemistry Section LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human DNA sequence homologous to v-mos, the transforming gene of Moloney murine sarcoma virus (MoMSV), contains a polypeptide coding sequence that is 75% homologous to the mouse cellular mos (c-mosmu). Neither the mouse nor human (c-moshu) sequence are capable of transforming NIH 3T3 cells. However, c-mosmu can be activated to transform mouse cells when linked to the long terminal repeat sequence (LTR) of MoMSV. Similar LTR-c-moshu recombinants are inactive in this assay. To locate the specific regions of the c-moshu coding region responsible for inactivity a series of mos gene hybrids were generated in E. coli. between c-moshu and v-mos. Analysis of v-mos/c-moshu hybrid recombinants have revealed that specific regions of the c-moshu locus, which when subsituted with v-mos specific sequences a novel way can actively function as a transforming gene. These analyses have provided for identifying domains in mos required for transforming activity. For example, we have identified a maximum of five amino acid differences between v-mos and c-moshu that could prevent the latter from being biologically active. These results demonstrate that the c-moshu oncogene requires both qualitative and quantitative changes in order to be activated as a transforming gene.

A dominant transforming gene has been identified from the human pancreatic cell line PANC-1. Better than 70% of this gene has been cloned in prokaryotic vectors. It appears to be a member of a family of human sequences related to the Kirsten ras oncogene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04899-11 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transforming Genes of Avian RNA Tumor Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Takis S. Papas Head, Carcinogenesis Regulation Section, LMO, NCI

COOPERATING UNITS *(if any)* Dept. of Pathology, UCLA, Los Angeles, CA; Biochemistry Dept., Georgetown University, Washington, D.C.; Dept. of Biology, Johns Hopkins University, Baltimore, MD; and Molecular Biology Dept., Univ. of California, Berkely, CA

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have determined the complete nucleotide sequence of the integrated proviral genome of avian myelocytomatosis virus (MC29), the complete nucleotide sequence of the chicken c-myc cellular locus and the complete nucleotide sequence of the human c-myc locus. Analysis of the genetic information of these three genes indicates: (i) an intervening sequence in both cellular loci can be identified by consensus splice signals; (ii) the c-myc locus can generate an mRNA whose termination signals are downstream from the translational termination signal; (iii) the three myc genes share the same reading frame, including translational termination signals; (iv) the homology is conserved only in the coding region; (v) most changes at the nucleotide level result in no change in the amino acid; and (vi) there are two distinct domains--the 5' unique domain which is different from the viral, and the 3' coding domain, which contains amino acids coded to the two exons whose sequences have been determined here. In the latter domain, the amino acid variation between v-myc and chicken c-myc is less than 2%, whereas that between the chicken c-myc and the human is 27%, with the variation concentrated in the region that flanks the splice points.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

G. F. Vande Woude	Chief	LMO, NCI
J. A. Lautenberger	Expert	LMO, NCI
K. P. Samuel	Visiting Vellow	LMO, NCI
D. K. Watson	Staff Fellow	LMO, NCI
N. C. Kan	Visiting Fellow	LMO, NCI
S. Aaronson	Chief	LCMB, NCI
S. Tronick	Chemist	LCMB, NCI
P. Reddy	Visiting Scientist	LCMB, NCI
R. Gallo	Chief	LTCB, NCI

Objectives:

The scope of this investigation is to delineate the relationship between virus gene expression and conversion of cells from normal to malignant state and to study the molecular anatomy of known tumor viruses and describe the mechanism by which subviral structures act in concert with cellular factors to regulate oncogenesis. To investigate the process by which viral oncogenes, as well as their cellular homologs, induce the activation of the metabolic processes and participate in malignant transformation. To delineate at the molecular level the mechanism by which oncogenes act in concert with cellular factors to induce oncogenesis. To introduce functionally modified oncogenes to specific target cells in an effort to analyze and alter the function of their normal counterparts. The technique of molecular cloning, DNA sequence analysis, and site mutagenesis will be used to implicate specific nucleotides in the transformation process.

Methods Employed:

A. Molecular Cloning. The integrated proviral genome of MC29 was initially cloned in λgt WES λB. In the present studies, two subclones of this DNA fragment were utilized for sequence analysis. A 1.4-kbp Xho I fragment that contained the 5' large terminal repeat (LTR) and gag sequences was subcloned in M13. The 2.9-kbp Bam HI fragment that contained the gag- and myc-specific sequence was subcloned in pBR322. The insert DNAs were purified by agarose gel electrophoresis and DEAE-cellulose (DE-52, Whatman) column chromatography after cleavage with appropriate restriction enzymes and were used in all subsequent analyses.

B. Nucleotide Sequence Analysis. Nucleotide sequence analysis of the 2.9-kbp Bam HI fragment was carried out by the method of Maxam and Gilbert. Appropriate restriction fragments were prepared and labeled at their 5' ends by using [γ-^{32}P]ATP and polynucleotide kinase as described by Maxam and Gilbert or at their 3' end by using cordycepin 5'-[α-^{32}P] triphosphate and terminal deoxynucleotidyl transferase according to Roychoudhury and Wu. The nucleotide sequence was determined by the procedure of Maxam and Gilbert.

C. Plasmid Construction Techniques. Plasmid DNAs were cleaved by the appropriate restriction enzymes and subjected to electrophoresis on polyacrylamide gels. Fragments were eluted from the gels by the method of Maxam and Gilbert. Vector DNA was prepared for ligation by cleavage with the appropriate restriction enzymes and treated with calf intestinal phosphatase (Boehringer Mannheim). Ligation of each isolated fragment (0.5µg) to vector DNA (1.6µg) was performed in 66mM Tris-HCl, pH 7.4, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.4 mM ATP, and 2.5 units/ml T4 DNA ligase (New England Biolabs). The reactions (0.04 ml) were incubated at 4°C for 18 h. Calcium chloride treated E. coli. DC646 cells were transformed as described by Cohen et al. and ampicillin resistant colonies were screened for plasmids as described above. To study gene expression, plasmid DNAs prepared from the transformed DC646 cells were introduced into N4830 or N4831 by the same procedure. Extensive use was made of published DNA sequences in the design of the recombinant plasmids. The sequences used for this work were: phage λ segments on plasmid pOG7, pBR322, the v-myc gene, the v-myb gene, and the v-ras gene.

D. Radiolabeling and Electrophoresis of Bacterial Proteins. E. coli cells were grown at 32°C in M56 minimal media supplemented with 0.5% glucose, 0.01% each of all amino acids except methionine and cystine, 0.01% biotin, 0.01% thiamine and 50 µg/ml ampicillin. When the OD_{590} of the cultures reached 0.2, the temperature was shifted to 41°C. Aliquots (150 µl) of the cells were taken 5 min before and at 10 min and 30 min after the temperature shift. These cells were added to 15 µl of media containing 2.5 µCi [^{35}S] cystine (NEN, 330 mCi/mmole) and incubated for 1.5 min. After labeling, some cultures were chased by adding unlabeled cystine to a final concentration of 0.5 mM. Cellular protein was precipitated with 10% (w/v) trichloracetilc acid, washed with acetone, and resuspended in 1.0% SDS/0.1% β-mercaptoethanol. The proteins were then resolved by electrophoresis on 10% SDS-polyacrylamide gels and visualized by autoradiography.

Major Findings:

A. The nucleotide sequence of the integrated proviral genome of avian myelocytomatosis virus (MC29) coding for gag-myc protein has been determined. By comparison of this nucleotide sequence with the helper virus as well as the c-myc region, it was possible to localize the junction points between helper viral and v-myc sequences. These studies demonstrate that (i) the large terminal repeat sequence of MC29 is very similar to that of Rous sarcoma virus; (ii) the viral genome has suffered extensive deletions in the gag, pol, and env genes; (iii) the gag region can code for p19, p10, and part of p27; (iv) the recombination between viral and cellular sequences occurred in the coding region of p27 such that the open reading frame extends for an additional stretch of 1,266 base pairs, resulting in a gag-myc hybrid protein; (v) the open reading frame terminated within the v-myc region 300 bases upstream of v-myc-helper viral junction; and (vi) the v-myc helper-viral junction at the 3' end occurred in the middle of env gene, rendering it defective.

B. Myelocytomatosis virus MC29 is a defective avian retrovirus with a hybrid transforming gene (Δgag-myc) consisting of a 1,358-bp sequence from the retroviral gag gene and a 1,568-bp sequence (v-myc) shared with a cellular

locus, termed c-myc. We have subjected to sequence analysis 2,735 bp of the cloned c-myc gene, which includes the v-myc-related region of 1,568 bp, an intervening sequence of 971 bp, and unique flanking sequences of 45 bp and 195 bp at the 5' and 3' ends, respectively. Analysis of the genetic information and alignment of the c-myc sequence with the known sequence of MC29 indicates that: (i) the two myc sequences share the same reading frame, including the translational termination signal; (ii) there are nine nucleotide changes between c-myc and v-myc that correspond to seven amino acid changes; (iii) the 971-bp intervening sequence of c-myc can be defined as an intron by consensus splice signals; (iv) the unique 5' sequence of c-myc could either extend its reading frame beyond the homology with v-myc or could be an intron because its junction with the myc region of the locus is a canonical 3' splice-acceptor site; (v) the v-myc contains 10 nucleotides at its 5' end not shared with the c-myc analyzed here and also not with known gag genes, probably derived from an upstream exon; and (vi) the c-myc locus can generate a mRNA whose termination signals have been identified to be located 83 bp and 119 bp from the point of divergence between the v-myc and c-myc. We conclude that the gene of the c-myc locus of the chicken and the onc gene of MC29 share homologous myc regions and differ in unique 5' coding regions, and we speculate, on this basis, that their protein products may have different functions. The hybrid onc gene of MC29 must have been generated from the c-myc gene by deletion of the 5' cellular coding sequence, followed by substitution with the 5' region of the viral gag gene.

C. We have determined the complete nucleotide sequence of human cellular c-myc, which is homologous to the transforming gene, v-myc, of myelocytomatosis virus MC29. Analysis of the genetic information and alignment with the known sequence of chicken c-myc and v-myc indicates: (i) an intervening sequence can be identified by consensus splice signals. The unique 5' sequence of c-myc and its junction with the v-myc region may be a canonical 3' splice acceptor. (ii) The c-myc locus can generate a mRNA whose termination signals are downstream from the translational termination signal. (iii) The three myc genes share the same reading frame, including translational termination signals. (iv) The homology is conserved only in the coding region. (v) Most changes at the nucleotide level result in no change in the amino acid. (vi) There are two distinct domains--the 5' unique domain, which is different from the viral, and the 3' coding domain, which contains amino acids coded by the two exons whose sequences have been determined here. In the latter domain, the amino acid variation between v-myc and chicken c-myc is less than 2%, whereas that between the chicken v-myc and the human is 27%, with the variation concentrated in the region that flanks the splicing points.

D. The genome of the avian myeloblastosis virus (AMV) has undergone a sequence substitution in which a portion of the region normally coding for the env protein has been replaced by cellular sequences. We have determined the complete nucleotide sequence of this region. Examination of the AMV oncogenic sequence revealed an open reading frame starting with the initiation codon ATG and terminating with the triplet TAG within the acquired cellular sequences and terminating with the triplet TAG at a point thirty-three nucleotides into helper viral sequences to the right of the helper-viral-cellular junction. The stretch of 795 nucleotides would code for a protein

of 265 amino acids with a molecular weight of 30,000 daltons. The eleven amino acids at the carboxy terminus of such a protein would be derived from the env gene of helper virus. Antibodies were prepared against synthetic peptides derived from the predicted amino acid sequences. One such antibody precipitated two magnesium proteins of apparent nucleotide weight of 30,000 daltons and 51,000 daltons.

E. A plasmid, pJL6, was constructed that contains a unique Cla I site twelve codons beyond the bacteriophage λ cII gene initiation codon. This site allowed us to fuse the carboxy-terminal portion of the cII gene. Transcription of the hybrid gene is controlled from the phage λp_L promoter. When this promoter is derepressed, E. coli cells harboring the chimeric plasmid produce a level of cII-myc fusion protein greater than 5% of total cellular protein. Antibodies raised by this protein form an immunoprecipitate with the MC29 gag-myc gene product, $P110^{gag\text{-}myc}$.

Significance to Biomedical Research and the Program of the Institute:

It is clear that the formation, the maintenance, and the expression of provirus are the central features of the life cycle of RNA tumor viruses. Many questions related to these aspects are unsettled. To elucidate the process of oncogenesis induced by these viruses, it is important that the structural organization of the transforming genes (oncogene) within the host chromosome and the process by which these genes are expressed and regulated.

Proposed Course:

Current research is being conducted toward defining the mechanism of action of malignant transformation in cells. Efforts are concentrated towards:

1. Greater emphasis will be placed on understanding how oncogenes are involved in malignant transformation. Analysis of the protein products of oncogenes in normal and malignant vertebrate cells will be pursued. Antibodies will be prepared from oncogenes expressed in our bacterial vectors. These antibodies will be used to immunoprecipitate proteins in vertebrate cells that phase antigenic proteins with proteins synthesized in bacteria. Targets of interest include myc-related proteins in normal chicken cells, bursal lymphoma, mouse plasmacytoma, and human Burkitt lymphoma.

2. Antibodies will be raised against the v-myb protein which was made in our bacterial vector. These antibodies will be utilized in turn to identify the transforming protein in different haematopoietic cells.

3. Oncogenic proteins will be analyzed for biological activity. The method o logy includes microinjection into cells or by incorporation into cells by hypoplasmic shock. At this point the most promising protein has been shown to possess the enzymatic properties of the viral protein. The bacterial myc and myb proteins also should be suitable for this analysis as well as any proteins synthesized from genes altered by directed mutagenesis.

4. Further characterization and structural analysis will be carried out on myc subgroup transforming viruses. MH_2 will be studied in great detail

since it is already cloned and partially sequenced in our laboratory. The transformation protein of this virus will be identified and studied in great detail.

5. We will attempt to identify the mechanism of overexpression of myc gene in certain tumor cells. We are testing several models such as enhancer rearrangement sequences or undermethylation.

Publications:

Baluda, M. A., Perbal, B., Rushlow, K. E. and Papas, T. S.: Avian myeloblastosis virus: A model for the generation of viral oncogenes from potentially oncogenic cellular genetic elements. Folia Biologica 29: 18-33, 1983.

Dalla Favera, R., Gelmann, E. P., Martinotti, S., Franchini, G., Papas, T. S., Gallo, R. C. and Wong-Staal, F.: Cloning and characterization of different human sequences related to the onc (v-myc) of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA 79: 6497-6501, 1982.

Lautenberger, J. A., Court, D. and Papas, T. S.: High level expression of Escherichia coli of the carboxy-terminal sequences of the avian myelocytomatosis virus (MC29) v-myc protein. Gene, 1983 (In Press).

Lautenberger, J. A., Kan, N. C., Court, D., Pry, T., Showalter, S. and Papas, T. S.: High-level expression of oncogenes in Escherichia coli. In Papas, T. S., Chirikjian, J. and Rosenberg, M. (Eds.): Gene Analysis Techniques, New York, Elsevier/ North Holland, Volume 3, 1983, (In Press).

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Tsichlis, P. N., Spyropoulos, D., Pry, T. W., Rushlow, K. E. and Papas, T. S.: The transforming sequences of avian myelocytomatosis virus (MC29). In Marchesi, V. T., Gallo, R. and Majerus, P. (Eds.): Differentiation and Function of Hematopoietic Cell Surfaces. New York, A. R. Liss, 1982, pp. 1-15.

Neel, B. G., Gasic, G. P., Rogleer, C. E., Skalka, A. M., Ju, G., Hishinuma, F., Papas, T., Astrin, S. M. and Hayward, W. S.: Molecular analysis of the c-myc locus in normal tissue and in avian leukosis virus-induced lymphomas. J. Virol. 44: 158-166, 1982.

Papas, T. S., Rosenberg, M., Chirikjian, J. G. (Eds): Gene Amplification and Analysis. Vol. 3, Expression of Cloned Genes in Prokaryotic and Eukaryotic Vectors. New York, Elsevier/North Holland, 1983 (in press).

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D. K., Baluda, M. A. and Reddy, E. P.: Complete nucleotide sequence of the transforming genes of avian myeloblastosis virus (AMV). J. Cell. Biochem. 20: 95-103, 1982.

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D. K., Baluda, M. A. and Reddy, E. P.: The nucleotide sequence of the transforming genes of avian myeloblastosis virus (AMV). In Scolnick, E. M. and Levine, A. J. (Eds.): UCLA Symposium on Tumor Viruses and Differentiation. New York, A. R. Liss, 1982, pp. 155-163.

Papas, T. S., Rushlow, K. E., Watson, D. K., Bader, J. P., Ray, D. and Reddy, E. P.: The transforming gene of avian myeloblastosis virus (AMV): Nucleotide sequence analysis and identification of its translational product. In Neth, Gallo, Greaves, Moore, Winkler (Eds.): Moderns Trends in Human Leukemia V, Berlin Heidelberg, Springer-Verlag, 1983, pp. 207-213.

Reddy, E. P., Reynolds, R. K., Watson, D. K., Schulz, R. A., Lautenberger, J. A. and Papas, T. S.: Nucleotide sequence analysis of the proviral genome of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA 80: 2500-2504, 1983.

Rhim, J. S., Trimmer, R., Huebner, R. J., Papas, T. S. and Jay, G.: Differential susceptibility of human cells to transformation by murine and avian sarcoma viruses. Proc. Soc. Exp. Bio. Med. 170: 350-358, 1982.

Rushlow, K. E., Lautenberger, J. A., Papas, T. D., Baluda, M. A., Perbal B., Chirikjian, J. G. and Reddy, E. P.: Nucleotide sequence of the transforming gene of avian myeloblastosis virus (AMV). Science 216: 1421-1423, 1982.

Watson, D. K., Psallidopoulos, M. C., Samuel, K. P., Dalla Favera, R. and Papas, T. S.: Nucleotide sequence analysis of the human c-myc locus, chicken homologue, and myelocytomatosis virus MC29 transforming gene reveals a highly conserved gene product. Proc. Natl. Acad. Sci. USA 80: 3642-3645, 1983.

Watson, D. K., Psallidopoulos, M. C., Samuel, K. P., Dalla Favera, R. and Papas T. S.: The myc genes. In Prasad, U., Levine, P. and Pearson G. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. University of Malaysia Press (In Press).

Watson, D. K., Reddy, E. P., Duesberg, P. H. and Papas, T. S.: Nucleotide sequence analysis of the chicken c-myc locus reveals homologous and unique coding regions by comparison with the transforming gene of avian myelocytomatosis virus MC29, Δgag-myc. Proc. Natl. Acad. Sci. USA 80: 2146-2150, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04963-07 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular and Biochemical Studies on p21 ras: Mechanism of Cell Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

T. Y. Shih Research Chemist, LMO, NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular and Developmental Biology, NIADDK; Laboratory of Molecular Virology and Carcinogenesis, Basic Research Program, Litton Bionetics, Inc., Frederick, Maryland

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The p21 proteins encoded by the ras genes of Harvey and Kirsten murine sarcoma viruses are directly responsible for the initiation and maintenance of virus-induced malilgnant transformation of the infected cells. This project seeks to characterize the p21 ras, to investigate the interaction of this protein with cellular components, and to study the structure-function relationship of this protein as a step to understand the biochemical pathway leading to conversion of normal cells into cancer cells. Large quantities of the Ha-p21 have been produced in E. coli in order to undertake detailed protein biochemistry studies. The biosynthesis of the p21 ras encoded by the EJ bladder ras gene has been studied. Structure-function relationship studies using the synthetic peptide and genetic engineering approaches have been undertaken. The hormonal responsiveness of the Ha-MuSV transformed cells was also investigated.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Linda S. Ulsh	Microbiologist	LMO, NCI
Paul R.J. Huang	Guest Researcher	LMO, NCI
James A. Lautenberger	Expert	LMO, NCI
Arun Seth	Visiting Associate	LMO, NCI
Takis S. Papas	Head, CRS	LMO, NCI
George Vande Woude	Chief	LMO, NCI

Objectives:

The scope of this project is to study the molecular biology and biochemistry of the ras genes and the encoded p21 transforming proteins in Harvey and Kirsten murine sarcoma viruses and in the related human neoplasia. The long range goal is to understand the molecular mechanism of cell transformation induced by these genes and their protein products. The immediate objective of this project is to characterize the biochemical properties of the p21^{ras} proteins, and to investigate the structure-function relationship of the p21 proteins employing biochemical and genetic engineering approaches. From these studies, we hope to learn the biochemical pathways leading to conversion of normal cells into malignant cancer cells and to develop the rational basis for chemoprevention and chemotherapy of the p21-related neoplasia.

Methods Employed:

1. Cell labeling and immunoprecipitation: Cells were labeled either with ^{35}S-methionine or ^{32}P-orthophosphate. The p21 proteins were identified by immunoprecipitation with antibodies directed against the p21. The immunoprecipitated proteins were analyzed by SDS-gel electrophoresis and visualyzed by autoradiography.

2. Peptide mapping: Peptides generated by protease digestion were analyzed by a 2° dimensional procedure on the thin-layer chromatography (TLC) plates. The first dimension was electrophoresis and the second dimension was chromatography.

3. Phospho-amino acid analysis: The ^{32}P-labeled p21 was hydrolyzed in 6 N HCl and the phospho-amino acids were identified by a 2° dimensional procedure.

4. Purification of the p21 from Ha-MuSV transformed cells: The p21 was purified from NIH3T3 cells transformed by Ha-MuSV. The purification procedures involved isolation of plasma membrane in a two phase solvent system; ammonium sulfate precipitation, DEAE-cellulose chromatography, and phenyl-Sepharose columns. The final purification was approximately two thousand fold and the p21 was about 90% pure.

5. Enzyme activity assays of the purified p21: Autophosphorylation activity of the p21 was assayed by incubating the purified p21 with γ-^{32}P-GTP. The phosphorylated proteins were visualized by SDS-gel electrophoresis. To assess

the potential protein kinase activity of the purified p21, candidate substrates including synthetic peptides were incubated with the p21 and the γ-32P-GTP.. The peptide products were analyzed by the high-performance liquid chromatography (HPLC) columns. The guanine nucleotide binding activity of the p21 was assayed by mixing p21 with ^{3}H-GDP; the ^{3}H-activity associated with p21 following immunoprecipitation was determined by liquid scintillation counting.

6. High-performance liquid chromatography (HPLC): Peptides corresponding to segments of the p21 sequence were synthesized on the solid matrixes. The peptides were analyzed by a reverse phase HPLC column using a linear acetonitrile gradient. To assay the phosphorylation products, the synthetic peptides were completely separated from the γ-^{32}P-GTP and other phosphorylated products in this HPLC system.

7. Antisera against the synthetic peptides: The synthetic peptides ranged from 10 to 17 amino acid residues in length, were conjugated with the carrier proteins, bovine serum albumin or keyhole limpet hemocyanin, by the bis-diazotized benzidine through the tyrosine residues. After mixing with adjuvant, rabbits were immunized with the antigens. To derive monoclonal antibodies, mice were also immunized after several boostings and antisera were screened against the Ha-MuSV transformed cell lysates labeled with ^{35}S-methionine for anti-p21 antibodies.

8. Production of the p21 protein in bacteria: The Ha-ras gene was inserted in a plasmid expression vector developed by Lautenberger et al. High level expression of the p21 (approximately 10% of total protein) was achieved in E. coli. Following lysis of the bacterial cells, the p21 protein was isolated by immunoprecipitation with antibodies directed against the p21. The biochemical activities were studied as the viral p21 isolated from transformed NIH3T3 cells.

Major Findings:

1. Biochemical properties of the p21 expressed in bacteria. Although the p21 protein has been extensively purified from the virus transformed cells, due to the extremely low level of the p21 protein in cells (approximately 0.01% to 0.1%), detailed protein biochemistry studies are limited. To obtain large quantities of p21 for enzymology study, the p21^{ras} gene of the Harvey murine sarcoma virus has been inserted into a plasmid expresion vector. High level expression (approximately 10% total protein) in E. coli of a fusion protein of 24,000 daltons containing most of the p21 sequences has been achieved. We have studied the biochemical properties of the p21 fusion protein in E. coli. The bacterial p21 was identified with an antibody directed against the Ha-p21. The immunoprecipitated p21 possessed the autophosphorylation enzyme activity by incubation with γ-^{32}P-GTP. In addition, the bacterial p21 also demonstrated the guanine nucleotide binding activity. These are the properties we previously observed for the p21 proteins in the virus-transformed cells. Unlike the eukaryotic cells, the bacterial cells lack most known protein kinases; therefore, the phosphorylation activity observed in E. coli is very significant. These observations strengthened the view that these biochemical activities are intrinsic properties of the p21^{ras}. The fact that the p21 protein produced in E. coli is biochemically active makes further charaterization of this protein more meaningful.

2. Biosynthesis of the p21^{ras} of EJ human bladder carcinoma cells. The p21 of the virus transformed cells is synthesized as a precursor in the free polysomes in the cytosol. Shortly after its synthesis, the pro-p21 is processed into a mature form which is found to be associated with the plasma membrane fraction. Fractionation of the processed p21 is also modified by autophosphorylation. In an attempt to understand the functional difference between the EJ p21 and its normal counterpart, the human c-ras^{H} p21 which differs slightly on the electrophoretic mobility in the SDS polyacrylamide gel, we have studied the kinetics of biosynthesis of these two p21s. The pulse labeling experiments indicated that both p21s were synthesized by a very similar kinetics and presumably involved the same synthetic pathway.

3. Structure-function studies of the p21 using the synthetic peptide approach. We have established that the p21 possesses an intrinsic enzymatic autophosphorylation activity. Whether or not p21 phosphorylated other substrate proteins is unresolved. A series of peptides of 10 to 17 amino acid residues long of the p21 phosphorylation site sequence were synthesized. These synthetic peptides were used as the phosphate acceptor substrates by incubating with the purified p21 from the Harvey virus-transformed cells using the γ-^{32}P-GTP as the phosphoryl donor. After isolation of the peptides by the HPLC columns no phosphorylation of any of these synthetic peptides could be demonstrated. Another attempt to identify the possible acceptor substrates in the cells was to raise the antibodies to the synthetic peptides. These peptides were chemically conjugated to carrier proteins such as bovine serum albumin or keyhole limpet hemocyanin. An immunization series with rabbits or mice was performed. Although very weak reactivities of the antisera to the p21 were detected the titers of antibodies were still too low to allow detection of possible cross-reacting proteins in cells.

4. Hormone responsiveness in MDCK cells transformed by Ha-MuSV. As an attempt to study the function of the p21, the hormonal responsiveness of the adenylate cyclase system has been investigated. The kidney cell line (MDCK) retains an adenylate cyclase system sensitive to glucagon, vasopressin, isoproterenol and prostaglandin E1. The stimulatory effect of glucagon was selectively lost in a cloned MDCK cell lines transformed by Ha-MuSV. Sensitivity to glucagon was restored by treatment with prostaglandin E1 or butyrate due to the reappearance of glucagon receptors. The parental MDCK line produced prostaglandins and in the transformed line, this function was abolished. These observations suggest that synthesis of glucagon receptors is controlled by endogenously produced prostaglandins in MDCK cells and that loss of glucagon receptors and their responsiveness in the transformed cells occurs as a consequence of the inability of these cells to synthesize this prostaglandin.

Significance to Biomedical Research and the Program of the Institute:

The long range goal of this program is to seek understanding of the biochemical mechanism leading to conversion of normal cells into cancer cells. The importance of this problem is that not only is it the knowledge required to understand the nature of malignant growth, but also at a practical level it would provide the rational basis for devising means for chemoprevention and chemotherapy. This is a scientific area which is still mostly sailing on the uncharted course and requires a great deal of original thinking and approaches. The study on

the ras genes and the encoded p21 proteins provided us the definite handle to investigate this problem. From our studies on the Harvey and Kirsten murine sarcoma viruses, we know that this p21ras protein is required for initiation and maintenance of the virus-induced malignant transformation of cells. By the same token, it is presumably true for malignant transformation induced by other cellular ras genes of the gene familily. Relevance of these p21 proteins in human neoplasia became more evident from the demonstration of the human oncogenes found in human bladder, colon and lung carcinomas. These human oncogenes are the cellular homologs of the ras genes of these sarcoma viruses. Studies on the p21 proteins will provide clues to find out the biochemical pathways involved in malignant transformation.

Proposed Course:

Available large quantity of the p21 protein produced in bacteria will enable us to do detailed protein biochemistry and to derive monoclonal antibodies with spectrum of specificity different from those currently available. These reagents will facilitate further studies on the functions of these proteins in cells. Comparative studies of the p21ras of viral, cellular or tumor oncogenes will provide us knowledge to understand the basis of ras gene induced oncogenesis. By employing the genetic engineering techniques, we wish to study the structure-function relationship of these proteins.

Publications:

Dhar, R., Ellis, R. W., Shih, T. Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D. and Scolnick, E. M.: The nucleotide sequence of the p21 transforming gene of Harvey murine sarcoma virus. Science 217: 934-937, 1982.

Lin, M. C., Koh, W. W. M., Dykman, D. D., Beckner, S. K. and Shih, T. Y.: Loss and restoration of glucagon receptors and responsiveness in a transformed kidney cell line. Exp. Cell Res. 142: 181-189, 1982.

Shih, T. Y.: Expression of the cloned p21 transforming gene (ras) of Harvey murine sarcoma virus. In Huang, P. C., Kuo, T. T. and Wu, R. (Eds.): Genetic Engineering Techniques: Recent Developments. New York, Academic Press, 1982, pp. 175-188.

Shih, T. Y., Stokes, P. E., Smythers, G. W., Dhar, R. and Oroszlan, S.: Characterization of the phosphorylation sites and the surrounding amino acid sequences of the p21 transforming proteins coded for by the Harvey and Kirsten strains of murine sarcoma viruses. J. Biol. Chem. 257: 11767-11773, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04970-07 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemistry of Cellular Transformation by Avian Tumor Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

John P. Bader Chief, Cellular Transformation Section LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Cellular Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	1.2	2.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Malignant transformation of cells by viruses often is induced by a virus-related protein which plays no role in virus reproduction. Sequence analysis of viral genomic regions responsible for transformation has allowed the projection of the amino acid sequences of two such proteins, and antibodies against certain of these sequences were developed. One antibody has allowed the tentative identification of the protein responsible for avian myeloblastosis, and another selects the protein encoded by MC29 virus, and a similar protein in nontransformed cells. The location of these proteins within the cell has been determined, and possible glycosylation and phosphorylation modifications were examined. In a continuation of earlier experiments on Rous sarcoma cells, certain metabolic differences related to transformation have been examined in detail. Although Na-K-ATPase was shown to be coupled to glycolytic ability, no substantial changes in the activity or efficiency of Na-K-ATPase were noted despite the increased glucose consumption and glycolysis of transformed cells. An increased production and utilization of ATP by transformed cells has not yet been explained.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Takis Papas	Chief, Cellular Transformation Section	LMO, NCI
Robert Balaban	Expert	LKEM, NHLBI
Dennis Watson	Staff Fellow	LMO, NCI

Objectives:

To identify the virus-coded protein responsible for inducing avian myeloblastosis and to identify its normal cellular homologue; to identify the normal cellular protein homologous to the transforming region of the MC29 virus-coded protein. To determine the intracellular location of these proteins and attempt to determine their cellular function and the mechanism by which they induce cancer. To determine the physiological basis for a number of recognized differences between tumor cells and normal cells, especially those differences relating to energy production and utilization methods.

Methods Employed:

A. Transformation of cells in culture by avian and murine sarcoma viruses, morphological resolution of transformed cells by microscopy, resolution of cellular organelles by supravital staining.

B. Isolation of cellular organelles, including microsomes, cell surface membranes, and nuclei.

C. Quantitative chemical determinations of protein and a variety of enzymes.

D. Complement fixation, immunopreciptation, immuno-affinity chromatography.

E. Paper chromatography.

F. Density gradients, polyacrylamide gel electrophoresis, agarose gel electrophoresis, autoradiography, fluorography.

G. Uptake of radioactive molecules into cells, incorporation of radioactive precusors into macromolecules, binding of radioactive molecules to specific cell-surface sites.

H. Chemical and enzymatic treatment of proteins.

Major Findngs:

The genome of avian myeloblastosis virus (AMV) consists of a portion encoding viral proteins and a portion similar to cellular DNA. In another laboratory this latter portion had been cloned in a microbial vector and the sequence of nucleotides determined. From the nucleotide sequence an amino acid sequence representing the AMV-specific protein (myb) was projected, specific peptides from

several areas were synthesized, and antibodies to these peptides were induced in rabbits. We have used these antibodies to identify the virus-induced protein in a line of avian myeloblasts. These proteins were found to be specific for the AMV-infected cells: 55,000 daltons (p^{55}), p^{50} and p^{31}. The p^{31} conforms with the molecular size of myb projected from the genome, but the other proteins could be myb conjugated to portions of viral structural proteins. None of these proteins could be labeled with glycosylation precursors, or radioactive phosphate, and presumably they are neither gl ycosylated nor phosphorylated. Another protein, p^{65}, is selected from a variety of transformed and nontransformed avian lines.

Similar experiments have been performed using antibodies induced against MC29 virus-peptides projected from analysis of the MC29 transformation region, myc. One antiserum, immunoselects p^{110}, a known conjugate of viral structural protein and myc protein. A naturally occurring duplex of this molecule has also been detected. The availabililty of this antibody was particularly exciting, since the expression of the cellular myc gene has been implicated in human and avian lymphomas, and in murine plasmacytomas. This antibody selects a protein, p^{95}, from avian lymphomas as well as chicken fibroblasts, but no selection from human or murine cells has been observed. The p^{95} is found in both the nucleus and cytoplasm, and is neither phosphorylated nor glycosylated.

Changes in the energy producing machinery of malignant cells have been recognized for decades but remain largely unexplained. The increased capacity for glucose uptake with no alteration in respiratory activity predicted an increased energy reservoir for tumor cells, contradicted when earlier experiments indicated decreased ATP levels in transformed cells. We developed a method for unequivocally eliminating ATPase activities in the measurements of adenosine nucleotide ratios, and found that cells transformed by Rous sarcoma virus had higher ATP/ADP levels then nontransformed cells, a result more consistent with the noted increased glucose consumption of these cells. However, elimination of glucose from the medium failed to bring transformed ATP/ADP to the level of nontransformed cells, and mitochondrial effectors likewise had no effect, indicating that increased glucose consumption alone is an inadequate explanation for increased ATP levels.

Glycolysis is metabolically coupled with Na-K-ATPase, and roles for both in tumorgenic potential have been suggested. We have shown that the ATP derived from glycolysis is more effectively used for Na-K-ATPase than the ATP from mitochondria, suggesting an effect of increased glucose consumption on Na-K-ATPase. However, we showed that the efficiency of Na-K-ATPase was neither impaired nor increased in tumor cells, and no direct experiments support the possibility that Na-K-ATPase is extraordinarily active in malignant cells. Therefore, while many aspects of energy production and utilization in tumor cells have been explored, no striking feature to explain malignancy has become prominent.

Significance to Biomedical Research and the Program of the Institute:

The identification of cellular proteins homologous to tumor-inducing viral proteins would be a major advancement in our understanding of the nature of cancer cells. It is likely that the tumor-inducing proteins will differ with respect to structure or quantity, the resolution of which would focus

experimental efforts into optimistic channels. The regulatory features of tumor cells directing or being affected by energy changes are unquestionably important to the malignant process of these cells, and pose possiblities for therapeutic intervention.

Proposed Course:

To put greater effort into the examination of the role of identified virus-coded proteins in the maintenance of malignant transformation and determine the reason for their tumorgenic potential in contrast to the normal function of their cellular counterparts. No short term resolution of the energy enigma in tumor cells seem likely, and we shall decrease our efforts in this area.

Publications:

Balaban, R. and Bader, J. P.: The efficiency of Na-K-ATPase in tumorigenic cells. Biochem. Biophys. Acta 730: 271-275, 1983.

Balaban, R. and Bader, J. P.: The relationship between glycolysis and Na-K-ATPase in cultured cells. Biochem. Biophys. Acta (In Press).

Papas, T. S., Rushlow, K. E., Watson, D. K., Bader, J. P., Ray, D. and Reddy, E. P.: The transforming gene of avian myeloblastosis virus (AMV): Nucleotide sequence analysis and identification of its translational product. In Neth, Gallo, Greaves, Moore, Winkler (Eds.): Modern Trends in Human Leukemia V, Berlin/Heidelberg Springer-Verlag, 1983, pp. 207-213.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05104-02 LMO

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure and Function of MSV Genome

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Mary Lou McGeady Staff Fellow, LMO, NCI

COOPERATING UNITS *(if any)*
Georgetown University, Washington, D. C.

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have tested the effect of in vitro cytosine methylation on the transforming activity of Moloney sarcoma virus (MSV). It was found that methylation of the MSV genome at HpaII or HhaI sites significantly reduced its ability to transform NIH3T3 cells in DNA transfection experiments. This reduction in transforming activity was reversed by the use of 5-Azacytidine, a specific inhibitor of methylation. A low level of transforming activity observed with methylated MSV DNA was apparently associated with the loss of methyl groups from MSV DNA during initial rounds of DNA replication in cells. Methylation of a certain region of the MSV LTR, i.e., the region at or slightly downstream from the start of transcription, and the mos gene (i.e. the 5' end) both inhibit transformation. Non-transformed cells which contained methylated copies of MSV DNA were isolated by a contransfection technique. The methylated MSV DNA in these cells was shown to be highly insensitive to DNase I digestion in contrast to non-methylated, actively expressed MSV genomes. After extensive exposure of the cells containing methylated MSV DNA to 5-Azacytidine, transformed cells were isolated which had lost methylation at a number of HpaII sites in the MSV DNA.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

G. F. Vande Woude	Chief, LMO	LMO, NCI
R. Ascione	Research Chemist	LMO, NCI

Objectives:

The objective of this project is to determine the effect of cytosine methylation on the expression of the transforming phenotype of the mos oncogene from Moloney sarcoma virus (MSV) both in the form of the v-mos gene and its cellular homologue, c-mos. Methylation of cytosine residues in genomic DNA has been described as a mechanism for the control of eukaryotic gene expression, with greater than 90% of the methylated cytosine residues occurring at CG sites. Many restriction endonucleases which contain CG as part of their recognition sequence do not cleave if cytosine is methylated. Therefore, with the use of such enzymes it is possible to determine the extent of methylation of a DNA sequence. The restriction endonucleases HpaII and MspI are particularly useful in this regard. Both enzymes recognize the sequence CCGG but HpaII will not cleave the DNA if the internal cytosine is methylated, whereas MspI will. It was determined that the normally nonexpressed endogenous c-mos gene is methylated at the HpaII sites in the 5' end of the cellular onc gene. Therefore, a study of the effect of methylation of mos on its expression is relevant in the case of both the cellular and the viral gene. In addition, a comparison of the efficiency of maintenance methylation of the mos gene, when found in a chromosomal versus an extrachromosomal state, should yield valuable information concerning the cytosine methylation process in eukaryotic cells.

Methods Employed:

Plasmid DNA containing the MSV DNA of interest was methylated in vitro with HpaII or HhaI methylase. The biological activity of the DNA was assayed by transfection into NIH3T3 cells and determining the number of foci of morphologically transformed cells. Cotransfection of methylated MSV DNA with, and selection for, the dominant marker Ecogpt was used to obtain nontransformed NIH3T3 cells containing methylated copies of MSV DNA. DNA from normal, transformed or Ecogpt selected cells was digested with restriction endonucleases and analyzed by the Southern blotting technique. Screening of cells for those producing mos containing RNA was done by the dot blot technique, and the RNA from some cell lines was analyzed by Northern blotting technique.

Major Findings:

Our previous studies demonstrated that when cloned Moloney sarcoma virus proviral DNA was methylated in vitro using a HpaII methylase and transfected into NIH3T3 cells, its efficiency of transformation was reduced by 70% compared to the control. When 5-Azacytidine, which can inhibit methylation of replicating DNA, was added to cells after transfection with methylated DNA, this treatment resulted in the

restoration of transforming activity and demonstrated that the decrease in MSV transforming efficiency was due to HpaII methylation. DNA from cells transformed by transfection of HpaII methylated DNA in the absence of 5-Azacytidine treatment was analyzed by Southern blotting. In every transformed cell line examined the MSV DNA was no longer methylated at HpaII sites, suggesting that the methylation was lost during DNA replication in cells, and that the non-methylated copies of MSV could be expressed in these cells. We have now been able to demonstrate that the loss of methylation at HpaII sites occurred predominantly between 48 and 72 hours after transfection.

The effect of HpaII methylation on the transcriptional control elements in the proviral LTR and structural mos gene was determined separately by contransfection experiments using two plasmids. One plasmid contained only the viral LTR and one contained only the v-mos region. When neither plasmid was methylated their cotransfection resulted in efficient transformation of NIH3T3 cells. Methylation of only the LTR containing plasmid resulted in 50% loss of transforming activity whereas methylation of only v-mos plasmid resulted in an 80% decrease in transforming efficiency. These same results were obtained when the plasmids were methylated with HhaI methylase or HpaII methylase prior to the contransfections. Methylation at both HpaII and HhaI sites was no more inhibitory to transformation than methylation at only one set of sites. Because there are regions of the genes which contain a site for only one of these enzymes we were able to exclude methylation of these regions as being necessary for inactivation of the transforming gene. By this method it appeared that methylation of the region at or slightly downstream from the start of transcription in the LTR and the 5' end of the mos gene was most inhibitory to expression.

To obtain cell lines containing methylated MSV proviral DNA it was necessary to select for cells which had taken up DNA but which did not express the transformed phenotype. This was accomplished by co-transfection of HpaII methylated MSV DNA with a plasmid containing Ecogpt (the E.coli XGPRT) and selecting for cells resistant to mycophenolic acid. Surviving colonies were picked and the cells were assayed for the presence of MSV DNA. While a significant number of colonies had DNA which was rearranged and/or did not retain the HpaII methylation pattern, two non-transformed cell lines were isolated which contained HpaII resistant copies of MSV DNA in a non-rescuable state. Although only HpaII methylated copies of MSV-DNA were transfected, after multiple passages of the cells the MSV DNA was methylated at HhaI sites as well. The methylated copies of MSV-DNA were found to be as resistant to DNase I treatment as the endogenous c-mos gene which is known to be highly resistant to this probe of chromatin structure. This is in contrast to actively transcribed copies of MSV-DNA in transformed cell lines which are not methylated and are highly sensitive to DNase I digestion.

The cells containing methylated copies of MSV DNA were grown in the presence of 5-Azacytizine for several weeks, and transformed cells were subsequently isolated by growth in soft agar. These transformed cell lines contain copies of MSV DNA with an altered resistance to HpaII digestion suggesting loss of methylation in MSV DNA resulting from the 5-Azacytidine treatment of cells.

We are currently studying the ability of a plasmid containing the polyomavirus origin of replication to replicate in cells constitutively producing polyomavirus T antigen, COP-5 cells. A plasmid containing the MSV LTR and mos gene as

well as the polyomavirus origin replicates following transfection into COP cells regardless of whether it was in vitro methylated with HpaII prior to transfection. In addition, the HpaII methylation pattern is conserved to a high degree in the replicated DNA.

Significance to Biomedical Research and the Program of the Institute:

The correlation between the presence of 5 methylcytosine in a DNA sequence and the lack of transcription of that sequence is established for several well-studied cellular genes. The work reported here extends these studies to oncogenes. The finding that the normally non-expressed endogenous c-mos gene has methylated HpaII sites at the 5' but not the 3' end of the gene suggested that methylation of the gene may be important in preventing its transcription. A direct test of the effect of methylation on mos expression, by methylating the DNA in vitro prior to transfection onto NIH3T3 cells, revealed that HpaII or HhaI methylation significantly reduced its biological activity. Isolation of cells containing methylated copies of MSV DNA has allowed us to determine that methylated MSV DNA is in a highly DNase I insensitive state, a chromatin state generally associated with non-transcribed regions of DNA. Extensive exposure of the cells to 5-Azacytosine was necessary to transform the cells. These results suggest the posibility that the activation of endogenous oncogenes may require changes in chromatin structure and that methylation of certain oncogenes may inhibit their expression.

Proposed Course:

We will continue the studies correlating chromatin structure with methylation state of the mos oncogene and its transcriptional state. This will be done in NIH3T3 cells where the transfected DNA is associated with high molecular weight DNA and in COP-5 cells where it appears as minichromosomes or extrachromosomal DNA.

Publications:

McGeady, M. L., Jappan, C., Ascione, R. and Vande Woude, G. F.: In vitro methylation of specific regions of the cloned Moloney sarcoma virus. Mol. Cell. Biol. 3: 305-314, 1983.

Wood, T. G., McGeady, M. L., Blair, D. G. and Vande Woude, G. F.: Long terminal repeat enhancement of v-mos transforming activity: Identification of essential regions. J. Virol. 46: 726-736, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05117-04 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Mechanisms Involved in Retrovirus Induced Oncogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

P. N. Tsichlis Expert, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.5	4.0	4.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The specificity of provirus DNA integration in Moloney murine leukemia virus induced rat thymic lymphomas was investigated. Two common regions for provirus integration in the rat genome (the Mlvi-1 and Mlvi-2 locus) were demonstrated. The cellular sequences in the vicinity of the integrated provirus are conserved. Their chromosomal localization in the mouse genome was determined. Sequences homologous to Mlvi-1 and Mlvi-2 appears rearranged in a variety of tumor systems.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

P. G. Strauss	Guest Researcher	LMO, NCI
C. Kozak	Staff Fellow	LVD, NIAID

Objectives:

The overall objective of this project is to define the molecular mechanism(s) of oncogenesis. Major emphasis has been placed on retrovirus induced oncogenesis which can serve as a model for the understanding of oncogenesis induced by a variety of agents.

Methods Employed:

Molecular cloning, Southern blotting and hybridization, DNA sequencing, and DNA transfection.

Major Findings:

1. There are two regions in the rat genome termed the Mlvi-1 and Mlvi-2 locus which have been shown to represent common substrates for MoMuLV provirus integration.

2. These two loci may represent new oncogenes, as they are unrelated to other known transforming genes.

3. Both Mlvi-1 and Mlvi-2 map along with c-myc in the mouse chromosome 15, which is known to be the subject of chromosomal abberations in mouse thymic lymphomas.

4. The MoMuLV provirus integrated in the two loci appears in the majority of cases to be intact and biologically active.

5. Hybrid provirus-host mRNA transcripts have been detected in about 50% of the tumors. However, the relationship of these transcripts to the cloned Mlvi-1 and Mlvi-2 sequences has not yet been determined.

In preliminary studies we have shown the following:

1. In some rat thymomas there are rearrangements of both the Mlvi-1 and Mlvi-2 locus that are not due to the integration of the MoMuLV provirus. In some of the tumors these rearrangements appear in addition to the rearrangements caused by the integrated provirus, suggesting that two separate processes may be occurring simultaneously.

2. We have detected rearrangements of the Mlvi-2 locus in mouse, cat and human lymphomas.

3. Sequences homologous to the Mlvi-2 locus have been detected in lower organisms (Drosophila melanogaster).

Significance to Biomedical Research and the Program of the Institute:

Understanding of the mechanism of oncogenesis in retrovirus induced tumors provides the conceptual framework necessary for the understanding of the complex mechanisms involved in nonvirus induced malignancies.

Proposed Course:

This intramural project will be terminated as the principal investigator is leaving the National Cancer Institute.

Publications:

Coffin, J. M., Conklin, K. F., Tsichlis, P. N. and Robinson, H. L.: Genetic analysis of pathogenic and nonpathogenic avian retroviruses: In Revoltella, R. P., Pontini, A. M., Bassilico, C., Rovera, S., Gallo, T. and Sharp, Y. S. (Eds.): Expression of Differentiated Functions in Cancer Cells. New York, Raven Press, 1982, pp. 423-434.

Coffin, J. M., Tsichlis, P. N., Conklin, K. F., Senior, A. and Robinson, H. L.: Genomes of endogenous and exogenous avian retroviruses. Virol. 126: 51-72, 1983.

Robinson, H. L., Coffin, J. M., Tsichlis, P. N., Shank, P. R., Schatz P. and Jensen. L.: Cancer induction by insertional mutagenesis: The role of viral genes in avian leukosis virus induced cancer. In O'Connor, T. E. and Rauscher, F. J. (Eds.): Oncogenes and Retroviruses: Evaluation of Basic Findings and Clinical Potential. New York, Alan Liss, 1983, pp. 37-42.

Skalka, A. M., Tsichlis, P. N., Malavarca, R., Cullen, B. and Ju, G.: Viral sequences determining the oncogenicity of avian leukosis viruses. In O'Connor, T. E. and Reuscher, F. J. (Eds.): Oncogenes and Retroviruses: Evaluation of Basic Findings and Clinical Potential. New York, Alan Liss, 1983, pp. 105-118.

Tsichlis, P. N., Donnehower, L., Hager, G., Zeller, N., Malavarca, R., Astrin, S. and Skalka, A. M.: Sequence comparison in the crossover region of an oncogenic avian retrovirus recombinant and its nononcogenic parent: Genetic regions that control growth rate and oncogenic potential. Mol. Cell. Biol. 2: 1331-1338, 1982.

Tsichlis, P. N., Hu, L. F. and Strauss, P. G.: Two common regions for proviral DNA integration in MoMuLV induced rat thymic lymphomas. Implications for oncogenesis. In Goldes, D. W. and Marks, P. A. (Eds.): UCLA Symposia on Molecular and Cellular Biology, New Series. New York, Alan Liss, Vol. 9. (In Press)

Tsichlis, P. N., Strauss, P. G. and Hu, L. F.: A common region for proviral DNA integration in MoMuLV induced rat thymic lymphomas. Nature 302: 445-449, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05120-04 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

High-level Expression of Oncogene Specified Proteins in Escherichia coli

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

James A. Lautenberger Expert, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A plasmid, pJL6, was constructed that contains a unique Cla I site twelve codons beyond the bacteriophage lambda cII gene initiation codon as well as an adjacent unique Hind III site. These sites allowed us to fuse the sequences from the avian myelocytomatosis virus (MC29) v-myc gene, the avian myeloblastosis virus (AMV) v-myb gene, and the Harvey murine sarcoma virus (Ha-MuSV) v-ras gene to the aminoterminal portion of the cII gene. Transcription of the hybrid genes is controlled from the lambda major leftward promoter. When this promoter is derepressed, Escherichia coli cells harboring the chimeric plasmid produce levels of fusion proteins that are greater than five percent of total cellular protein. Antibodies raised by the cII-myc fusion protein form an immunoprecipitate with the MC29 gene product, P110gag-myc. The cII-ras fusion protein is precipitated by monoclonal antibodies directed towards the Ha-MSV p21 (v-ras protein) binds GDP, and is capable of autophosphorylation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged onthis Project:

Takis Papas	Head, Carcinogenesis Regulation Section	LMO, NCI
Nancy Kan	Visiting Fellow	LMO, NCI
Dennis Watson	Staff Fellow	LMO, NCI
Donald Court	Head, Molecular Control and Genetics Section	LMO, NCI
Thomas Shih	Research Chemist	LMO, NCI

Objectives:

The scope of this investigation is to delineate the relationship between oncogene expression and the conversion of cells from a normal to a malignant state. The nature of the protein products of the oncogenes is especially emphasized in this study because they remain more illusive than the DNA sequences that constitute the genes themselves. Expression of the proteins in bacteria should facilitate the analysis of their biochemical properties and their effect on living cells. This is because these proteins can be easily produced in large quantities from bacteria. These techniques, when coupled with directed mutagenesis, can provide great insight into the relationship between structure and function of the protein products of oncogenes.

Methods Employed:

1. Preparation of plasmid DNA. Plasmid DNAs were prepared as described by Birnboim and Doly (Nucl. Acids Res. 7: 1513-1523, 1979) from 5-ml cultures for preparation of restriction fragments. Plasmid DNA from the larger cultures was further purified by ethidium bromide-CsCl banding.

2. Plasmid Construction Techniques. Plasmid DNAs were cleaved by the appropriate restriction enzymes and subjected to eletrophoresis on polyacrylamide gels. Fragments were eluted from the gels by the method of Maxam and Gilbert (Methods Enzymol. 65: 499-560, 1980). Vector DNA was prepared for ligation by cleavage with the appropriate restriction enzymes and treated with calf intestinal phosphatase (Boehringer Mannheim). Ligation of each isolated fragment (0.5µg) to vector DNA (1.6µg was performed in 66 mM Tris-HCl, pH 7.4, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.4 mM ATP, and 2.5 units/ml T4 DNA ligase (New England Biolabs). The reactions (0.04 ml) were incubated at 4°C for 18 h. Calcium chloride treated E. coli DC646 cells were transformed as described by Cohen et al. (Proc. Natl. Acad. Sci. USA 6: 2110-2114, 1972) and ampicillin resistant colonies were screened for plasmids as described above. To study gene expression, plasmid DNAs prepared from the transformed DC646 cells were introduced into N4830 or N4831 by the same procedure. Extensive use was made of published DNA sequences in the design of the recombinant plasmids.

3. Radiolabeling and Electrophoresis of Bacterial Proteins. E. coli cells were grown at 32°C in M56 minimal media supplemented with 0.5% glucose, 0.01% each of all amino acids except methionine and cystine, 0.01% % biotin, 0.01% thiamine

and 50µg/ml ampicillin. When the OD_{590} of the cultures reached 0.2, the temperature was shifted to 41°C. Aliquots (150 µl) of the cells were taken 5 min before and at 10 min and 30 min after the temperature shift. These cells were added to 15 µl of media containing 2.5 µci [^{35}s]cystine (NEN, 330 mCi/mmole) and incubated for 1.5 min. After labeling, some cultures were chased by adding unlabeled cystine to a final concentration of 0.5 mM. Cellular protein was precipitated with 10% (w/v) trichloroacetic acid, washed with acetone, and resuspended in 1.0% SDS/0.1% β-mercaptoethanol. The proteins were then resolved by electrophoresis on 10% SDS-polyacrylamide gels and visualized by autoradiography.

4. Preparation of Bacterial Extracts. Unfractionated extracts were prepared from cells grown at 32°C in supplemented M56 media to an OD_{590}=0.3. The cultures were then induced by shaking another 60 min at 41°C. The cells were pelleted by centrifugation, resuspended in 1/40 volume supplemented M56 media and heated for 20 min at 95°C after being brought to a final concentration of 0.7% SDS and 0.07% β-mercaptoethanol. Alternatively, cells were pelleted by centrifugation and resuspended in 50 mM Tris-HCl, pH 8.0, in 25% sucrose. Lysozyme was added to a final concentration of 2 mg/ml. After 5 min at 0°C, $MgCl_2$ was added to 5 mM final concentration, followed by DNase 1 to 60 µg/ml. The cells were lysed by the addition of 1% NP40/0.5% sodium deoxycholate/0.1 M NaCl/0.01 nM Tris-HCl, pH 7.2/1 mM EDTA and centrifuged at 12,000 g for 10 min. The pelleted material was washed with 1 M NaCl/10 mM in 1% SDS/0.0% β-mercaptoethanol by being heated for 10 min at 95°C. This resuspended material is designated the "high-salt-pellet fraction."

5. Computer analysis of DNA sequences. A package of Fortran programs has been written to be used in the laboratory MINC 11/23 minicomputer. These include: an editor to create and alter files that contain DNA sequences; a program to search for restriction sites; a program to translate DNA sequences into amino acid sequences; a program to create hard copy dot matrix homolgy diagrams of the type described by Maizel and Lenk (Proc. Natl. Acad. Sci. USA 78: 7665-7669, 1981) on the laboratory LA34-VA graphics printer. A high-speed communications interface and the NIH DCRT CLINK program have allowed the efficient use of programs run on the NIH DEC/10 and IBM 370 mainframes. Frequently used programs on these machines include: the SEQ and Queen and Korn programs for DNA sequence analysis; the Lipman and Wilbur DNA and protein sequence alignment program; the DNA and protein data base homology search program of these workers; the protein hydrophobility and secondary structure analysis program of R. Feldmann; and the Genbank retrieval system of R. Feldmann. The later program has saved the laboratory a large amount of effort since we have been able to make use of several lengthy published sequences without having to manually enter them into our computer.

Major Findings:

1. A plasmid, pJL6, was constructed that was of general usefulness in the efficient expression of oncogene proteins in E. coli. The insertion of the carboxy-terminal portion of the MC29 v-myc gene into this plasmid allowed the high-level synthesis of a 23,500 dalton protein. Five lines of evidence indicate that this protein is the expected protein containing myc sequences.

(i) The size of the protein corresponds well with the number of codons between the cII initiation codon and the v-myc termination codon. Translation of each of the other v-myc frames would lead to a much shorter peptide. (ii) The protein is not seen in temperature shifted cells harboring the plasmid vector without an insert. (iii) Synthesis of significant levels of the protein requires heat inactivation of the phage λ repressor. (iv) There is some, but much less, synthesis of this protein in cells that do not contain the λ gene N product. In the absence of this protein, most messages initiated at the P_L promoter are terminated at the t_{R1} site upstream from the cII translation initiation codon. (v) Rabbits immunized with the presumed cII-myc fusion protein develop antibodies that immunoprecipitate the MC29 $P110^{gag\text{-}myc}$.

2. A major portion of the gene for the Harvey murine sarcoma virus (Ha-MuSV) $p21^{ras}$ was placed into the pJL6 expression vector. Bacteria expressed high levels of the protein product of this gene. The protein when expressed in bacteria was immunoprecipitated by monoclonal antibodies directed against Ha-MuSV $p21^{ras}$. The bacteria protein also shared the known biochemical properties of the viral $p21^{ras}$ because it was found to bind GDP and was capable of autophosphorylation.

3. Dr. Nancy Kan has been able to use pJL6 to express as protein in bacteria the avian myeloblastosis myb gene. Dr. Arun Seth has used the vector to express Moloney murine sarcoma virus mos sequences.

4. The alignment program of Lipman and Wylbur shows that the U3 region of the LTR of avian myeloblastosis virus is more closely related to the U3 region of ev-1 and RAV-0 than to the U3 region of RSV or MC29. This is suprising since AMV is a very virulent exogenous virus while RAV-0 is a much less virulent endogeneous virus and ev-1 is a common genetic locus that appears to have little or no deleterious effect on the animal.

5. Significant homology could readily be visualized when the protein sequence of the v-raf gene determined by Dr. George Mark was compared to that of other sarcoma viruses by the dot matrix homology plots generated by the LMO MINC 11/23 computer. This is of interest because the v-raf product does not possess protein kinase activity. The usefulness of amino acid comparisons was demonstrated since no homology was apparent in the corresponding DNA plots.

Significance to Biomedical Research and the Program of the Institute:

To understand more fully how oncogenes are involved in malignant transformation it will be necessary to study the protein products of these genes. The expression of such proteins in bacteria will aid such studies in several ways. In many cases such proteins have been shown to be enzymatically active. In these cases they can also be analyzed for biological activity. Even when only part of an oncogene is expressed, the protein product is useful because it can raise antibodies that may cross react with the "normal" or "abnormal" protein products of oncogenes in mammalian cells. Such a study is especially of interest for the myc gene since this gene is a preferred integration target for avian leukosis virus in bursal lymphoma and is rearranged in murine plasmacytoma and human Burkitt lymphoma.

Proposed Course:

1. Analysis of the protein products of oncogenes in normal and malignant vertebrate cells. The protein products from oncogenes placed in bacterial expression plasmids will be used to raise antibodies in rabbits against these proteins. These antibodies will be used to immunoprecipitate proteins in vertebrate cells that share antigenic properties with the proteins synthesized in bacteria. Such vertebrate proteins presumably will be the product of the oncogenes within the vertebrate cell analyzed. The validity of this method has been demonstrated since antibodies directed against the bacterial myc protein immunoprecipitate the MC29 P110$^{gag\text{-}myc}$. Targets of interest include myc-related protein in normal chicken cells, avian leukosis virus-induced chicken bursal lymphomas, murine plasmacytomas, and human Burkitt lymphoma. It may be necessary to express c-myc sequences from the host species to make these antibodies. Presently, Dr. Dennis Watson has used the pJL6 expression vector to produce a protein containing human c-myc sequences. The myb related proteins that should be accessible by this method include the normal c-myb protein found in leukemic myeloblasts induced by AMV. The antigen used in the later study will be the cII-myb protein produced by bacteria containing the plasmid pNKcII-myb 1. This plasmid was constructed by Dr. Nancy Kan who inserted AMV myb sequences into the pJL6 expression vector.

2. Mutational analysis of the Ha-MuSV ras gene. Deletions into the Ha-MuSV gene will be made by the method of Barnes and Beven (Nucl. Acids Res. 11:349-368, 1983). This method involves: (i) cloning a gene into an M13 derivitive constructed by these workers (WB2348); (ii) linearizing the DNA at random places by the use of nucleases; (iii) ligation of linkers onto the linearized DNA; (iv) cleavage by an enzyme that will cut the linkers and a single specific site on the DNA; and (v) recircularization of the DNA by the action of DNA ligase. The result is an ordered set of deletions that extend from the unique restriction site to random location in the DNA. The deleted DNA can be inserted into the pJL6 expression vector and the protein products can be analysed for enzymatic or biological activity. As a variation of this procedure, deletions that extend into the same position from each end can be recombined so as to produce "linker scanning mutants." These mutants possess a substitution of small regions by the sequences of oligonucleotide linkers. Analysis of the protein products of such mutants could help pinpoint the functional regions of the p21^{ras} protein.

3. Analysis of oncogene proteins for biological activity. Proteins expressed in bacteria from oncogene sequences by the use of the vector pJL6 will be analyzed for their effect on living cells. These methods include microinjection into cells (in collaboration with Dr. George Scangos, Johns Hopkins University) or incorporation into cells by hypoplasmic shock (Rechsteiner et al. Cell 29: 33-42, 1982). The later analysis will be carried out in collaboration with Dr. Hayden Coon of NCI. At present the most promising protein for these studies is p21^{ras} because the bacterial protein has been shown to possess the enzymatic properties of the viral protein. The bacterial myc and myb proteins also should be suitable for this analysis as well as any proteins synthesized from genes altered by directed mutagenesis.

4. Expression of the complete MC29 p110$^{gag-myc}$ as protein. A Cla I-Bam HI fragment containing the MC29 gag sequences and all of the myc sequences not already in pJLcIImycl will be inserted into pJLcIImycl by the use of linkers. The protein synthesized from this gene should have more of the properties of P110$^{gag-myc}$ than the protein synthesized just from the carboxy-terminal end of the myc gene.

5. Analysis of the AMV LTR promoter. While both AMV and RSV are virulent exogenous viruses, there is a wide difference in the tumors they produce and in the specificity they have for transformation target cells. AMV can only transform hematopoietic cells in vitro while RSV can transform fibroblasts. While much of this is likely due to the nature of the viral oncogenes, it is of interest to determine if the promoters in the LTR region of these viruses differ. Dr. Louis Laimins, Laboratory of Molecular Virology, and collaborators have constructed an assay for promoter activity in cells by transiently introducing into these cells a plasmid containing the putative promoter coupled to a bacteria chloramphenicol acetyl transferase gene. The later gene is very useful since the enzyme does not occur in vertebrate cells and is quite easy to assay. Dr. Laimins has already analyzed the RSV LTR by this method and found it to be active in a wide variety of vertebrate cell lines from several tissue types. When the AMV LTR is analyzed by the same methods it will be of great interest to determine if its activity is more restricted as to species and cell type of the host cell. The study with AMV should also provide evidence as to the nature of possible enhancer elements in the avian retroviruses. This question is of interest for these viruses because, unlike the murine sarcoma viruses, the avian retrovirus LTR do not have lengthy internal repeats.

Publications:

Lautenberger, J. A.: A program for reading DNA sequence gels using a small computer equipped with a graphics tablet. In Soll, D. and Roberts, R. J. (Eds.) The Applications of Computers to Research on Nucleic Acids. Oxford, UK, IRL Press, 1983, pp. 27-30.

Lautenberger, J. A., Court, D. and Papas, T. S.: High level expression of Escherichia coli of the carboxy-terminal sequences of the avian myelocytomatosis virus (MC29) v-myc protein. Gene, 1983 (In Press).

Lautenberger, J. A., Kan, N. C., Court, D., Pry, T., Showalter, S. and Papas, T. S.: High-level expression of oncogenes in Escherichia coli. In Papas, T. S., Chirikjian, J. and Rosenberg, M. Gene Analysis Techniques, North Holland Elsevier, 1983, (In Press).

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Tsichlis, P. N., Spyropoulos, D., Pry, T. W., Rushlow, K. E. and Papas, T. S.: The transforming sequences of avian myelocytomatosis virus (MC29). In Marchesi, V. T., Gallo, R. and Majerus, P. (Eds.): Differentiation and Function of Hematopoietic Cell Surfaces. New York, A. R. Liss, 1982, pp. 1-15.

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D. K., Baluda, M. A. and Reddy, E. P.: Complete nucleotide sequence of the transforming genes of avian myeloblastosis virus (AMV). J. Cell. Biochem. 20: 95-103, 1982.

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D. K., Baluda, M. A. and Reddy, E. P.: The nucleotide sequence of the transforming genes of avian myeloblastosis virus (AMV). In Scolnick, E. M. and Levine, A. J. (Eds.): UCLA Symposium on Tumor Viruses and Differentiation. New York, A. R. Liss, 1982, pp. 155-163.

Reddy, E. P., Reynolds, R. K., Watson, D. K., Schulz, R. A., Lautenberger, J. A. and T. S. Papas: Nucleotide sequence analysis of the proviral genome of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA 80: 2500-2504, 1983.

Rushlow, K. E., Lautenberger, J. A., Papas, T. D., Baluda, M. A., Perbal B., Chirikjian, J. G. and Reddy, E. P.: Nucleotide sequence of the transforming gene of avian myeloblastosis virus (AMV). Science 216: 1421-1423, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05156-04 LMO

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression of MSV Genetic Information

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Thomas Gordon Wood Senior Staff Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The essential region of the LTR responsible for enhancement of the transforming activity of v-mos and activation of the transforming potential of c-mos is a sequence in the unique 3' region of the LTR containing the 73-bp tandem repeat. A single copy of the tandem repeat does enhance the transforming activity of v-mos, while transfection of DNA clones containing less than one copy of the 73-bp sequence result in a transforming activty equivalent to transfection of v-mos alone. A sequence within the normal mouse DNA preceding c-mos prevents activation of the transforming potential of c-mos by a 3' LTR. A series of deletion mutants were utilized in defining a 200 bp DNA fragment, 1.3 kb upstream from c-mos, that functions in a cis-acting fashion to prevent activation of c-mos. Insertion of this cis-acting element into the viral sequences preceding v-mos prevents enhancement of the transforming activity of v-mos by a 3' LTR.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

G. F. Vande Woude	Chief, LMO	LMO, NCI
M. L. McGeady	Staff Fellow	LMO, NCI
D. G. Blair	Expert	LMO, NCI

Objectives:

The objective of this project is to identify and characterize genetic elements required for activation and expression of genes capable of neoplastic transformation. Our specific goals are to assess the role of the retroviral long terminal repeat (LTR) in the expression of oncogenic sequences, to identify the nucleotide signals that regulate the biogenesis and processing of mRNA from these genes and to determine the mechanism through which cellular sequences located upstream from the mouse c-mos gene prevent activation of the transforming potential of c-mos by a proviral LTR.

Methods Employed:

High molecular weight DNA was isolated from cells transformed by DNA transfection. After digestion with restriction endonucleases, the DNA was subjected to electrophoresis on agarose gels and analyzed by Southern technique (E. M. Southern, J. Mol. Biol. 98: 503, 1975). Hybridization analysis of DNA blots employed radioactive probes specific for various DNA segments which comprise the transfected DNAs.

The construction of recombinant DNA clones employed standard techniques for restriction digestion, DNA fractionation and ligation, and used pBR322 as the cloning vector. DNA transfection-transformation assays were performed as described by Blair et al. (Proc. Natl. Acad. Sci. USA 77: 3504, 1980).

Major Findings:

The molecular elements of the Moloney sarcoma virus (MSV) proviral genome responsible for cell transformation are the acquired v-mos sequence and the proviral LTR. We have shown that in DNA transfection analysis the transforming activity of v-mos is enhanced with equivalent efficiency by introducing a single LTR in either a 5' or 3' position relative to v-mos. We have demonstrated that RNA transcripts containing v-mos sequences are expressed in these transformed cells and shown that a single copy of the LTR is present in the integrated copy of the transfected DNA. To identify the essential LTR sequences required for enhancement, we have tested the transforming activity of a series of recombinant DNA clones containing various sequences derived from the LTR and introduced 3' to v-mos. These experiments have shown that the essential element within the LTR responsible for the enhancing properties is a region containng the 73 base pair (bp) tandem duplication. If LTR sequences encoding the polyadenylation signal but not the 73 bp tandem repeats are introduced 3' to v-mos, the transforming efficiency is equivalent to the level observed with transfection of v-mos DNA alone. Both

copies of the tandem repeat are not required for enhancement. A single copy of the repeat does enhance the tranforming activity of v-mos. However, the transforming efficiency is less with a single copy of the repeat sequence than the level observed with tandem repeats. Further deletions that yield less than one complete copy of the 73 bp repeat resulted in a transforming efficiency equivalent to transfection of v-mos alone, suggesting that at least one full copy of the tandem repeat is required for enhancement.

The mouse cellular homologue to v-mos, c-mos, is present as a single-copy gene per haploid genome and is evolutionarily conserved in vertebrates. In contrast to its viral counterpart, c-mos does not induce morphological transformation in DNA transfection assays. The transforming potential of c-mos is efficiently activated when an LTR is inserted 5' to c-mos but not when the LTR is introduced 3' to c-mos. This is in contrast to results obtained for LTR enhancement of the transforming activity of v-mos. In an effort to explain the low transforming activity observed with an LTR introduced 3' to c-mos the RNA transcripts expressed in cells transformed by this recombinant DNA were examined. In one transfectant a 3.8-kb c-mos containing RNA transcript was detected by probes representing either c-mos or the U3 region of the LTR. This is consistent with the presence of LTR sequences 3' to c-mos. However, a probe representing the 3 kb of normal mouse sequences preceding c-mos did not hybridize to the 3.8 kb RNA transcript. Southern analysis of cellular DNA from this transfectant demonstrated that the mouse sequence 5' to c-mos had been deleted from the integrated copy of the transfected DNA. These results suggested that the loss of mouse sequences preceding c-mos may be necessary for activation of the transforming potential of c-mos. To determine the effect of these normal mouse sequences, a series of recombinant DNA clones were constructed, each containing an LTR 3' to c-mos but with varying regions of the mouse sequence 5' to c-mos. Results from tests to determine the transforming activity of each of these recombinant DNA clones suggested that a 200 bp fragment of the mouse sequence, located 1.5 kb upstream from c-mos, functions in a cis-acting fashion to prevent activation of the transforming potential of c-mos by a 3' LTR.

To determine if this mouse sequence can prevent enhancement of the transforming activity of v-mos by a 3' LTR, a DNA fragment containing the cis-acting mouse sequence was introduced into the viral sequences preceding v-mos. The transforming efficiency of a recombinant DNA containing an LTR 3' to v-mos was reduced 90% by introducing this mouse sequence immediately 5' to v-mos. However, this reduction is dependent upon the position in the viral sequences 5' to v-mos that the mouse sequence is inserted and to some extent on its orientation relative to v-mos.

Significance to Biomedical Research and the Program of the Institute:

The acute transforming retroviruses have been useful in demonstrating the role of specific cellular sequences, termed oncogenes, in malignant transformation. A portion of the genome of these viruses is homologous to host chromosomal sequences that represent the cellular progenitor (c-onc) to the viral encoded oncogene (v-onc). We have examined one such oncogenic sequence, mos, which is responsible for the transforming activity of Moloney sarcoma virus (MSV). We have shown that the transforming activity of v-mos is enhanced by a single LTR from either a 5' or 3' relative position. The cellular homologue to v-mos,

c-mos, does not induce cell transformation. However, the transforming potential of c-mos can be activated by insertion of a proviral LTR. The normal biological functions of c-mos and the other oncogenes that have been identified are unknown. Since these genes are evolutionarily conserved, they may represent essential genetic elements in normal cells and, under the appropriate genetic controls, may perform a necessary cellular function in the absence of a pathological response. Disruption of normal cellular restraints allowing uncontrolled expression of an oncogene results in neoplastic transformation. Studies to identify and characterize genetic elements that control gene activation and RNA transcription wil benefit our understanding of gene expression.

Proposed Course:

During the next year, we plan to continue our studies on the role of the LTR in gene expression; however, our emphasis will be concentrated more on a separation of promoter and enhancer functions. Furthermore, by reconstructing proviral-like structures containing one complete LTR and a partial LTR sequence at the opposing position, we intend to assess the coordinate effects of LTR sequences on the expression of an intervening DNA sequence.

We have initiated a study to characterize the subgenomic v-mos containing mRNA expressed from both the HT1 and m1 strains of MSV and to identify the nucleotide signals involved in the expression of v-mos subgenomic mRNA.

A detailed analysis of the cis-acting element present in the mouse sequence preceding c-mos will also be a major objective. A further characterization of the ability of this sequence to influence gene expression is required with particular emphasis on the mechanism involved. Furthermore, we intend to examine the effect of this cis-acting element on the expression of other mammalian genes and hopefully to initiate a similar analysis of the DNA sequences preceding the human homologue to c-mos.

Publications:

Wood, T. G., Blair, D. G. and Vande Woude, G. F.: Moloney sarcoma virus: Analysis of RNA and DNAs structure in cells transformed by subgenomic proviral DNA fragments. In Robberson, D. L. and Saunders, G. F. (Eds.): Perspectives on Genes and the Molecular Biology of Cancer. New York, Raven Press, 1983, pp. 299-306.

Wood, T.G., McGeady, M. L., Blair, D. G. and Vande Woude, G. F.: Long terminal repeat enhancement of v-mos transforming activity: Identification of essential regions. J. Virol. 46: 726-736, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05186-03 LMO

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression and Regulation of Avian RNA Tumor Viruses and Transforming Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
K. Samuel Visiting Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have determined the complete nucleotide sequence of the human cellular analog (c-myc) of the transforming gene (v-myc) of avian myelocytomatosis virus (MC29). We have also cloned a rearranged fragment flanking the 5' region of the c-myc gene of the beta lymphoid cell line, NC37. This cell line has previously been shown to overproduce myc mRNA. Possible mechanisms for regulating transcription of c-myc involving DNA rearrangement and modification upstream from the 5'-axon are being investigated. This region shows a different pattern of cytosine modification in NC37 cells than in normal human DNA. Finally, we have screened two lower phyla eukaryotes, C. elegans (a nematode) and D. discoideum (slime mold) for myc-related sequences. Southern blot hybridization experiments show that both organisms contain myc-like sequences in their genome.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Takis Papas	Head, Carcinogenesis Regulation Section	LMO, NCI
James Lautenberger	Expert	LMO, NCI
Dennis Watson	Staff Fellow	LMO, NCI
Nancy Kan	Visiting Fellow	LMO, NCI
Christos Flordellis	Visiting Fellow	LMO, NCI

Objectives:

The scope of these investigations is (a) to study the sequence organization of the c-myc locus in human and lower eucaryotic organisms, (b) to identify a biological function(s) for the myc gene product in normal and neoplastic cells, (c) to investigate possible mechanism(s) of transcriptional activation of c-myc during neoplastic transformation, and (d) to study the evolutionary relationship of myc to its non-chordate precursor in the most primitive organism in which this gene is detected.

Methods Employed:

A. Cloning

1. The human c-myc locus was isolated by screening a human DNA library with the MC29 (v-myc) probe using standard recombinant DNA techniques. One clone, λ-LMC41, was subcloned into the plasmid pBR322 at the Eco RI/Hind III sites. The 8.4 kb insert with the entire c-myc locus was used as a source of DNA fragments for DNA sequencing.

2. A rearranged ~3.5 kb Pst/Pst DNA fragment spanning the 5' flanking sequence and up to the first Pst I site within the 5' portion of the first (or 5') exon of the human β-Lymphoid cell line, NC37, has been cloned. A total Pst I digestion of NC37 cell DNA was separated on a 0.8% low melting agarose gel, and the size fragments between 2.0 kb and 4.0 kb range were extracted and purified. This DNA was then used for ligation into Pst I site of pBR322 plasmid that was treated with calf intestinal phosphatase using standard DNA ligation techniques. The ligated DNAs were used for transformation of calcium-treated C600 bacteria, and colonies selected on tetracycline agar plates. These colonies were hybridized to a 700 bp probe specific for the upstream 5' flanking region by standard colony hybridization techniques.

3. DNA modification of the 5' flanking region of c-myc in NC37 and normal human placenta DNA was studied using the restriction endonucleases Hpa II and Msp I to digest high molecular weight cellular DNAs, separating on 0.8% or 1.0 agarose gels, blotting onto 0.45 μm or 0.20 μm pure nitrocellulose filters, and hybridizing with specific 5'-flanking c-myc probes. The enzyme Hpa II will not cleave DNA in which the C in the dinucleotide 5'-CpG-3' is methylated.

4. Standard bacteriophage vectors containing cellular DNA libraries of the nematode (C. elegans) and slime mold (D. discoideum) were screened by plaque hybridization to a 1.5-kb Pst/Pst v-myc probe under relaxed conditions. After three cycles of plaque purification, clones showing positive hybridization signals for myc were picked and their DNAs extracted by standard techniques.

B. DNA Sequencing Strategy

The normal human c-myc locus, originally subcloned into pBR322, was used for sequence analysis by the chemical sequencing technique of Maxam and Gilbert. Appropriate restriction endonuclease fragments were purified from agarose gels using standard gel purification techniques. The DNA fragments were labeled either (i) at their 5'-termini with [γ-^{32}P] ATP and polynucleotiode kinase, or (ii) at their 3'-ends using [α-^{32}P] cordycepin phosphate and deoxynucleotidyl transferase. Some DNA fragments with recessed 3'-termini were end-labeled with [$\alpha 3^{2}$P] dNTP and E. coli Klenow DNA polymerase. The resulting end-labeled fragments were either recut with another restriction enzyme or strand separated, and their sequence ladders read from 5%, 8%, or 20% sequencing gels.

Major Findings:

1. Examination of the nucleotide sequence of the human c-myc gene reveals (a) the presence of two exons interrupted by non-coding (intervening) sequences, and (b) an open reading frame and an ATAA translational termination sequence; but we have not yet identified an ATG translational initiation codon in the domain of the sequenced regions of c-myc.

2. We have found that the c-myc gene in the NC37 β-lymphoid cell line has undergone rearrangement at the 5'-flanking region of one of its alleles, and is only detectable upon hybridization of Pst-I and Sst-I digested cell DNA with 5'-flanking probes. We are not yet certain whether this gene is also translocated, as is the case of several Burkitt's and non-Burkitt's β-lymphomas. We have also found that the state of methylation at the 5'-flanking sequences of c-myc is different from the normal homolog. A mechanism for transcriptional control involving DNA modification of c-myc is under continuing investigation. Previous studies have shown that NC37 cells overproduce c-myc mRNA. We have cloned and are presently characterizing a DNA fragment (an 3.5-kb Pst-I piece) which contains the rearranged myc sequence.

3. Cloning of myc-related sequences in C. elegans and D. discoideum:

Using a complete Bam HI library of D. discoideum DNAS, we have isolated recombinant phages which contain putative myc or myc-related sequences. Under relaxed hybridization conditions (e.g., 30% formamide, 30-35°, in 5XSSC) Southern blot analysis with the 1.5-kb Pst/Pst v-myc probe reveals that both C. elegans and D. discoideum contain several restriction enzyme fragments that light up positively with our probe.

Significance to Biomedical Research and the Program of the Institute:

Specific activation of the c-myc gene in human β-lymphomas and other β-lymphoid cell lines and tissues have been noted. Both c-myc gene rearrangement and chromosomal translocation in these cells may play an important role in human oncogenesis. Investigation of the structure and organization of normal and rearranged human c-myc gene, and its interaction with the immunoglobulin gene family (in Burkitt's and non-Burkitt's β-cells) will greatly enhance our ability to control or modulate the expression of this oncogene. Identifying the common precursor(s) of myc in lower eukaryotes could make possible the identification of its functional role(s) during evolution.

Proposed Course:

Ongoing and proposed experiments will involve:

1. Characterization of the rearranged cloned NC37 myc fragment by restriction enzyme mapping and DNA sequencing.

2. Investigation of other upstream signals other than DNA modification (e.g., enhancer or modulator) sequences that may control myc mRNA expression in NC37 cells.

3. Perform a detailed study of NC37 myc mRNA initiation and expression using Northern blotting techniques and nuclease S1 analysis; DNA probes of defined regions (intron, exon, recombination junction) flanking the myc exon will be employed.

4. Analysis of NC37 myc gene product using myc-specific anti-sera and, eventually, myc-specific antibody to quantitate and investigate the structure of the myc protein.

5. To characterize the C. elegans and D. discoideum myc-like sequences in the clones isolated using standard restriction enzyme mapping, DNA sequencing, and DNA R-looping techniques; by comparison with the human, mouse, and chicken c-myc genes, the evolutionary divergence of these sequences in lower eukaryotes will be investigated.

Publications:

Watson, D. K., Psallidopoulos, M. C., Samuel, K. P., Dalla Favera, R. and Papas T. S.: The myc genes. In Prasad, U., Levine, P. and Pearson G. (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Maylaysia, University of Malaysia Press (In Press).

Watson, D. K., Psallidopoulos, M. C., Samuel, K. P., Dalla Favera, R. and Papas, T. S.: Nucleotide sequence analysis of the human c-myc locus, chicken homologue, and myelocytomatosis virus MC29 transforming gene reveals a highly conserved gene product. Proc. Natl. Acad. Sci. USA 80: 3642-3645, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05187-03 LMO

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Nature of DNA Rearrangements in the Mlvi-1 Locus in MoMuLV Induced Rat Thymomas

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
P. G. Strauss Guest Research, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Mlvi-1 locus is a cellular DNA region that represents a common provirus integration substrate in MoMuLV induced rat thymomas. The provirus integrated in this locus in three independent tumors was cloned and it was found to be intact and biologically active. However, the rearrangements observed in the Mlvi-1 locus in several additional tumors are not due to provirus DNA integration.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

P. N. Tsichlis Expert LMO, NCI

Objectives:

The overall objective of this project is to define the mechanism(s) by which certain cellular DNA regions may be involved in tumor induction and/or progression.

Methods Employed:

Molecular cloning, Southern blotting and hybridization, and DNA transfection.

Major Findings:

1. The MoMuLV provirus integrated in Mlvi-1 appears in the majority of cases to be intact and biologically active.

2. In some rat thymomas there are rearrangements of the Mlvi-1 locus that are not due to the integration of the MoMuLV provirus. In some of the tumors these rearrangements appear in addition to the rearrangements caused by the integrated provirus suggesting that two separate processes affecting the same locus may be occurring simultaneously in the same tumor.

Significance to Biomedical Research and the Program of the Institute:

Understanding the molecular mechanisms of oncogenesis in a mammalian system will provide further insight into the understanding of human cancer.

Proposed Course:

This intramural project will be terminated as the principal investigator is leaving the National Cancer Institute.

Publications:

Tsichlis, P. N., Hu, L. F. and Strauss, P. G.: Two common regions for proviral DNA integration in MoMuLV induced rat thymic lymphomas. Implications for oncogenesis. In Goldes, D. W. and Marks, P. A. (Eds.): UCLA Symposia on Molecular and Cellular Biology, New Series. New York, Alan Liss, Vol. 9. (In Press)

Tsichlis, P. N., Strauss, P. G. and Hu, L. F.: A common region for proviral DNA integration in MoMuLV-induced rat thymic lymphomas. Nature 302: 445-449, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05221-03 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Recombination in Prokaryotic and Eukaryotic Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

S. Bear Staff Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

Laboratory of Experimental Pathology, NIADDK

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Molecular Control and Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The interaction of a phage encoded topoisomerase I with specific sites for DNA recombination is being studied by: 1) genetic analysis of the inhibition of this reaction by various topoisomerase I mutants and 2) biochemical analyses using attachment sites cloned onto a plasmid vector. Functional domains of the protein and recombination intermediates are being identified.

Control regions of the genome of HSV-1 have been identified in the dDNA that occur as reiterated sequences in virion particles. An origin of replication and packaging sites are evident. When lambda HSV hybrid clones are transfected into eukaryotic cells with HSV helper, substantial editing of the lambda sequences is seen.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

D. Court	Head, Carcinogenesis Regulation Section	LMO, NCI
A. Colberg-Poley	Postdoctoral Fellow	LEP, NIADDK

Objectives:

A. To examine the interaction of a bacteriophage encoded DNA topoisomerase type I with a specific recombinationally active DNA substrate. This topoisomerase I is the product of the lambda int gene, and is required for the site specific DNA recombination event that occurs when λ DNA integrates into, or is excised from, the E. coli chromosome. The integration event occurs between a specific site on the λ chromosome, attP, and a site on the E. coli chromosome, attB, and results in an integrated prophage flanked by virus/host hybrid sites, attL on the left and attR on the right. In addition to the phage encoded int protein, integration and excision require host factors (himA and hip). Excision, the recombination event between attL and attR that results in excision of the prophage also requires a second phage encoded protein, Xis. These recombination events are multi-step processes involving: a) recognition by the toposiomerase and host factors of the DNA substrates, b) binding of the proteins, c) synapsis of the two DNA substrates, d) cleavage of the DNA, and e) recombination of the DNA molecules and release from the synapse. A chi-form, or Holliday structure, has been proposed to be the intermediate form of the two DNA molecules during site specific recombination. To study the different steps of the recombination event and to identify the proposed intermediates of recombination, we are utilizing bacteriophage with mutations in the int gene.

B. To identify control regions in the genome of Herpes simplex virus type 1 (HSV-1) present in the DNA of defective HSV-1 particles. This DNA is isolated from the virion capsids as a repeated 9.5 kb sequence and most probably contains active replication and packaging sites. In addition, it is known to carry two promoters for immediate early messenger RNA synthesis.

Methods Employed:

A. Standard genetic, biochemical, and recombinant DNA techniques were employed to study the phenomenon of negative complementation: the inhibition of wild-type int by a mutant int. This inhibition is not observed with all point mutants or with some deletion mutants. In some cases, even if the mutant int protein cannot promote recombination alone, the mutant can act to inhibit the wild type int from completing the recombination. A set of 36 int mutants previously generated are being tested for their negative complementation potential in both integration (PxB) and excision (LxR). Also, in place of the wild type int, we are using three "super int" products (from int mutants that produce a more active int) in the same genetic tests. In this way, the mutants are grouped genetically according to their inhibition phenotype. Prototype int mutants will be selected from each group of negative complementing phage and the DNA from the inhibited recombination event subjected to a biochemical analysis to detect recombination intermediates in the following way:

The specific sites for recombination, attP and attB, have been cloned in inverted orientation in the plasmid vector pBR322. In addition, the hybrid attachment sites attL and attR have also been cloned in inverted orientation in pBR322. These cloned sites act as substrates for the recombination event in vivo (integration PxB; excision LxR). Int (mutant and wild-type) and xis proteins are provided by a phage infection of the cells carrying either plasmid. Host factors himA and hip are provided by the E. coli cells. Recombination results in DNA rearrangements in the plasmid and can be demonstrated by restriction endonuclease digestion of the plasmid DNA and examination of the DNA fragments after gel electrophoresis. An intermediate of the recombination event will also be detectable by a characteristic migration in gel electrophoresis.

B. To study the control regions on this HSV fragment it has been cloned. The 9.5 kb Eco RI dDNA fragment (major class I) and the phage vector (λgtWES.B) carrying the 9.5 kb Eco RI fragment (λWES::12-7) have been described previously (Z01CP04882-06 LMV and Z01CP05102-03 LMV). Origin minus HSV DNA fragments (not derived from dDNA populations) have also been cloned into bacteriophage vectors. The hybrid plamid (pBR325:12-7) has also been constructed in this laboratory.

To test for an origin of replication and a possible packaging site this hybrid phage or plasmid DNA is transfected into VERO cells along with helper HSV. When HSV plaques are evident (48 hrs) both total DNA and DNA from herpes virus capsids are isolated. Supernatants are passaged, and after 1,3 and 5 passages, the virion DNA is isolated again. These DNA are restricted with Hpa I and electrophoresed in agarose. The gels are dehydrated and hybridized to either a ^{32}P labelled λ or pBR325 vector DNA. Clones containing an origin of replication and packaging sequence will be perpetuated. This results in an amplification of the adjoining vector sequences.

Major Findings:

A.

1. Chi-form recombination intermediates have been generated in vitro using the plasmids carrying the attachment site substrates.

2. 7/36 phage with int mutations negatively complement an LxR reaction by the biochemical analysis.

3. 2/10 phage tested with int mutations negatively complement a PxB reaction by the genetic analysis.

4. 1/36 phage with int mutations negatively complements a "super int" in an LxR reaction.

5. Inhibition of the reaction seen by genetic analysis is confirmed by the biochemical analysis of plasmid DNA.

6. Xis inhibits a PxB reaction. This is detected both in the genetic and biochemical analyses.

7. DNA sequences representing the coding regions for the int gene have been cloned into an expression vector utilizing the lambda P_L promoter.

8. E. coli host factors himA and hip have been shown to be utilized during packaging of lambda DNA, a reaction that also involves site specific cleavage of DNA by a phage encoded protein (ter).

B. At forty-eight hours there is no significant difference in the λ sequences detected among the experimental groups that received cloned HSV fragments. Transfection with vector alone or with HSV alone produced no sequences hybridizing to the λ probe. However, in two separate experiments, at fifth passage, DNA isolated from virions shows λ sequences only in the group transfected with the λ hybrid carrying the 9.5 kb Eco R1 insert. It was also observed that much of the λ DNA has been deleted and rearranged, and that about half of the 9.5 kb HSV insert is lost. This serves to define more definitively the sequences required for replication and packaging of these hybrid clones. Plasmid sequences are also detected at fifth passage in the experimental group transfected with pBR325: 12-7. However, no major rearrangements were seen in the plasmid DNA amplified and detected at fifth passage.

Significance to Biomedical Research and the Program of the Institute:

A. DNA recombination is similar throughout eukaryotic and prokaryotic life forms. Integration of viral DNA into the genome of its cellular host is seen not only in prokaryotic bacteriophage but in the eukaryotic DNA and RNA tumor viruses. In the RNA tumor viruses, these recombination events are key elements in the disruption of the cellular physiology that leads to the generation of tumors. In addition, chromosomal anomalies resulting from such recombination events as translocations and deletions have been identified in a wide range of disease states. An understanding of the process of DNA recombination in prokaryotes provides a basis for work in eukaryotic systems.

B. These studies indicate that an origin of replication and packaging sequences are present in the DNA that is represented as a concatemer in defective HSV particles. In addition, "deletion mutants" of the original phage:HSV clone are rapidly generated just as defective (deletion) particles are generated from full-length HSV. An understanding of the mechanism of the generation of these deletion mutants will contribute to the understanding of the replicative life cycle of HSV. This will also contribute to the effective use of an HSV replication origin and packaging sequences as part of eukaryotic cloning.

Proposed Course:

A. Genetic analyses will proceed to group the int mutants by negative complementation phenotype. This will allow for selection of mutants to: 1) examine by the biochemical analysis for recombination intermediates and 2) clone the mutant int gene into an expression vector to make high levels of the protein. With purified protein experiments can be designed to determine at which step in the recombination process the different int mutants are inhibitory. These experiments may lead to determination of active sites on the int protein since the genetic lesion has been physically mapped. DNA sequence changes of these mutants will also be determined.

B. In order to understand the mechanism by which the deletion/rearrangement mutant clones are generated from the parent λgtWES:12-7 clone they are being subcloned into the vector pBR322. This will allow for an analysis of the λ and HSV sequences remaining.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05238-02 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologues

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Dennis Watson Staff Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

Biochemistry Department, Georgetown University, Washington, D.C.
Laboratory of Viral Carcinogenesis, NCI

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To determine the functional relationship between the MC29 (avian mycelocytomastosis virus) onc gene and its cellular prototypes, we have determined the sequence of the chicken and human c-myc genes and compared them to the MC29 transforming gene (v-myc). Both cellular genes contain sequences homologous to v-myc in two major exons and interrupted by a single intron with no viral homology. While the total number of viral-specific nucleotides in chicken c-myc is identical to the number in v-myc, the human c-myc has additional nucleotides, concentrated in the 5' exon. The two exons of the cellular genes and the viral gene have a common reading frame and protein terminator signal. The protein predicted from the reading frame suggests myc encodes a conserved gene product. The homology seen between the cellular genes is conserved only in the coding region. Both cellular genes differ from the v-myc by additional 5' sequences, substituted by the Δgag of MC29 in myc protein. This may be the most significant difference between the cellular genes and the viral gene, perhaps eliciting functional differences between the gene products.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Takis Papas	Head, Carcinogenesis Regulation Section	LMO, NCI
James Lautenberger	Expert	LMO, NCI
Kenneth Samuel	Visiting Fellow	LMO, NCI
Nancy Kan	Visiting Fellow	LMO, NCI
Christos Flordellis	Visiting Fellow	LMO, NCI

Objectives:

The purpose of this investigation is to determine the relationship between onc genes and their normal cellular homologs. Structural analysis of these genes will allow us to better understand their biological functions.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cell by treatment with proteinase K followed by phenol-chloroform extraction. Preparation of vector DNA from phage λ derivatives by phenol extraction of CsCl banded phage.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments of agarose gels.

3. Preparations of DNA probes using purified myc-specific DNA, to be nick translated using E. coli DNA polymerase.

4. Preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of myc probes.

5. Construction of recombinant phage libraries by litigation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging.

6. Isolation of phage from the libraries containing virus-related sequences by hybridization of cDNA probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure.

7. Subcloning of isolated c-myc DNA fragments into pBR322.

8. DNA sequencing analysis of cloned DNA by the method of Maxam and Gilbert and by the dideoxyribonucleotide method of Sanger and associates. For the latter method, the cloned DNA is recloned into a filamentous phage.

9. RNA extraction from cultured cells using guanadine hydrochloride extraction. Isolation of RNA after cellular fractionation. Separation of polyA-RNA by several (2-3) cycles of purification through oligo(dT) cellulose. Analysis of RNA on formaldehyde-agarose gels by Northern analysis.

10. Immunoprecipitation of S^{35}-methonine labeled human and chicken cell lysates with myc specific antisera using protein A-sepharose and analyzed by SDS-polyacrylamide gel electrophoresis.

11. S^{35}-methionine labeling of expressed protein in bacteria utilizing human myc fused to λ cII in pJL6 (vector constructed by Dr. James Lautenberger).

Major Findings:

1. The human c-myc gene is larger than the chicken c-myc gene. The 5' exon encodes for 257 amino acids, 23 longer than the chicken homologue. Both 3' exons encode 187 amino acids.

2. The greatest variation between the c-myc loci occurs in the 5' exon, primarily as the result of insertions and deletions, most occurring close to the splicing region.

3. While the amino acid variation between v-myc and chicken c-myc is less than 2%, the variation between chicken c-myc and human c-myc is 27%.

4. Analysis of chicken c-myc sequences 5' to the myc-specific exons allows identification of additional exons in the normal cellular gene which have been replaced by Δgag in v-myc.

5. Synthetic peptide have been used to make antibodies against myc protein. Antibodies have been made that successfully precipitate p110, the viral transforming protein, from Q8 cells.

6. By restriction enzyme digestions and Southern blot analysis, we have investigated polymorphism of myc. While the DNA prepared from normal human leukocyte DNA does not show any polymorphism, DNA from blood and tumor cells from leukemic patients reveals gene rearrangement.

7. Northern analysis of $polyA^+$ RNA from chicken fibroblasts has allowed identification of additional exons 5' to the two viral homologous chicken exons.

8. The cloned human c-myc gene has been modified and re-cloned into an expression vector, pJL6. Successful overproduction of the carboxy terminal amino acids of human c-myc has been accomplished.

Significance to Biomedical Research and the Program of the Institute:

Transformation of cells by acute leukemia is of great importance in defining the gene responsible for leukemogenesis. Analysis of the genomic structure of viral genes and their cellular homologs is of immense importance to better understand the mechanism of the leukemic process.

Proposed Course:

1. The sequence of c-myc 5' to myc-specific sequences will be further analyzed. The complete sequence will allow identification of the cellular initiation signal, thus allowing assignment of the amino acids of the normal cellular gene.

2. Because of the homology between v- and c-myc, the open reading frame of v-myc has been used to predict amino acid sequence of the carboxy terminal region. Synthetic peptides have been used to obtain antibodies. These will be used to identify the cellular myc protein from chicken and human cells.

3. In addition to antibodies against synthetic peptides, the expressed human myc protein will be isolated from gels and used as antigen.

4. The extent of homology betwen c-myc genes of chicken and humans will be assessed by heteroduplex analyses of cloned DNA and by R-loop analyses of cellular RNA and cloned DNA.

5. Analysis of the expression myc-related sequences using Northern analysis of polyA+ and polyA- will be continued, using defined upstream probes to identify and charcterize the entire myc gene in chicken and human.

6. Cat, mouse and hamster DNA will be restricted by multiple restriction enzymes, followed by Southern transfer and hybridization to detect myc homologous sequences. Using enzymes that result in species-specific myc homologous DNA fragments, somatic cell hybrid panels will be analyzed to determine the chromosomal location of myc in these species.

Publications:

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D. K., Baluda, M. A. and Reddy, E. P.: Complete nucleotide sequence of the transforming genes of avian myeltoblastosis virus (AMV). J. Cell. Biochem. 20: 95-103, 1982.

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D. K., Baluda, M. A. and Reddy, E. P.: The nucleotide sequence of the transforming genes of the avian myeltoblastosis virus (AMV). In Scolnick, E. M. and Levine, A. J. (Eds.): UCLA Symposium on Tumor Viruses and Differentiation. New York, A. R. Liss, 1982, pp. 155-163.

Papas, T. S., Rushlow, K. E., Watson, D. K., Bader, J. P., Ray, D. and Reddy, E. P.: The transforming gene of avian myeloblastosis virus (AMV): Nucleotide sequence analysis and identification of its translational product. In Neth, Gallo, Greaves, Moore, Winkler (Eds.): Modern Trends in Human Leukemia V, Berlin/Heidelberg, Springer/Verlag, 1983, pp. 207-213.

Reddy, E. P., Reynolds, R. K., Watson, D. K., Schulz, R. A., Lautenberger, J. A. and Papas, T. S.: Nucleotide sequence analysis of the proviral genome of avian myelocytomatosis virus (MC29). Proc. Natl. Acad. Sci. USA 80: 2500-2504, 1983.

Watson, D. K. and Moudrianakis, E. N.: Purification and characterization of a chromatin-associated enzyme: The H2A protease. Biochemistry (In Press).

Watson, D. K., Psallidopoulos, M. C., Samuel, K. P., Dalla-Favera, R. and Papas, T. S.: Nucleotide sequence analysis of the human c-myc locus, chicken homologue, and myelocytomatosis virus MC29 transforming gene reveals a highly conserved gene product. Proc. Natl. Acad. Sci. USA 80: 3642-3645, 1983.

Watson, D. K., Psallidopoulos, M. C., Samuel, K. P., Dalla-Favera, R. and Papas, T. S.: The myc genes. In Prasad, U., Levine, P. and Pearson, G., (Eds.): Nasopharyngeal Carcinoma: Current Concepts. Malaysia, University of Malaysia Press (In Press).

Watson, D. K., Reddy, E. P., Duesberg, P. H. and Papas, T. S.: Nucleotide sequence analysis of the chicken c-myc gene reveals homologous and unique coding regions by comparison with the transforming gene of avian myelocytomatosis virus MC29, Δgag-myc. Proc. Natl. Acad. Sci. USA 80: 2146-2150, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05239-02 LMO

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural Analysis of the Avian Myeloblastosis Virus Genome

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Nancy C. Kan Visiting Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

MH2 is a member of the defective avian leukemia viruses carrying myc-related oncogenes. This group of viruses also includes MC29, CMII and OK10. Southern blotting of MH2-infected quail DNA revealed a 6.5 kb Eco RI fragment that hybridized to MC29 proviral DNA. This fragment was cloned from a lambda phage library constructed from a size-fractionated RI digest of MH2-infected quail DNA. DNA sequence analysis of the RI fragment indicated that it contained 99% of MH2 proviral genome 5.3 kb in length. Restriction enzyme and heteroduplex mapping showed that 3' to the gag-myc junction between MC29 and RSV, MH2 continued to carry a few hundred bases of gag sequence plus an additional 1 kb sequence not present in MC29 and RSV. The latter probably represented the 5' region of c-myc gene. Most of the gene coding for the envelope protein of MH2 has been replaced by the v-myc region found in MC29. The AMV transforming gene has also been inserted between the ClaI site and the BamHI site of the expression vector pJL6. The fusion protein was predicted to carry 13 amino acids specified by the lambda cII gene and the entire myb region except its first five amino acids to give a molecular weight of 32,000 daltons. When the lambda Pl promoter on the hybrid plasmid was derepressed, E. coli harboring the plasmid produced a high level of a 32K protein. This protein was not synthesized in E. coli grown under repressed condition.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Takis S. Papas	Head, Carcinogenesis Regulation Section	LMO, NCI
James Lautenberger	Expert	LMO, NCI
Dennis Watson	Staff Fellow	LMO, NCI

Objectives:

The scope of this investigation is to delineate the relationship between virus gene expression and conversion of cells from normal to malignant state and to study the molecular anatomy of known tumor viruses and describe the mechanism by which subviral structures act in concert with cellular factors to regulate oncogenesis. To investigate the process by which viral oncogenes, as well as their cellular homologues, induce the activation of the metabolic processes and participate in malignant transformation. To delineate at the molecular level the mechanism by which oncogenes act in concert with cellular factors to induce oncogenesis. To introduce functionally modified oncogenes to specific target cells in an effort to analyze and alter the function of their normal counterparts. The technique of molecular cloning, DNA sequence analysis, and site mutagenesis will be used to implicate specific nucleotides in the transformation process.

Methods Employed:

(1) Preparation of recombinant plasmid DNA by precipitating chromosomal DNA with high salt, followed by CsCl-ethidium bromide banding of supercoiled DNA.

(2) Preparation of 5'-end labeled DNA fragments using $\gamma[^{32}P]$ATP and polynucleotide kinase. Preparation of 3'-end labeled DNA fragments using $\alpha[^{32}P]$NTP and E coli DNA polymerase large fragment.

(3) DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert.

(4) Gel electrophoresis analysis of DNA fragments on agarose or polyacrylamide gels.

(5) Computer analysis of DNA and protein sequences. The program of Queen and Korn is used to list DNA sequences, locate restriction sites, predict amino acids, and find repeated sequences. The program of J. Maizel is used for graphic demonstrations of sequence homology between DNA sequences. The program of M. Davehoff is used to find other DNA sequences and compare protein sequences.

Major Findings:

The pursuit of this project has led to findings in five areas. (1) 99% of MH2 proviral genome has been cloned from MH2-infected quail DNA and the provirus is shown to be 5.3 kb in length. (2) The two LTR's of MH2 provirus are not completely identical. The Eco RI site in the 5' LTR is not present in the 3' LTR. (3) Electron microscopic analysis of heteroduplex structures formed between MH2

and MC29 proviral genomes reveals a single-stranded loop of MH2 1.5 kb in length and located near the gag-myc junction in MC29. (4) DNA sequence analysis indicates that MH2 provirus differs from MC29 in that it carries a nondefective p27 gag protein, has lost most of its env gene, and contains a region of 1 kb which may represent additional myc sequences not present in MC29. (5) The AMV transforming gene has been subcloned into the expression vector pJL6. E. coli harboring the plasmid produces a high level of the 32K cII-myb fusion protein.

Significance to Biomedical Research and the Program of the Institute:

It is clear that the formation, the maintenance and the expression of provirus are the central features of the life cycle of RNA tumor viruses. Many questions related to these aspects are unsettled. To elucidate the process of oncogenesis induced by these viruses, it is important to understand the structural organization of the transforming genes within the host chromosome and the process by which these genes are expressed and regulated.

Proposed Course:

(1) In order to determine whether the recombination site between AMV and MAV overlaps with the RNA splicing site which is used to generate the subgenomic RNA species of AMV and MAV, the S1 nuclease mapping experiment will be performed. A DNA fragment containing the putative RNA splicing site will be labeled at the 5'-ends and after strand separation, hybridized to poly A-containing RNA purified from either AMV-nonproducer cells or MAV-transformed cells. The mixture will then be subjected to S1 nuclease digestion. The nucleotides at the RNA splicing site will be determined by electrophoresing the RNA-protected DNA fragment in parallel with the same fragment subjected to DNA sequencing reactions on a denaturing gel.

(2) In order to localize the other recombination site between AMV and MAV, DNA sequence at the 3'-end of the cloned MAV provirus will be determined. This region also includes the 3'-LTR, the junction between the 3'-LTR and the cellular sequence, and the region between the envelope gene and the 3'-LTR. The latter sequence is of particular interest because it may provide some clue to the problem that MAV itself causes a variety of malignant diseases in birds.

(3) Attempts will be made to synthesize the AMV transforming protein in E. coli. The plasmid vector pCQ2 constructed by C. Queen will be used. It contains the PR promoter of phage lambda and a temperature-sensitive repressor cI gene so that the PR-promoted transcription occurs only at 42°C. Proteins made in E. coli will be labeled with ^{35}S-methionine and analyzed on SDS-polyacrylamide gels. The origin of the viral proteins will be confirmed by immunoprecipitation with antisera against AMV transforming protein.

Proposed Course:

(1) The complete nucleotide sequence of cloned MH2 provirus will be determined. Emphasis will be made on the origin of the 1 kb or so nucleotide sequence which is located near the gag-myc junction and is not present in RSV and MC29. For example, the extra cellular sequence in MH2 can be used as a probe to detect new cellular genes and their transcripts. The relationship between the c-myc gene

homologous to MC29 v-myc region and MH2-related cellular genes will be explored. On the other hand, computer programs will be used to determine if the extra cellular sequence in MH2 is related to the 5' region of c-myc genes in chicken, mouse and man. This comparison could identify intron-exon junctions and help define the complete normal c-myc genes from Drosophila to vertebrates. (2) It has been reported that in MH2-transformed cells, in addition to the viral RNA, there is a spliced subgenomic RNA species which is transcribed into a 57K protein containing only myc sequences. Computer programs will be used to search the nucleotide sequence of MH2 provirus for possible RNA donor and acceptor sites, as well as methionine codons which may serve as initiators for the 57K protein. However, in order to determine precisely the RNA splice site, S1 nuclease mapping experiments will be performed. (3) Since a large portion of the AMV transforming protein has been successfully synthesized in E. coli, attempts will be made to purify the 32K dalton protein in large quantity. Anti-myb antiserum directed against it will then be used to immunoprecipitate viral proteins in AMV-transformed cells. Similar experiments will be performed to clone the MH2 transforming gene into the expression vector pJL6. MH2 transforming protein is more oncogenic than that of MC29 probably because MH2 carries additional cellular sequences to
that in MC29.

Publications:

Lautenberger, J. A., Kan, N. C., Court, D., Pry, T., Showalter, S. and Papas, T. S.: High-level expression of oncogenes in Escherichia coli. In Papas, T. S., Chirikjian, J. and Rosenberg, M. (Eds.): Gene Analysis Techniques, North Holland/Elsevier, Volume 3, 1983. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05286-02 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transforming Genes in Human Tumors and Chemically Transformed Human Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

C. S. Cooper Visting Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The presence of dominant transforming genes in cell lines derived from human tumors and in human cell lines that have been transformed by chemical carcinogens in vitro has been investigated. High molecular weight DNA samples prepared from a teratocarcinoma cell line, a fibrosarcoma cell line and from human cells that were transformed by MNNG were applied to mouse NIH3T3 cells using the calcium phosphate precipitation technique. After transfection the cells were either reseeded and scored for foci of transformed cells after 10-14 days or injected into athymic nude mice, which were examined periodically for the presence of tumors. The results obtained show that the DNA prepared from the MNNG transformed cells and the teratocarcinoma cells were able to morphologically transform NIH3T3 cells and that cells transfected with DNA from the MNNG transformed cells and the fibrosarcoma give rise to tumors in nude mice. Both the morphologically transformed NIH3T3 cells and the tumors possessed DNA highly repeated human Alu sequences.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Donald G. Blair	Expert	LMO, NCI
George F. Vande Woude	Chief, LMO	LMO, NCI

Objectives:

The objectives of this project are 1) to identify and characterize dominant transforming genes present in cell lines derived from human tumors and from human cells that have been transformed in culture by chemical carcinogens; 2) to clone these transforming genes; and 3) to characterize the cloned DNA and compare their sequences with those of analogous DNA sequences isolated from non-transformed cells.

Methods Employed:

Transforming genes present in high molecular weight DNAs prepared from transfected cells were detected using the standard transfection/transformation assay as well as a new technique in which transfected NIH3T3 cells were injected directly into athymic nude mice. Southern blotting using a cloned probe specific for highly repetitive human DNA sequences was used to determine whether transformed NIH3T3 cells and tumors induced in nude mice contained human DNA sequences.

Major Findings:

The presence of dominant transforming genes in cell lines derived from a human teratocarcinoma and human fibrosarcoma as well as in human cells that were transformed in vitro by MNNG has been examined. The results show that DNA from the teratocarcinoma and the MNNG transformed cells were able to morphologically transform NIH 3T3 cells with efficiences of 0.02 and 0.05 focus forming units per g DNA respectively while DNA prepared from nontransformed human cells from calf thymus had little or no activity (< 0.005 focus forming units per µg DNA). The particular morphologies of the foci of transformed cells induced by the DNAs from the teratocarcinoma and chemically transformed cell lines were characteristic and different but both of the types of primary transfectant exhibited anchorage independence in soft agar and were able to induce tumors in immunocompetent as well as athymic nude mice.

DNA samples prepared from the teratocarcinoma and MNNG primary transfectant all contained high levels of the repeated human Alu sequence and were used in a second round of transfection experiments to produce secondary transfectants. All DNA samples induced foci with an efficiency of 0.13-0.33 focus forming units per µg DNA. Samples of DNA prepared from a series of MNNG-HOS secondary transfectants were digested with EcoRI, blotted and probed for the presence of human Alu sequences. The results showed that the levels of Alu sequences were considerably lower than those found in DNA sample from primary transfectants and that some particular sequences of DNA that hybridized with the Alu probe were present in most secondary transfectants.

Southern blots of DNA from MNNG-HOS, PA1 and HT1080 transfectants were probed to see whether they contained the human homologous of the onc-genes of the Harvey and Kirsten sarcoma virus. The probes failed to detect human DNA sequences in the transfectants under conditions where they hybridize to sequences in human DNA.

The presence of dominant transforming genes was also investigated in experiments in which NIH3T3 cells that had been transfected with human tumor DNA were injected into athymic nude mice, which were then examined for the presence of tumors. The results showed that cells that were transfected with DNA from either MNNG transformed tumor cells or a human fibrosarcoma cell line induced tumors in nude mice in 5-6 weeks while no tumor appeared after 8 weeks when cells that had been transfected with calf-thymus DNA were injected into nude mice. Furthermore DNA obtained from many of these tumors contained high levels of human Alu sequences. Analyses of the human Alu sequences associated with the transforming genes revealed that the nude mouse assay and the NIH3T3 transfection assay detected the same MNNG-HOS transforming gene.

Significance to Biomedical Research and the Program of the Institute:

The results of the studies described here show that a human fibrosarcoma cell line and a human teratocarcinoma cell line contain transforming genes. Thus, the induction of at least some human fibrosarcoma and teratocarcinoma may induce the activation of a dominant transforming gene. The results also show that an MNNG transformed human cell contains a dominant transforming gene and since attempts to detect similar active DNA sequences in the cell from which this transformant was derived by treatment with the chemical carcinogens were unsuccessful it is therefore conceivable that chemical carcinogens may be able to activate potential transforming genes present in human cells.

Proposed Course:

The human transforming genes present in these studies will be cloned by standard cloning procedures.

Publications:

Blair, D. G., Cooper, C. S., Oskarsson, M. K., Eader, L. A. and Vande Woude, G. F.: New method for detecting cellular transforming genes. Science 218: 1122-1125, 1982.

Blair, D. G., Cooper, C. S., Oskarsson, M. K., Eader, L. A. and Vande Woude, G. F.: Tumorigenesis by transfected cells in nude mice: A new method for detecting cellular transforming genes. In O'Connor, T. E. and Rauscher, Jr., F. J. (Eds.): Oncogenes and Retroviruses: Evaluation of Basic Findings and Clinical Potential. New York, Alan R. Liss, Inc., 1983, pp. 79-90.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05287-02 LMO

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA Replication, Integration and Gene Expression of MSV

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
T. Robins Staff Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have developed a simple and rapid system for molecularly cloning unitegrated retroviral DNA directly from eukaryotic cells. This "shuttle" vector was constructed by incorporating both plasmid vector DNA sequences from pBR322 and the origin of DNA replication from polyoma virus into the genome of HT-1 MSV. This system has allowed us to molecularly clone infectious, non-permuted, circular forms of unitegrated retroviral DNA. We believe this system will prove useful in elucidating the mechanisms by which retroviruses integrate into the host genome and thereby incorporate cellular proto-oncogenes into viral genomes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

G. F. Vande Woude Chief, LMO LMO, NCI

Objectives:

The immediate goal of this research is to devise a system by which we can better understand the replication and integration of oncogenic retroviruses. This system allows us to molecularly clone directly from eukaryotic cells both the unitegrated and integrated forms of retroviral DNA. This property provides a simple and rapid way to analyze the structure of replicative intermediates in the retrovirus life cycle.

The long term objective of this research is to use retrovirus integration as a model system to study DNA replication in normal eukaryotic cells.

Methods Employed:

We have used the standard recombinant DNA techniques to introduce new genetic elements into the molecularly cloned genomic DNA of HT-1 MSV. These chimeric plasmids can then be used to reconstitute a replicating transforming retrovirus by re-introducing them back into eukaryotic cells (NIH3T3) by standard DNA-mediated-gene-transfer techniques. The retroviruses derived from these cells is used to infect the appropriate cell type and the low-molecular weight DNA (unintegrated viral DNA) can be extracted from the "Hirt" supernatant. The plasmids isolated from these eukaryotic cells can be recovered in prokaryotic cells by DNA transformation of E. coli.

Major Findings:

We have constructed and tested the first prototype of a novel retrovirus "shuttle" vector which allows the direct molecular cloning of unintegrated circular retroviral DNA from eukaryotic cells. This was accomplished by constructing a chimeric plasmid which incorporated DNA sequences from both pBR322 and polyomavirus into the genome of HT-1 MSV. The DNA sequences from pBR322, containing the ampR and Col E1 origin of DNA replication allow the plasmid to be propagated and recovered in E. coli. The DNA sequences from polyomavirus are required for DNA replication in permissive mouse (COP-5) cells that constitutively express the polyoma large T-protein. The remainder of the plasmid is comprised of DNA sequences from the proviral DNA of HT-1 MSV which are necessary to generate a rescuable transforming retrovirus.

We have demonstrated that cotransfection of this plasmid containing a single long terminal repeat sequence (LTR) with molecularly cloned Moloney murine leukemia virus is able to rescue a replicating and transforming MSV. Furthermore, when this virus is used to infect COP-5 cells, the unintegrated circular viral DNAs are amplified. This unintegrated circular viral DNA can then be directly recloned by DNA transformation into E. coli. Analysis of these recovered plasmids indicates

that some molecules are essentially identical to the plasmid initially transfected and contain either one or two LTRs. These two different circular viral DNA intermediates are characteristic of retrovirus replication. Thus, this demonstrates that the sequences present in the original chimeric plasmid can be transcribed and rescued as infectious retrovirus and that the viral DNA can subsequently be recloned directly from the unintegrated circular viral DNA in COP-5 cells.

Analysis of one recovered plasmid indicates that the vector has transduced approximately 2.5 kb of mouse cell DNA. This plasmid appears to be the result of a recombinational event between a site in the mouse cell DNA upstream of the integrated vector DNA and a site at or near the 5' end of the 3'terminal LTR. Therefore, the newly acquired mouse cell DNA sequences have been incorporated into the retroviral genome. Morever, this demonstrates that it may be possible to use this vector to transduce segments of cellular DNA from eukaryotic cells.

Significance to Biomedical Research and the Program of the Institute:

Although oncogenic retroviruses probably do not play a direct role in the majority of human cancers, they have provided us with enormous insight into the possible mechanisms underlying this disease.

Retrovirus research has uncovered the existence of proto-oncogenes present in normal cells which clearly have the potential to transform cells. Morover, there now appears to be a variety of ways by which the oncogenic potential of these genes can be activated. We believe that the research program described here and the retrovirus "shuttle" vector which we have constructed will give us new insights into the interactions of these cellular proto-oncogenes with retroviruses.

Proposed Course:

Investigation of the use of this vector for three major areas of research will continue.

I. cDNA Cloning Vehicle:

Retroviruses, by virtue of the fact that their life cycle contains an RNA genome, have been demonstrated to be able to remove introns from structural genes inserted into their proviral DNA by recombinant DNA techniques. Therefore, it should be possible to insert any structural gene into this vector and subsequently expect to recover the corresponding cDNA of this gene by directly recloning the circular proviral DNA from COP-5 cells.

II. Transient Expression Vector:

One extraordinary feature of this vector is its ability to amplify up to 10-100 fold the number of unintegrated circular proviral DNA copies in infected COP-5 cells. If this circular proviral DNA can be transcribed then it should be able to transiently express at high levels the proteins which are coded for by this virus. This may be very important for proteins such as the mos gene product of

MSV which is apparently toxic to cells and produced in very small quantities even in MSV transformed cells.

III. Integration Intermediate:

Retroviruses replicate through a DNA intermediate which integrates into the host chromosome. It is not known which of the unintegrated viral DNA forms, either the linear form or one of the two circular viral DNA forms containing either one or two LTRs, is directly involved in the integration process. The unique character of the vector described here has allowed us to molecularly clone an infectious nonpermuted circular viral DNA of MSV. We can now begin to ask questions regarding the function of these molecules in infected cells and their possible role in the integration process.

Publications:

Maguire, R. T., Robins, T. S., Thorgeirsson, S. S. and Heilman, C. A.: Expression of cellular myc and mos genes in undifferentiated B cell lymphomas of Burkitt and non-Burkitt types. Proc. Natl. Acad. Sci. USA 80: 1947-1950, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05288-02 LMO

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cell Interaction, cAMP and Control of Developmental Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
D. Blumberg Senior Staff Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A very simple model system, the cellular slime mold Dictyostelium discoideum, is being used to study mechanisms which control developmental gene activation during normal differentiation. Post-aggregation Dictyostelium cells transcribe an additional 26% of their genome which is not expressed in earlier pre-aggregation stage cells. Our previous studies have indicated that cell-cell interaction is a necessary prerequisite for the synthesis and stability of the late messenger RNAs. Following activation the actual rate of transcription and the subsequent stability of many of the messenger RNAs transcribed off of this portion of the genome are further regulated by a cyclic AMP-mediated process. We are currently utilizing both cDNA and genomic clones of individual members of this group of late genes to explore basic aspects of their sequence and structural organization in chromatin with the long-term objective of understanding the nucleoprotein interactions which are important in the activation of their expression and subsequent regulation by cyclic AMP.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

None.

Objectives:

The aim of these studies is to understand mechanisms which control developmental gene activation during normal differentiation. A very simple model system, the cellular slime mold Dictyostelium discoideum, is being employed.

The predominant feature of the developmental cycle of Dictyostelium discoideum is the aggregation of unicellular free-living amoeba into a multicellular organism. Differentiation of amoeba within the newly formed aggregates generates the three distinct cell types found in the mature fruiting body: spore cells, stalk cells and basel disks. Dictyostelium exhibits many features of development seen in more complex eukaryotic organisms. Specific cell-cell contacts are found, a homogeneous population differentiates into discrete cell types, and there is specific cell migration and pattern formation. These morphogenetic changes are accompanied by major changes in the pattern of gene expression. In particular, post-aggregation Dictyostelium cells contain 2,000-3,000 new messenger RNA species that are absent from earlier preaggregation stage cells. These new aggregation dependent sequences compose 30% of the mass of the messenger RNA in the late cells and, together with their heteronuclear RNA precursors, represent the transcription products of an additional 26% of the single copy portion of the genome.

The initiation of transcription on this portion of the genome is dependent upon cell-cell interaction. Additionally, both the rate of transcription and subsequent stability of the mRNAs transcribed off of this portion of the genome are then further regulated by a cAMP-mediated process.

The major objective of our work is to elucidate the molecular mechanisms by which these genes are coordinately activated and to understand the controls which regulate the transcription rate and stability of these mRNAs in response to environmental stimuli--in this case cell contact and cAMP. In order to achieve this objective, several closely integrated approaches are being employed. First, we are exploring basic aspects of the structural organization of these genes in chromatin with the long-term objective of understanding the nucleoprotein interactions which are important in activation of gene expression.

Secondly, we are utilizing cDNA and genomic clones of individual members of this late group of genes in order to map aspects of the structural organization (i.e., sequences not in nucleosomes, attachment points to the nuclear matrix) onto restriction endonuclease and DNA sequence maps of the genes.

Finally, complementary studies to be initiated by Dr. Masakazu Oyama, a Fogarty Fellow, who is expected to arrive in July 1983, will focus on the development of a transformation system which will allow us to reintroduce mutated forms of these genes back into cells in order to define genetically those sites and

structures essential for both the initial activation of the genes in response to cell contact as well as for their cAMP-mediated regulation.

Methods Employed:

Standard molecular cloning techniques are being employed to isolate and map recombinant DNAs encoding constitutively expressed and developmentally regulated genes. Two endonucleases, DNase I and micrococcal nuclease, are being used to probe the structural organization of genes in chromatin while a variety of agarose and polyacrylamide gel systems are being utilized in one and two dimensions in order to resolve nucleoprotein complexes, histones or DNA and RNA species.

Major Findings:

1. Analysis of Basic Structural Aspects of Dictyostelium Chromatin From Growing Cells. Dictyostelium's chromatin offers a unique opportunity to study the structure of actively transcribed genes or genes which will become active in response to a developmental stimuli. Unlike the chromatin from higher cells, in which less than 20% of the DNA is ever transcribed, nearly 80% of the Dictyostelium genome is transcribed. Fifty percent is transcribed during growth and an additional 30% is active during late development. Thus, analysis of bulk Dictyostelium chromatin is essentially an analysis of bulk active chromatin. Additionally, because of the low complexity of the genome, one can probe the structural organization of single-copy genes in nucleoprotein complexes. In spite of these attractive advantages, very little is known of the basic structural organization of Dictyostelium's chromatin. Thus, our experiments have had to begin at the very elementary level of working out methods and procedures for preparing nuclei from Dictyostelium cells and making intact chromatin.

A. Dictyostelium's DNA is Found in Nucleosomes. As in other eukaryotes, the bulk of Dictyostelium's DNA is found in nucleosomes. Digestion of nuclei with micrococcal nuclease, followed by purification of the DNA and analysis on agarose gels, reveals a repeated ladder of bands with a spacing of approximately 165 nucleotides. Unlike the nucleosome repeat ladder observed in higher eukaryotes, the Dictyostelium pattern is somewhat more diffuse. Higher level resolution of the repeat ladder on acrylamide or formamide high percentage agarose gels reveals discontinuities and irregularities in the repeat pattern that are not observed in the chromatin of other cells. The repeat pattern has been analyzed for individual genes which fall into three transcriptional classes: (1) genes which are transcribed throughout growth and development, (2) genes which are not transcribed in the growing cells but are expressed only after the cells have formed the tight cell to cell contacts, and are ultimately expressed only in prespore cells, and (3) genes which are expressed at a very low level in growing cells and induced to a higher level of transcription at the time when cell contact is formed, and are ultimately expressed only in prestalk cells. The genes which are transcribed actively in the growing cells from which the chromatin was prepared do not show the distinct nucleosome repeat ladder. Instead, they are present as a smear over the entire molecular weight range of the micrococcal nuclease ladder. By contrast, the two classes of genes, those not transcribed and those transcribed at a very low rate in these cells, give a very clear, crisp nucleosomal repeat pattern. Thus, those

genes that are being actively transcribed display a higher level of heterogeneity in the size DNA fragment that is protected in nucleosomes, while those genes which are not transcribed or are transcribed at a very low rate in these cells, show a very uniform positioning of the nucleosomes over the DNA allowing micrococcal nuclease to cut at discrete points which gives rise to precise bands. We are currently comparing the repeat pattern of the prespore and prestalk genes in order to determine whether at this level of packaging there are any differences between the organization of genes which are to be expressed in one cell type vs. those to be expressed in the other major cell type.

B. Identification of Two Major Mononucleosome Species in Dictyostelium Chromatin. Mononucleosome core particles liberated from Dictyostelium nuclei by digestion with micrococcal nuclease have been analyzed on low ionic strength polyacrylamide gels. Two major mononucleosome species are detected. The more rapidly migrating mononucleosome species (MN-A) is retarded in its mobility relative to the mononucleosome core particles from HeLa or drosophila cells (MN-1). In fact the MN-A species migrates at a position similar to that of the mononucleosome species associated with HMG proteins in HeLa cells. The more slowly migrating mononucleosome species from Dictyostelium (MN-B), co-migrates with the mononucleosome species of HeLa or drosophila that contains core particle plus the linker region and histone H-1 (MN-2). Analysis of the DNA associated with the mononucleosome species by second dimension polyacrylamide gel electrophoresis reveals that the MN-A mononucleosome is associated with DNA in the size range of 145 to 170 base pairs, while the MN-B species contains DNA of 178 to 198 base pairs. These DNA sizes are equivalent to the sizes of DNA found associated with the MN-1 and MN-2 mononucleosome species found in other eukaryotes. Thus, the slower migration of the Dictyostelium MN-A particle relative to the mononucleosome core particles found in HeLa cells is most likely due to the protein composition of the particle. Analysis of the proteins associated with the two mononucleosome species from Dictyostelium utilizing second dimension polyacrylamide gels indicates that both histones H-2A and H-2B are considerably larger than their equivalent counterparts in higher cells. Preliminary results indicate, however, that the MN-A and MN-B species in Dictyostelium may not be strictly equivalent to the MN-1 and MN-2 species in higher cells. The mononucleosome core particle plus linker region, MN-2, is a precursor of the mononucleosome core particle MN-1. This does not appear to be the case in Dictyostelium; MN-B is not converted to MN-A, even under prolonged digestion conditions. Additionally, in preliminary experiments we have not been able to detect histone H-1 associated with the more slowly migrating MN-B species in Dictyostelium, although we can detect significant amounts of histone H-1 associated with nucleosome species which we had previously assumed to be dinucleosomes. Using improved conditions for isolating chromatin from Dictyostelium and higher resolution gel systems, we are extending our analysis of the mononucleosome particles and attempting to identify a particle which may be a candidate for containing both the mononucleosome core as well as the linker region. Experiments are also underway to improve the release of proteins from the mononucleosome species and to facilitate their detection on the second dimension gels so that we can better analyze the mononucleosome particles for association with non-histone chromosomal proteins and for minor modifications of the various histone species.

C. Dictyostelium Histones Differ Substantially from Histones Found in Higher Organisms. Analysis of Dictyostelium's histones utilizing both SDS

polyacrylamide gels and acid urea gels reveal significant differences between the histones from Dictyosteluim and those from other systems. As indicated above, both histones H-2A and H-2B are significantly larger than the histones found in mammalian or insect cells. Additionally, histone H-3 would appear to be comprised of two different molecular weight species. Like its counterpart in higher cells, histone H-4 can exist in a variety of different acetylated forms. Further analysis that the Dictyostelium histones using triton acid urea and CTAB acid urea gels is underway to resolve minor charge variants of the histone species. We are also seeking to confirm our identity of the various histones using immunological approaches. In particular, the histones are being electroblotted to nitrocellulose paper and reacted with antibodies to the better characterized histones from other cell types as well as to antibodies raised against certain histone modifications such as antibodies to ubiquitin and ubiquitin conjugates.

2. Relationship of Basic Aspects of the Structural Organization of Dictyostelium's Chromatin to the Functional Organization of its Genes.

A. Hybridization Mapping of the Major Mononucleosome Species: DNA associated with the two major mononucleosome species, MN-A and MN-B, is being analyzed for its ability to hybridize to DNA encoding messenger RNA species that are transcribed actively in the growing cells or only transcribed during the late portion of development and preferentially expressed in either prespore or prestalk cells. The mononucleosome particles are resolved on a low ionic strength polyacrylamide gel system. The DNA is released and electrophoresed in a second dimension gel, electrotransferred to activated papers, and hybridized to gene-specific probes. Very preliminary results may indicate a preferential hybridization of some actively transcribed sequences to the more slowly migrating MN-B mononucleosome.

B. Sensitivity of Structural Gene Regions to DNase I and Existence of Adjacent Hypersensitive Sites. Genes which are being or have been transcribed in a particular cell are found in a structural organization in chromatin which renders them more sensitive to digestion by DNase I. Since the late genes which specify the differentiation functions in Dictyostelium are completely inactive in growing cells and require a very specific aspect of cellular interaction to be activated, we are interested to know whether these genes existed in a DNase I sensitive or insensitive configuration in chromatin. This question takes on added interest since this differentiation pathway in Dictyostelium is a reversible pathway. Disruption of cell interactions very rapidly and specifically leads to a shutoff of transcription of the late genes, as well as to rapid degradation of the late messenger RNA species. So far, there is no known instance of a gene which, upon ceasing to be transcribed, loses its DNase I sensitive configuration. However, in most systems where these questions have been addressed the cells have been undergoing terminal differentiation. Thus, it was of interest to see how genes in a pathway of reversible differentiation are packaged with respect to the DNase I sensitive configuration. Nuclei from growing Dictyostelium cells were digested with increasing concentrations of DNase I. The DNA was extracted, deproteinized, redigested with a restriction endonuclease which generated fragments known to map to the structural region of genes either expressed actively in the growing cells or only expresssed during late development and therefore inactive in the growing cells. The rate at which these fragments disappeared as a

function of DNase I digestion was measured. Under conditions of very low ionic strength where the higher order chromatin structure is lost and the DNA exists as a 10 nanometer fiber--the beads on a string configuration, there is no difference in sensitivity to DNase I between constitutively expressed, actively transcribed genes and the inactive late genes in the chromatin from the growing cells. These results indicate that at the level of the individual mononucleosome, one cannot detect a difference in DNase I sensitivity between active and inactive structural gene regions. We are currently repeating these experiments under conditions where the higher order structure of the chromatin, the 300 Angstrom fiber, is not disrupted and looking to see whether there is a difference in the higher order folding of the chromatin that would lead to a differential sensitivity of the different classes of genes.

We are also comparing the sensitivity of these protein encoding genes to DNase I with the sensitivity of genes encoding the ribosomal RNAs that are known to be transcribed at a significantly higher rate.

In contrast to the relatively uniform sensitivity of the protein coding regions of active and inactive genes, a region immediately adjacent, presumably to the 3' end of one of the inactive late genes, shows a substantially increased rate of digestion with DNase I. Efforts are underway to map and sequence this hypersensitive region. Previous work from other labs looking at Dictyostelium actin and discoidin genes has revealed that both 3' and 5' noncoding regions contain long stretches, several hundred base pairs in length, of dA,dT either alternating or in long homopolymer stretches. A question which arises is whether the hypersensitivity that we observe on the 3' side of the structural gene region results from a failure to package these highly AT-rich regions in nucleosomes.

Significance to Biomedical Research and the Program of the Institute:

Abnormalities in differentiation and developmental gene expression are characteristic of the malignant cell. Because of the unique features of its developmental program, Dictyostelium provides a powerful and simple system for exploring mechanisms which control eukaryotic developmental gene expression.

Proposed Course:

Our present research program is directed toward two major goals: 1) defining at a molecular level the mechanism by which a quarter of the Dictyostelium discoideum genome is brought from a transcriptionally inactive state to an active one; and 2) Defining the physical basis for the cell contact/cAMP-mediated instability of the late aggregation dependent mRNAs.

It is hoped that by understanding how these basic changes in gene expression occur that the role of cell-cell interaction and cAMP in eliciting these responses can ultimately be understood.

Publications:

Blumberg, D. D., Chung, S., Landfear, S. M. and Lodish, H. F.: Cell-Cell contact, cAMP and gene expression during differentiation of the cellular slime mold dictyostelium discoideum. In Weber, R. and Burger, M. M. (Eds.):

Embryonic Development: Part B, Cellular Aspects. New York, Alan R. Liss, 1982, pp. 167-182.

Lodish, H. F., Blumberg, D. D., Chisolm, R., Chung, E., Coloma, A., Landfear, S., Barklis, E., Lefebvre, P., Zuker, C. and Mangiarotti, G.: Control of gene expression. In Loomis, Jr., W. F. (Ed.): Dictyostelium discoideum: A Developmental System, Ed. 2. New York, Academic Press, 1982, pp. 325-352.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05294-02 LMO

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Isolation of a Human Transforming Gene from a Teratocarcinoma

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
M. Tainsky Staff Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human teratocarcinoma cell line PA-1 was found to contain a dominant trans forming gene when analyzed in the NIH3T3 tissue culture DNA transformation assay. Isolation of the transforming DNA sequences by molecular cloning is in progress. Two of the four RI fragments present in a secondary focus NIH3T3 cell have been cloned. These will be used to isolate the complete transforming region from a genomic library prepared from this cell line.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

C. Cooper	Visiting Fellow	LMO, NCI
G. F. Vande Woude	Chief, LMO	LMO, NCI

Objectives:

One approach to the study of the molecular mechanism involved in neoplastic transformation is to isolate by molecular cloning dominant transforming genes, DNA sequences which are capable of morphological transformation of mouse fibroblasts, NIH3T3 cells, in culture. Using DNA isolated from a human teratocarcinoma cell, we have been able to transform NIH3T3 cells in a DNA mediated transfection assay. DNA from these primary transformants was also capable of DNA mediated transformation of NIH3T3 cells. An appropriate secondary transformation was chosen for isolation, by molecular cloning, of the transforming gene sequences.

Methods Employed:

DNA extracted from a human teratocarcinoma cell line, PA-1, and used in a standard NIH3T3 DNA-mediated transformation assay was found to cause transformed cell focus formation. DNA samples extracted from these primary foci, as well as subsequent secondary foci, were found to be active in the NIH 3T3 assay and to have retained human DNA sequences as judged by hybridization to a human ubiquitous repeat DNA sequence, Blur 8, on Southern analysis. Initially, two of these restriction endonuclease RI fragments from a secondary focus were cloned into the bacteriophage vector λgt WES λB. These represented part of the total human DNA in this mouse cell focus. These sequences were used as probes to clone additional human DNA sequences from a representative genomic library of this transformed mouse cell prepared in the bacteriophage vector λ EMBL 3.

Major Findings:

A cell line was derived from acitic fluid from a patient with malignant ovarian teratoma. DNA from this human teratocarcinoma cell line, PA-1, was found to be active in the DNA mediated transformation assay using NIH3T3 cells, a mouse fibroblast cell line. A cell line, 26-7a, derived from a second round of transformation was found to contain human DNA sequences and DNA extracted from it was active in the transformation assay. Southern analysis of DNA from 26-7a contain four human RI DNA fragments which hybridize to a human highly repeated DNA sequence probe, Blur 8. Two fragments were cloned into the bacteriophage vector λgt WES λBas follows. 26-7a cell DNA was cleaved with RI and size fractionated through a sucrose gradient. Southern blot analysis was employed to localize fractions containing human sequences already known from analytical experiments. Two fractions were chosen for further use. DNA was ligated into λgt WES λB RI arms and in vitro packaged into phage particles. These phage were screened for the presence of human cross sequence by hybridization to phage plaque lifts. Two of the fragments were cloned by this approach. One was 2.6 kilobase pairs (Kbp) and the second was 3.6 Kbp. These 2 cloned fragments were subcloned into

tne plasmid vector pBR322 and labeled by nick translation. No significant homology other than to the human repeated sequences (Blur 8-like) was observed between the 2.6 Kbp and 3.6 Kbp fragments. A representative genomic library was prepared from DNA from 26-7a by partial Sau 3A-I digestion, followed size fractionation through sucrose gradients. DNA in the size range of 16-23 Kbp was ligated to BamHI arms of the bacteriophage vector λ EMBL-3. This library was screened by plaque hybridization using labeled 2.6 and 3.6 Kbp DNA fragments as the probe. Twenty-one phage clones were isolated which were related to these probes. Restriction endonuclease analysis and DNA cross homology analysis with other known oncogenes is in progress. In addition, these clones are being tested for transforming activity in the DNA mediated transformation assay using NIH3T3 cells.

Significance to Biomedical Research and the Program of the Institute:

Transforming genes such as the one which will be isolated in these experiments have been the object of much recent study. A number of such genes have been isolated from human tumors and some have been found to be related to the ras family of well studied rat oncogenes. It will be of great interest to determine if the transforming gene of PA-1 is ras related and therefore implying a similarity in the molecular mechanism involved in carcinogenesis in diverse tumor types such as bladder, lung and colon carcinomas, fibrosarcomas, and ovarian teratocarcinomas. The nature of the PA-1 transforming protein will add basic information concerning the mechanism of carcinogenesis.

Proposed Course:

Further cloning experiments are required for the isolation of the remaining human DNA sequences associated with the transforming gene from this human teratocarcinoma, PA-1. Using the new genomic library which has recently been constructed, cloned sequences will be tested for transforming activity. Once isolated the structural gene and control elements will be located and compared to those in a normal human genome. This structural gene will be compared to other oncogenes by DNA sequence analysis. From this sequence synthetic peptides can be prepared for the purpose of preparing antisera to the oncogene product. This antisera can be used to determine the role of this oncogene in human neoplasia.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05295-02 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Activation of onc Genes in Viruses and Human Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Donald G. Blair Expert, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The provirus of the Moloney murine sarcoma virus (MSV) contains an MSV-specific transforming sequence (v-mos) whose transforming efficiency can be enhanced by MSV long terminal repeat (LTR) sequences linked either 5' or 3'. Enhancement of v-mos transformation by LTR sequences linked 3' requires only those U3 sequences containing the 73 bp repeat. Promoter sequences and the polyadenylation site derived from unique 5' and 3' LTR sequences are not required. Although v-mos transformation is efficiently enhanced by a 3' LTR, c-mos linked to a 3' LTR transforms poorly. A sequence approximately 1.2 kb 5' to c-mos has been identified which prevents the enhancement of c-mos transformation by a 3' LTR. This sequence apparently blocks the enhancer function of the 73 bp repeat sequence. The human cellular homologue (hu-mos) of mos does not transform NIH3T3 mouse cells even when linked to LTR sequences. Certain recombinants between hu-mos and v-mos containing sequences derived from both parents are able to transform, indicating that under appropriate conditions hu-mos can act as a transforming gene. A dominant gene has been identified from the human pancreatic carcinoma cell line PANC-1. This gene appears to be a member of the family of human sequences related to the ras oncogene band on Kirsten murine sarcoma virus.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Thomas G. Wood	Senior Staff Fellow	LMO, NCI
Marianne K. Oskarsson	Chemist	LMO, NCI
Colin S. Cooper	Visiting Fellow	LMO, NCI
Michael Tainsky	Senior Staff Fellow	LMO, NCI
Bryan O'Hara	Visiting Fellow	LMO, NCI
George F. Vande Woude	Chief	LMO, NCI

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses and the function of specific gene products of MSV in this process.

To define the functions of specific portions of the Moloney murine sarcoma virus (MSV) genome in MSV transformation and to identify specific genetic sequences necessary to activate the transformation potential of normal cell sequences of mouse and human origin.

To develop screening and selection systems to identify and isolate human transforming DNA sequences from primary human tumor and tumor cell line DNAs. To identify, isolate and characterize such sequences and their gene products. To characterize the normal cellular homologues of such sequences and to determine the mechanism by which their oncogenic potential is activated.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells, DNA and RNA isolation from tumor tissues and tissue culture, Southern and Northern blotting using radioactive probes prepared from cloned DNAs, calcium-phosphate-DNA transfection, measurement of tumorigenicity of cell lines in nude and other strains of mice, immunoprecipitation and protein gel analysis to detect antibodies to specific cellular proteins.

Major Findings:

1) The entire MSV proviral LTR is not required to enhance transformation when linked 3' to v-mos. Previous studies had shown that a proviral LTR linked 3' to v-mos was capable of efficiently enhancing the transforming potential of v-mos approximately 300-fold over that observed for v-mos alone. To determine the regions of the LTR required for this enhancement, a series of recombinants were constructed containing various portions of the LTR linked 3' to v-mos. Clones containing unique 5' sequences, including the cap site, and the site of poly A addition in the 3' proviral LTR were essentially inactive in DNA transfection assays. However, a construct containing a single copy of the 73 bp repeat sequence located in the unique 3' portion of the proviral LTR transformed efficiently. These results demonstrate an LTR located 3' to v-mos functions as an enhancer to activate v-mos transformation and that polyadenylation signals and U5 derived promoter signals do not play a significant role.

2) A sequence in normal mouse DNA located ~1.2 kb 5' to the end of the c-mos region inhibits the ability of an LTR from MSV to enhance transformation. Previous results had shown that whereas linkage of an MSV proviral LTR 5' to c-mos efficiently activates its transforming potential, a clone designated LS1, in which the LTR is linked 3' to normal mouse sequences containing c-mos, transforms with a dramatically reduced efficiency. Analysis of the mos-containing polyadenylated RNA present in LS1 transformed cells, as well as the structure of integrated transfected c-mos sequences present in these cells, indicated that sequences 5' to c-mos had been lost in these transfected cells. These observations suggested that activation might require the deletion of normal mouse sequences 5' to c-mos which could be playing an inhibitory role. To test this hypothesis a series of clones containing different amounts of normal mouse sequences 5' to c-mos and an LTR linked 3' to c-mos were constructed and analyzed for their ability to transfect and transform mouse cells. The results indicate that ~1.2 kb from the 5' end of c-mos and immediately adjacent to a Sac I site are responsible for the low transforming activity of LS1. This suggests that sequences exist which can act to negate the effect of enhancer sequences which have been identified in a wide variety of genes and viruses.

3) Recombinants between v-mos and the human mos homologue (hu-mos) are biologically active. Previous results had shown that the human homologue of mos, the MSV-transforming sequence, was unable to transform mouse cells in DNA transfections even when linked to LTR sequences both 5' and 3' of the hu-mos homologue. In a further attempt to determine if hu-mos possessed a transforming potential, a series of recombinants were selected in E. coli which contained both v-mos (mouse) and hu-mos sequences. These clones had recombined at unique sites within regions of homology between the hu-mos and v-mos sequences. Our results indicate that several of these recombinants are able to transfect and transform mouse cells. This indicates that the human-mouse homologue does possess transforming potential, but that some block exists to its effective expression in mouse cells which can be overcome by the replacement of hu-mos sequences with v-mos sequences.

4) The biologically active transforming gene present in the human pancreatic carcinoma cell line PANC-1 is a member of the human ras^K family. The human pancreatic carcinoma cell line PANC-1 contains a dominant transforming gene which was initially identified by the ability of cells transfected by this tumor DNA to induce tumors in nude mice. These cells contained large amounts of human DNA as detected by hybridization to the human Alu family repeat clone, Blur 8, in Southern blot analysis of DNA from tumors and tumor-derived cells. Secondary and tertiary transformants could be derived from this tumor DNA which contained reduced amounts of human DNA as measured by Alu hybridization. Analysis with several cloned viral oncogenes showed that all transformants contained sequences homologous to the Kirsten ras gene but not to Ha-ras or to mos. The Alu hybridization pattern observed was also consistent with this active human gene being a member of ras^K family. Cloning of Alu containing fragments from a partial Mbo1λ library of a secondary PANC-1 transfectant has resulted in the isolation of approximately 75% of the ras^K gene present in these cells.

Significance to Biomedical Research and the Program of the Institute:

The process by which specific promoter and activator sequences "turn on" inactive sequences represents one potentially relevant mechanism of human carcinogenesis. Our increased understanding of how this process occurs, what specific types of sequences are required, and how these sequences act to overcome factors and sequences blocking gene expression should enable us to be more able to affect and manipulate these events both in vivo and in vitro. It should allow us to reproduce and study the process of spontaneous or chemically induced activation in vitro and to identify normal genetic sequences which can be activated to express a potential oncogenic phenotype. We should be able to isolate sequences which function as activators and promoters of oncogenic and other cellular sequences. The isolation of both human oncogenic and human promoter/activator sequences will allow us to develop systems to study how such elements function and interact in a human genetic and cellular background.

Development of techniques of DNA transfection using human tumor DNA has for the first time allowed the identification of specific human DNA sequences which may be involved in the initiation and maintenance of the transformed phenotype in human cancers. Screening techniques based on morphological transformation of a few cell lines in tissue culture, however, may severely limit the spectrum of transforming sequences which can be detected. Tumor formation in nude mice represents a definitive, biologically significant transformation marker. It provides a nonsubjective, highly selective screening technique which can be used to test large numbers of samples easily. Since in some cases morphological transformation and tumorigenesis represent separable manifestations of the transformed state, it offers the possibility of detection of new classes of transforming sequences not previously recognized. Once detected, such sequences can be identified, cloned and analyzed by conventional techniques.

Proposed Course:

Additional constructs containing portions of the proviral LTR and v- and c-mos will be analyzed to further map and confirm the nature of the minimum enhancing sequence and to define its limits of action. Analysis of the factors affecting the inhibitory activity of the normal mouse sequences present 5' to c-mos will be continued. Attempts to identify its mechanism of action will be made by analyzing RNA expression in cells containing cloned genes linked to this sequence. Clones containing this sequence placed between two enhanced sequences will be analyzed to determine if both 5' and 3' enhancement is sensitive to its action.

Additional v-mos/hu-mos recombinants which have recombined at different sites will be analyzed for biological activity. The structures of the sequences present in cells transformed by these recombinants will be analyzed to determine if any further gene rearrangements have occurred. The nature of hu-mos protein expression will be analyzed utilizing antibodies prepared against the predicted hu-mos peptide sequence to determine if either the level or sites of protein expression differ between the recombinants and v-mos transformed cells. The ability of these recombinants to transform human cells will be tested.

The cloning of the ras^k gene from PANC-1 will continue. Once the entire gene has been cloned, attempts will be made to reassemble an active transforming gene and to attempt to determine what alterations have been made in the normal cell gene to produce an active transforming gene. Sequence analysis of selected regions will be utilized to further analyze changes present in the ras^k gene of PANC-1.

Transfection analysis of DNA isolated from fresh tumor material will be continued in attempts to isolate other human transforming sequences. A preselection of transfected cells using a dominant drug resistance marker cotransfected with human tumor DNA will be utilized to increase the sensitivity both of the focus induction and the tumor induction assay in NIH3T3 cells. Mouse tumors induced in vivo by various carcinogens will be analyzed utilizing the tumor induction assay. The nature of active genes identified in these tumors will be analyzed to determine if mechanisms of action of various carcinogens can be correlated with the active genes recovered. Studies on the optimization of the mouse tumor assay system will continue to determine if the level of sensitivity and the number of genes are detectable by the assay.

Publications:

Blair, D. G., Cooper, C. S., Oskarsson, M. K., Eader, L. A. and Vande Woude, G. F.: New method for detecting cellular transforming genes. Science 218: 1122-1125, 1982.

Blair, D. G., Cooper, C. S., Oskarsson, M. K., Eader, L. A. and Vande Woude, G. F.: Tumorigenesis by transfected cells in nude mice: A new method for detecting cellular transforming genes. In O'Connor, T. E. and Rauscher, Jr., F. J. (Eds.): Oncogenes and Retroviruses: Evaluation of Basic Findings and Clinical Potential. New York, Alan R. Liss, Inc., 1983, pp. 79-90.

Blair, D. G., McClements, W. L. and Vande Woude, G. F.: Use of retroviral sequences in co-transfection to activate and rescue an onc gene. In Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, 1982, pp. 152-157.

Stanker, L. H., Horn, J. P., Gallick, G. E., Kloetzer, W. S., Murphy, Jr., E. C., Blair, D. G. and Arlinghaus, R. B.: Gag-mos polyproteins encoded by variants of the Moloney strain of mouse sarcoma virus. J. Virol. 126: 336-347, 1983.

Wood, T. G., McGeady, M. L., Blair, D. G. and Vande Woude, G. F.: Long terminal repeat enhancement of v-mos transforming activity: Identification of essential regions. J. Virol. 46: 726-736, 1983.

Woodworth, A., Oskarsson, M., Blair, D. and Vande Woude, G.: Biological properties of the human DNA sequence homologous to the mos transforming gene of Moloney sarcoma virus. In Weinstein, I. B. and Vogel, H. J. (Eds.): Genes and Proteins in Oncogenesis. New York, Academic Press, Inc., 1983, pp. 233-240.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05342-01 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Expression of mos Gene at Elevated Levels

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Roberta Black Staff Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Characterization of the Moloney murine sarcoma virus v-mos protein has been hindered by expression of extremely low levels in transformed cells. In order to analyze the v-mos gene product, a mammalian expression system is being developed in which v-mos protein will be produced at elevated levels. Specifically, plasmid vectors have been constructed in which v-mos has been placed under the control of the murine beta-globin major promoter such that v-mos protein will be synthesized either as a single protein or as a hybrid fusion protein with beta-globin. Plasmid DNA containing v-mos as described above and plasmid DNA containing the gene coding for the enzyme adenine phosphoribosyl transferase (aprt) have been simultaneously introduced into murine erythroleukemia cells lacking aprt (MEL aprt-) using Ca-PO4 medicated DNA coprecipitation. Upon induction of differentiation, it is predicted that the exogeneously added beta-globin promoter will be activated to express v-mos protein at elevated levels. This system will provide a method by which to explore the nature of v-mos protein both in vivo, in these MEL cells, and in vitro, after isolation and purification. In addition, such a powerful eukaryotic expression system would be generally applicable for use in studies of other gene products.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

G. F. Vande Woude	Chief, LMO	LMO, NCI
M. Tainsky	Staff Fellow	LMO, NCI

Objectives:

The objective of this work is to develop a eukaryotic expression system in which the product of the v-mos gene is expressed at high levels. A secondary aim of this project is to develop an expression system for the above purpose which is adaptable for use in studies of other gene products.

Methods Employed:

The v-mos gene from the plasmid pHT10 and the mouse β major globin gene from λ gt WES.MBG2 (Tilghman, S.M. et al (1977) PNAS 74: 4406) were used to construct, in pBR322, plasmids containing the v-mos gene under the control of the β-globin major promoter. In one arrangement, a 1.9 kb BglII-BamH1 fragment containing v-mos and 5' MuLV flanking region was inserted into a single BamH1 site within the second exon of the β-globin major gene. A second construction contains a 360 bp fragment representing the promoter region of β-globin and a 1.2 kb fragment representing the v-mos coding region. Each of the plasmid DNAs was contransfected into MEL aprt$^-$ cells with plasmid DNA containing the hamster aprt gene (pHaprt-2) using the Ca-PO_4 mediated DNA coprecipitation technique. MEL aprt$^+$ colonies were isolated using selective media containing azaserine and adenine. DNA was extracted from expanded cell colonies and used in DNA blotting experiments to identify aprt and v-mos containing clones. Elevated expression of v-mos is predicted upon induction of cells to differentiate with DMSO or sodium butyrate.

Major Findings:

Cell colonies with an aprt$^+$ phenotype have been obtained after transfection of MEL aprt$^-$ cells with pHaprt-2 and after contransfection with pHaprt-2 and one of the v-mos containing plasmids. Three to four days after addition of sodium butyrate to aprt$^+$ cells, hemoglobin production can be visualized in cell pellets, thus indicating that the cells have been successfully induced. Blot hybridization indicates the presence of pBR-containing sequences in the aprt$^+$ clones. DNA blotting experiments are in progress to identify those clones in which v-mos under the control of the β-globin promoter has been successfully cotransfected with the aprt gene.

Significance to Biomedical Research and the Program of the Institute:

Upon induction of erythroleukemia cells, globin represents 10-25% of the total cytoplasmic proteins expressed. In addition, the background of cellular proteins is low due to a general decrease in macromolecular synthesis. This eukaryotic expression system, therefore, has the potential to produce large quantities of a protein, the coding region of which is under the control of the β-globin promoter.

Specifically, this system will facilitate biochemical analyses of the v-mos gene product by providing significant quantities of protein with which to work.

Proposed Course:

Preliminary results will be confirmed and extended. The optimum conditions for activation of v-mos expression will be determined. Upon obtaining significant amounts of v-mos protein we will investigate its nature.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05343-01 LMO

PERIOD COVERED
April 1, 1983 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologs

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Christos Flordellis Visiting Fellow, LMO, NCI

COOPERATING UNITS *(if any)*
Biochemistry Department, Georgetown University, Washington, D.C.

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither .
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

MC-29 and MH2 are members of a group of viruses (avian defective leukemia viruses) carrying myc-related oncogenes.

Analysis of the genomic structure of viral genes and their cellular homologs and study of expression of myc-related sequences is of great importance to better understand the mechanisms responsible for leukemogenesis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Takis Papas	Head, Carcinogenesis Regulation Section	LMO, NCI
James Lautenberger	Expert	LMO, NCI
Dennis Watson	Staff Fellow	LMO, NCI
Kenneth Samuel	Visiting Fellow	LMO, NCI
Nancy Kan	Visiting Fellow	LMO, NCI

Objectives:

The purpose of this investigation is to determine the functional relationship between onc genes and their normal cellular homologs. Structural analysis along with study of RNA transcripts of these genes from both normal and transformed cells will allow us to better understand their biological functions.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells by treatment with proteinase K followed by phenol-chloroform extraction. Preparation of vector DNA from phage λ derivatives by phenol extraction of CsCl banded phage.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments of agarose gels.

3. Preparation of DNA probes using purified MH2-specific DNA, to be nick translated using E. coli DNA polymerase.

4. Preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of MH2 probes.

5. Construction of recombinant phage libraries by litigation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging.

6. Isolation of phage from the libraries containing virus-related sequences by hybridization of cDNA probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure.

7. Subcloning of isolated c-MH2 DNA fragments into pBR322.

8. DNA sequencing analysis of cloned DNA by the method of Maxam and Gilbert and by the dideoxyribonucleotide method of Sanger and associates. For the latter method, the cloned DNA is recloned into a filamentous phage.

9. RNA extraction from cultured cells using guanadine hydrochloride extraction. Isolation of RNA after cellular fractionation. Separation of polyA-RNA by several (2-3) cycles of purification through oligo(dT) cellulose. Analysis of RNA on formaldehyde-agarose gels by Northern analysis.

Major Findings:

This project was just initiated.

Significance to Biomedical Research and the Program of the Institute:

Transformation of cells by acute leukemia is of great importance in defining the gene responsible for leukemogenesis. Analysis of the genomic structure of viral genes and their cellular homologs is of immense importance to better understand the mechanism of the leukonic process.

Proposed Course:

1. The complete nucleotide sequence of MH2 provirus will be determined. We will compare this sequence with those of c-myc genes in chicken, mouse and man. This will allow us to identify intron-exon junctions and help define the complete normal c-myc genes in vertebrates.

2. Expression of myc-related sequences will be examined using Northern analysis of polyA$^+$ RNA. Total cellular, nuclear and cytoplasmic RNA from normal and transformed cells will be isolated. The RNA will be seperated to poly (A^+) and poly(A^-) species by several cycles over oligo(dT)-cellulose. The RNA molecules will be fractionated on formaldehyde-agarose gels, transferred to nitrocellulose filter and hybridized to probes representing sequences mapping intron and extron of myc-specific cellular DNA.

3. S1 nuclease mapping experiments will be performed in order to precisely determine the initiation of transciption in normal and cultured cells transformed with MC29 and MH2 sequences.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05344-01 LMO

PERIOD COVERED

January 1, 1983 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of Cellular DNA Sequences Required to Transform Human Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Bryan M. O'Hara Visiting Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.85	0.85	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The identification of cellular DNA sequences determining tumorigenicity has relied almost solely on the transfection assay for production of foci in NIH3T3 cells. This method raises serious questions regarding the importance of such isolated genes in human systems and it may also be greatly restricted in the type of transforming genes it can detect. We are developing a system utilizing a more appropriate human cell line as recipient in transfection and to use the transfected cells in both focus and tumor formation assays. The first objective was the identification of a human cell line which was an efficient recipient for DNA transfer and which was non-tumorigenic in the athymic nude mouse without being immunologically rejected. Certain hybrids of human fibroblasts and the normally tumorigenic HeLa cell line fulfill these criteria, particularly as they retain a cryptic tumorigenic potential and are theoretically capable of being rendered tumorigenic by one or a small number of further changes. To obtain a positive control, these lines are being tested for focus and tumor formation following transfection with cloned viral onc genes. The parents of the hybrids, the hybrids and their rare tumorigenic derivatives are being examined for mRNA changes using viral onc gene probes to identify the mechanism of suppression of tumorigenicity in the hybrids.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Donald G. Blair Expert LMO, NCI

Objectives:

To develop an assay using human cells which will allow detection of focus and tumor formation following transfection of the human cells with viral onc genes.

To use the assay to test the activity of cellular transforming genes previously isolated using NIH3T3 cell-assays.

To use the assay to screen human tumor cell DNAs for cellular transforming genes.

To determine if identical transforming genes are detectable in cells of different species.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells, DNA and RNA isolation from tumor tissues and tissue culture, Southern and Northern blotting using radioactive probes prepared from cloned DNA, calcium-phosphate-DNA transfection, measurement of tumorigenicity of cell lines in nude mice, immunoprecipitation and protein gel analysis to detect specific cellular proteins.

Drug selection of transfected cells utilizing media supplemented with G418 (Schering) for neomycin resistance and with mycophenolic acid and xanthine for the presence of the gpt gene. Parents of the human hybrid cell lines, the hybrids and their derivative lines were obtained from Harold Klinger, Albert Einstein College of Medicine.

Major Findings:

HeLa/Normal Human Fibroblast Hybrids are Efficient Recipients in DNA Transfection. We have screened selected non-tumorigenic cell lines for their ability to act as recipients in DNA transfer. Of those tested, we have identified two HeLa/fibroblast hybrids and SV80 (an SV40-transformed human fibroblast cell line) which can be transfected efficiently. Using two bacterial genes selectable in eukaryotic cells as dominant workers (gpt and neo), it was found that between .01 and .1% of the cells could be transformed. Thus, these cell lines are sufficiently competent in DNA transfection assays to detect transfer of genes present as single copies in eukaryotic cellular DNA. We have initiated attempts to transform these transfectable lines while we are continuing to screen other potential recipients. Cells have been transfected with mixtures of gpt and different cloned viral onc genes. Cells which have taken up the gpt marker and which should also have taken up viral onc genes were isolated through selection for gpt. These were plated for focus formation and were also injected into mice.

Significance to Biomedical Research and the Program of the Institute:

A number of cellular genes have been isolated which cause NIH3T3 cells to form foci in monolayer culture and to form tumors in nude mice. The NIH3T3 cells are suitable for this purpose presumably because they have proceeded to a penultimate step in the process of transformation and may need only one additional change to render them transformed. Also, they are efficient recipients in DNA transfection, a process by which new genes can be introduced into large numbers of cells. However, there are two major drawbacks to the use of this recipient line. Being of murine origin, it is questionable what role genes which transform them play in human systems. Also, they appear to be relatively more sensitive to some classes of cellular transforming genes, i.e., members of the ras family. There are many primary human tumors and tumor cell lines which do not transform them and these may possess transforming genes inactive in NIH3T3 cells. For these reasons we are seeking to develop focus and tumor formation assays using human cell lines. These lines must be efficiently transfectable, which human lines generally are not and they must be non-tumorigenic but with some indication that they have progressed to a condition which is close to being transformed. Using such lines it may be possible to detect new transforming genes which are not effective on NIH3T3 cells. They can also be used to confirm the functionality of previously isolated genes in human cells. Any genes which are detected in this way can be cloned using established procedures.

Proposed Course:

Initially, human cell hybrids and SV80 cells will be transfected with mixtures of the gpt gene and a bank of viral onc genes. Following selection for the gpt gene, the human cells will be assayed for focus formation and for tumorigenicity in athymic nude mice. Considerable progress has been made towards these ends. If foci or tumors result with any of the viral onc genes or mixtures of these, this will form the basis for studies using primary human tumor DNAs. Human tumor line DNAs which are active on NIH3T3 cells, such as the cloned human cellular EJ transforming gene, will also be tested initially. We will confirm that the viral onc genes are present in the transfected cells and that they are transcribed and translated. If the sequences are present and expressed, but no cell transformation is observed, then further analysis will be undertaken to determine the reasons for their failure to transform human cells.

The non-tumorigenic HeLa/fibroblast hybrids provide an opportunity to investigate the role in transformation of cellular onc genes of which viral onc gene analogues are available. We have the parents of the hybrids (the tumorigenic HeLa cell line and a normal human fibroblast) and tumorigenic derivatives of the hybrids. This group of related cells will be examined for differences in mRNA populations using viral onc gene probes. It may be possible to relate specific changes in mRNA expression to certain phenotypes, thus implicating particular cellular onc genes in the process of transformation.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05345-01 LMO

PERIOD COVERED: October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Repetitive DNA Sequences Flanking the Mouse c-mos Locus

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Friedrich Propst Visiting Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Laboratory of Molecular Oncology

SECTION: Tumor Biochemistry Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In the course of looking for its transcriptional activity, the DNA region of the mouse genome containing the c-mos proto oncogene, which is homologous to the transforming gene of Moloney murine sarcoma virus (v-mos), has been characterized with respect to location and identification of repetitive DNA sequences. The c-mos gene itself appears to be a single copy gene and no other related genes have been detected by Southern blot hybridization techniques. A cluster of three members of the B1 family, a mouse repititive DNA family, was identified by Southern blot hybridization with labeled total mouse genomic DNA as a probe and by DNA sequencing. These 3 members of the B1 family are located 0.6 kb, 2.8 kb, and 5.5 kb downstream from the putative 3' end of the c-mos gene. The precise location and identification of another copy of a non-B1 repetitive DNA family located between the last two B1 repeats is currently under study. This non-B1 repetitive DNA family apparently comprises 5000-10,000 copies per diploid genome. No repetitive DNA sequences were as yet detected within 3 kb of upstream flanking sequences of the c-mos gene by using labeled total mouse genomic DNA as a probe. A DNA clone containing the c-mos gene and about 8 kb of 5' flanking region has been isolated from a genomic library. This clone will be characterized in a similar manner and by heteroduplex analysis with the analogous region of the human genes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

George F. Vande Woude Chief LMO, NCI

Objectives:

Characterization of the mouse c-mos locus with respect to homologous RNA transcripts; localization and identification of repetitive DNA sequences. Comparison of the mouse and the human c-mos locus by heteroduplex analysis and/or hybridization experiments to detect possible cross homology upstream from the c-mos gene which is defined by homology with v-mos. Characterization of the mouse c-mos gene with respect to the possible presence of related genes in the mouse genome.

Methods Employed:

Standard methods of the molecular biology laboratory such as cloning and subcloning of genes into bacteriophage λ and derivatives of pBR322, restriction enzyme analysis and Southern hybridization analysis of genomic and cloned DNA, screening of genomic libraries, DNA sequencing and heteroduplex analysis.

Major Findings:

RNA transcripts present in crude preparations of Moloney murine leukemia virus (MoMuLV), which hybridize to 3' flanking DNA of the mouse c-mos gene, have been shown to do so by virtue of homology to repetitive DNA sequences. Three copies of the mouse repetitive DNA family B1 map 0.6 kb., 2.8 kb and 5.5 kb downstream from the putative 3' end of the c-mos gene. These repeats have been identified by sequencing the appropriate DNA fragments and/or by hybridization to specific probes. Another copy of a non-B1 repetitive DNA family (copy number 5000-10,000 copies per diploid genome) maps between the last two B1 repeats. The genome of the mouse apparently does not contain genes closely related to c-mos. This was shown by Southern blot hybridization with a c-mos specific probe under conditions where the human mos gene (76% homology) and the quail mos gene could be easily detected.

Significance to Biomedical Research and the Program of the Institute:

The c-mos gene has as yet not been found to be expressed in normal mouse cells. However, by appropriate recombination either in vitro or during the in vivo generation of Moloney murine sarcoma virus the gene can be activated. Transfection of these recombinants into mouse NIH3T3 cells transforms the cells into tumor cells. In these cells RNA transcripts homologous to the mos gene are detected. Cells from a single spontaneous mouse plasmacytoma also contain mos-related RNA. The mos gene in these cells is rearranged by recombination with an endogenous retrovirus-like DNA sequence which is repeated about 1000 times in the genome. On the other hand, it was shown that clusters of repetitive DNA can cause genetic instability and rearrangements. Therefore, localization and identification of repetitive DNA sequences at the c-mos locus might lead to

further understanding of the activation of the gene in tumor cells. For future studies of the mos gene itself and its possible transcription in normal cells, it is important to know that there are apparently no related genes in the mouse genome. The comparison with the human analog might yield a better definition of the c-mos gene which is as yet only defined by the homology with the transforming gene of a tumor virus.

Proposed Course:

As mentioned above, the complete characterization of the c-mos locus of the mouse genome in terms of repetitive DNA sequences and cross homology with the human mos locus is in progress. Future studies will concentrate on the transcription of the c-mos gene in normal cells. Since the c-mos gene is highly conserved in evolution, it is unlikely that it is an inactive gene. The transcriptional activity of the gene might be very low or is restricted to a very peculiar cell type or certain (developmental) circumstances. Finding this cell type or these circumstances might allow correlation of mos expression with a very defined phenotype, thereby leading to insight of the function of the c-mos product in normal cells and the way it transforms cells.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 CP05346-01 LMO

PERIOD COVERED
March 7, 1983 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
High level expression of cII - mos fusion protein in E.coli.

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Arun Seth Visiting Associate, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Tumor Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have constructed a recombinant plasmid (pA28) in which the transcription of the v-mos gene can be induced under the control of lambda pL promoter to amplify the v-mos gene product to greater than 5% of the total cellular protein. This was accomplished by inserting the entire v-mos gene of Mo-Murine Sarcoma virus (HT-1) at cla I site of pJL6, a plasmid vector that contains lambda pL promoter and sequences coding for NH2-terminal portion of the lambda cII gene. In the resulting plasmid (pA28) the transcription begins at lambda pL promoter and the translation initiates at ATG of the lambda cII gene and terminates at TGA of v-mos to yield a predicted cII-mos hybrid protein. SDS-gel electrophoresis of the total cellular proteins indicates that the fusion is a polypeptide of molecular weight (MW) 41,000 daltons.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

G. F. Vande Woude Chief LMO, NCI

Objectives:

One of the main goals of our laboratory is to determine the biochemical functions of the transforming protein of Mo-murine sarcoma virus. It has been well documented that the mos gene product of MSV is responsible for inducing fibrosarcomas in vivo and cellular transformation in vitro. However, the precise molecular mechanism of cellular transformation caused by $p37^{mos}$ remains unclear, because of a lack of sufficient quantities of purified protein. One general way of cirumventing the difficulty of obtaining pure and large amounts of transforming protein may involve the expression of oncogene in E. coli. In an attempt to obtain significant amounts of mos protein in bacteria we have used an efficient plasmid vector (pJL6) which contains the following features:

1. A λ pl promoter, to amplify the transcription of cloned genes.

2. An efficient protein initiation signal and the region of cII gene encoding the NH_2-terminal portion of protein, to obtain the cII mos fusion protein.

3. Two unique restriction sites (cla I and HindIII) at the junction of cII gene and pBR322 sequences, to facilitate insertion of genes in the vector.

Methods Employed:

Cloning of the mos gene into the plasmid vector (pJL6) was performed using standard recombinant DNA techniques. For analysis of plasmid encoded protein the cell extracts from induced and uninduced bacterial cultures were analysed by SDS-gel electrophoresis and fluorography or staining.

Major Findings:

Cellular transformation by MSV is caused by a viral coded protein with molecular weight (MW) of 37,000 daltons. The transforming protein ($p37^{mos}$) represents approximately 0.0005% of the total cellular protein in MSV transformed cells and has never been detected in the normal cells. $p37^{mos}$ is extremely difficult to purify from the transformed cells. We therefore have constructed a plasmid vector (pA28) that directs the synthesis of cII-mos fusion protein in E. coli. A rough estimate suggests that the hybrid protein represents 5% of the total bacterial protein.

Although the bacterial polypeptide (cII - mos) is expressed as a fusion protein and contains sixteen additional amino acids, we anticipate that small changes at the NH_2-terminus will not seriously interfere with the functions of the transforming protein. Previously several fused proteins have been shown to be enzymatically active and biologically functional.

Significance to Biomedical Research and the Program of the Institute:

It has been shown that several transforming proteins $p60^{src}$, $p21^{ras}$, $p120^{abl}$ have protein kinase activity and it is very likely that this activity plays a major role in the malignant transformation of cells. However, no detectable protein kinase has been observed in $p37^{mos}$. Since the $p37^{mos}$ is present in scarce amount in transformed cells, it is extremely difficult to isolate the protein. In an attempt to resolve this problem we have shown the construction of a plasmid which expresses large amounts of cII - mos fusion protein in bacteria. The approach presented here will allow us to purify and study the transforming protein in greater detail and should also help to elucidate the precise role of $p37^{mos}$ in cellular transformation. The antisera raised against bacterial full length protein may also prove to be a very useful reagent to detect mos related proteins in MSV-transformed cells.

Proposed Course:

During the course of next year, we intend to fully characterize the plasmid encoded protein and also compare its properties with the $p37^{mos}$. Protein kinase, autophosphorylation and nucleotide binding activities of bacterial mos protein will also be explored. Furthermore, after purification we will micro-inject the protein into NIH3T3 cells to verify the role of mos in cellular transformation.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05347-01 LMO

PERIOD COVERED

May 1, 1983 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Gene Regulation Mediated by Transcription Initiation and Termination

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Mohammed Zuber Visiting Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Molecular Control and Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Regulation of early gene expression in phage lambda is mediated by antitermination of transcripton. However, the mechanism of transcription termination itself is not well understood. Towards a better understanding of the transcription termination antitermination mechanism, the Rho dependent t-Rl terminator along with its regulatory sequences was cloned onto a plasmid. Deletions of various lengths into the terminator segment were generated in vitro; DNA sequencing was then done to determine the deletion end points. After cloning these deletions onto an expression vector between a promoter and its structural gene, their effect on the expression of a distal gene is determined. Deletions that remove the terminator (or part of it) express the distal gene at higher levels than when transcription terminates at the terminator.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Donald Court Head, Molecular Control and Genetics Section LMO, NCI

Objectives:

We are chiefly interested in gene regulation in phage lambda at the level of transcription initiation and termination. Studies involve examination of the regulatory functions and DNA sequences which affect initiation and termination.

Transcription antitermination positively regulates the early gene expression in phage lambda. The antitermination machinery comprises both phage and host encoded proteins. The phage encoded N gene product somehow modifes the transcription process at a specific sequence on the phage genome called nut site (for N utilization) so that the RNA polymerase can read through the transcription terminator signal at the rho dependent t_{RI} terminator site. A host protein nusA is also involved in the termination-antitermination process.

Two types of terminators exist. Those that can terminate with RNA polymerase alone and those that require a host protein Rho in addition. Although the structure of Rho independent terminators is well understood, very little is known about the Rho dependent terminators. The project we have undertaken emphasizes experimentation towards a better understanding of the transcription termination/antitermination mechanisms at the Rho dependent t_{RI} terminator.

Methods Employed:

Standard microbial, genetic, biochemical and recombinant DNA techniques.

Major Findings:

1. The Rho dependent t_{RI} terminator of phage lambda along with two regulatory sequences box A and nut R was cloned into a plasmid. These regulatory sequences are the proposed sites for host nus A action and for λN action respectively.

2. In vitro deletions extending to different lengths into the transciptional regulatory sequences were made with exonuclease III and S1 endonuclease.

3. DNA sequencing determined the deletion end points.

4. The deletions were cloned onto an expression vector in between a promoter and its structural gene to examine the effect of the terminator and the respective deletions on expression of the gene downstream.

5. A pair of strains was constructed. One expressed λN product from a prophage. The other lacked λN function. The effect of N function on the

normal terminator and the deletions is examined. Deletions that remove the nut R site are the same in N^+ or N^- conditions.

Significance to Biomedical Research and the Program of the Institute:

The expression of certain genes are turned on in cancer cells. Our studies are aimed at understanding the molecular basis of how genes are turned on and off. We are using E. coli and λ as model systems. Information learned here is applied to problems concerning cancer development through interaction with other groups in the Laboratory of Molecular Oncology.

Proposed Course:

1. Studies are in progress to determine the effect of the different deletions we have isolated and sequenced on:

    A. The box A - nus recognition site
    B. The nut R - N recognition site
    C. The t_{RI} - Rho dependent terminator

2. To make more precise in vitro deletion mutants by T4 polymerase and S1 endonuclease to better locate box A, nut R and t_{RI}.

3. To perform in vitro transcription experiments to determine the effect of the terminator and the deletions into it on transcription.

4. To study the lambda promoter for repressor establishment (p_{RE}) and its dependence on the positive activator protein, cII.

5. To clone a newly recognized promoter into plasmid and phage vectors and to study its effect on the late gene expression in phage lambda.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05357-01 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interactions Between E. Coli K12 and Bacteriophage

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

N. Jahan Visiting Fellow, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Molecular Control and Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Bacteriophage lambda when expressed from a prophage causes several changes in host physiology (transformation). Two regions of the prophage DNA encode genes (hin) which cause this effect. One of these regions is cloned in a plasmid and defines an open reading frame of 186 bp. The effects caused by hin can all be related to membrane changes in the host.

Another phage function(s) causes the induction of certain host proteins which are a subset of the E. coli heat shock proteins. This function of lambda as well as the hin function are all located in the same PL-transcription unit.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

D. Court Head, Carcinogenesis Regulation Section LMO, NCI

Objectives:

We are interested in a type of transformation of E. coli by phage λ . A specific set of genes have been defined and mapped on λ that cause this physiological change in the host. In addition, we are studying the changes wrought on the cellular transport systems and cell membrane itself by λ hin expression. λ hin function exerts broad pleiotropic effects upon the cell:

1. It reduces the uptake of several precursors (i.e., uridine, thymidine, etc.) for macromolecular synthesis.

2. It reduces cAMP levels in the cell and thereby affects expression of genes under control of cAMP and its receptor protein, CRP.

3. It causes a small increase in ppGpp levels, 2-3 fold.

4. It enhances preferentially the stability of the PL transcript.

The mechanism of action of hin is not yet understood. It is clear that two different segments under the control of the P_L promoter can independently cause these effects, but within each region it is not clear that one gene is responsible.

We are also examining a separate phenomena caused by another λ function in the p_L operon that induces several of the E. coli heat shock proteins and reduces transcription of other cellular proteins.

Methods Employed:

Standard microbial, genetic, biochemical and recombinant DNA techniques.

Major Findings:

1. One hin gene has been precisely mapped. The sequence of a potential gene in this region has been determined. It has an open reading frame capable of making a protein 62 amino acids in length.

2. A strong Rho-independent transcription-termination signal is located within the open reading frame cited above. This is the first example of this type of terminator within a gene.

3. This hin gene has been cloned on a plasmid. We will study its effect in the absence of other λ genes and use in vitro mutagenesis to isolate mutants of hin on the plasmid.

Significance to Biomedical Research and the Program of the Institute:

The interaction and influence of viruses on their hosts is a common property both in prokaryotic and eukaryotic systems. Our studies are aimed at understanding how host functions are altered by the expression of viral genes in cells. We believe many phenomena observed in both prokaryotic and eukaryotic cells may have common explanations.

Proposed Course:

1. To clone each gene involved in hin from λ to a plasmid so that each can be examined independently.

2. To determine the mechanism by which hin causes its effect.

3. To determine how heat shock genes are activated by λ.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP08718-05 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transcriptional and Post-transcriptional Control of the Lambda Int Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

D. L. Court Research Biologist, LMO, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Molecular Control and Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The lambda int gene product, Int, recombines phage and bacterial DNA at a specific site during the integration step of lysogeny. This recombination involves a type I topoisomerase activity of integrase. Regulation of integrase synthesis is complex. (1) Transcription of the gene can occur from either of two promoters. Lambda cII protein activates transcription initiation near int at pI. The lambda N gene product prevents RNA polymerase from terminating transcription at several terminators between pL and int. (2) The expression of integrase is also subject to post-transcriptional regulation by a site, sib, which is located beyond int in the b region of lambda. Expression of int from pL is inhibited by sib, whereas that from pI is not. The negative control of int expression by sib is termed retroregulation. Retroregulation of int is caused, in part, by processing of the pL transcript at the sib site by RNase III of Escherichia coli.

The RNase III processing occurs in a region of extensive dyad symmetry in the DNA. Part of this symmetric element is also the transcription terminator (tI) which stops transcription initiating from the pI promoter but does not stop transcription from the pL promoter because of the N-antitermination activity on PL. The shorter pI transcript forms a stable stem and loop structure at its 3' end in the region of symmetry but lacks the entire dyad symmetry required for RNA processing that is formed in the longer pI transcript. Processing removes the stem and loop structure from the pL transcript and forms a 3' end that we believe is far more sensitive to nuclease attack than the end of the pI transcript.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

S. Bear	Staff Fellow	LMO, NCI
G. Guarneros	Visiting Scientist	LMB, NCI
A. Oppenheim	Expert	LMB, NCI
P. Guzman	Guest Researcher	LMO, NCI
G. Olmeda	Guest Researcher	LMO, NCI

Objectives:

The regulation of the λ int gene has been determined. It can be transcribed from either of two promoters PI and PL. PI requires λ cII protein in addition to RNA polymerase to initiate. It transcribes int and terminates at a site tI 260 bases beyond the gene. This RNA synthesizes high levels of int protein. PL also transcribes int but is prevented from terminating at tI by λ N gene product which makes polymerases initiating at PL (but not PI) non-terminating. The PL transcripts do not synthesize int. A site on the PL transcript inhibits int expression. It is a site for endoribonuclease (RNaseIII) located 260 bases beyond int. RNA processing here sensitizes the int m-RNA to a proposed 3'-5' exonuclease in E. coli. The terminated PI transcript is not processed (the RNaseIII site is not formed) and is not sensitive to the exonuclease. This posttranscriptional control of int from a site located beyond the gene is called retroregulation.

Our primary objective now is to determine why the terminated P_I transcript is stable to mRNA degradation and the processed p_L transcript becomes susceptible. Can the processed p_L transcript be made resistant again by placing upstream a strong stem structure in the RNA which exists at the end of the p_I transcript?

Major Findings:

(1) cII activates the promoters PI and PE to allow RNA polymerase to initiate.

(2) Int synthesis occurs from PI transcripts that terminate at tI 260 bases beyond the int gene.

(3) Int synthesis is blocked from the PL transcript that extends beyond the tI terminator. Inhibition of int synthesis from this transcript is caused by RNaseIII (endoribonuclease) processing.

(4) The int mRNA of the PL transcript is degraded when processing occurs. A 3' to 5' exonuclease has been postulated.

(5) We have defined within +/-10 bp the RNase III recognition site by using Bal31 to generate a deletion map.

(6) RNaseIII protein cuts the PL transcript at this site in vitro. Mutants in the site are not processed.

(7) RNaseIII does not process the terminated P_I transcript in vivo, and only partially processes it in vitro at high nuclease concentrations.

(8) Insertion of foreign DNA between sib and int prevents retroregulation. We postulate distance and time prevent the nuclease from being effective or that specific sites block the progress.

(9) An ELISA assay for int has been developed to monitor accurately changes in int levels under different conditions.

(10) Preliminary studies indicate that the xis gene product or its translation affects the level of int protein made in the cell.

Significance to Biomedical Research and the Program of the Institute:

In cancer cells the expression of some genes is permanently turned on by mechanisms that we don't yet understand. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using E. coli and λ as model systems. Application of knowledge gained in bacterial systems has been applied to understanding gene control in complex systems.

Proposed Course:

(1) To determine the mechanism of transcription termination. (2) To analyse the mechanism of posttranscriptional control mechanisms, i.e., endo and exo ribonuclease control systems in E. coli. (3) To determine why some mRNA's are more resistant than others to degradation by nucleases.

Publications:

Court, D. and Oppenheim, A.: Deletion analysis of the retroregulatory site for the λ int gene. J. Mol. Biol. 166: 233-240, 1983.

Court, D., Schmeissner, U., Rosenberg, M., Oppenheim, A., Guarneros, G. and Montanez, C.: Processing of λ int RNA: A mechanism for gene control. In Schlessinger, D. (Ed.): Microbiology. Washington, D.C., American Society of Microbiology, 1983, pp. 78-81.

Gottesman, M., Oppenheim, A. and Court, D.: Retroregulation: Control of gene expression from sites distal to the gene. Cell 29: 727-728, 1982.

ANNUAL REPORT OF

LABORATORY OF MOLECULAR VIROLOGY

NATIONAL CANCER INSTITUTE

October 1, 1982 through September 30, 1983

The Laboratory of Molecular Virology (1) analyzes the mechanism of gene expression in normal and transformed eukaryotic cells; (2) uses viruses as tools for probing cellular regulatory mechanisms; (3) develops and applies biological, biochemical and immunological procedures to obtain evidence for the mechanism by which cellular proteins are recognized by the immune system; (4) plans and conducts research on transforming proteins to define their properties in normal cells and their potential role in the development of neoplasms; and (5) investigates the mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells.

The Virus Tumor Biology Section (1) characterizes the nucleotide sequence from regions of viral and cellular DNA thought to be involved in gene expression; (2) investigates the properties of cellular and viral transforming genes and their protein products; (3) evaluates the effects of viral infection on cellular control mechanisms in transformation and in lytic responses; and (4) develops eukaryotic viral vectors to study gene expression.

The Cell Physiology Section (1) investigates the molecular elements essential for cellular transformation; (2) studies the properties of cell surface molecules in expression of the cellular phenotype; (3) uses recombinant DNA techniques and molecular genetics to study the elements involved in gene regulation; and (4) employs prokaryotic host-vector-systems to examine sequences involved in efficient gene expression and protein production.

A principle goal in the Laboratory of Molecular Virology is the elucidation of signals associated with gene expression. In particular, our interest has been directed toward regulatory events which take place at the level of transcription and processing of RNA. We have been involved in the elucidation and analysis of novel genetic elements, enhancer sequences, which appear to be responsible for controlling the rate at which particular genes are transcribed. We have demonstrated the existence of these enhancer sequences not only in the genomes of DNA viruses such as SV40 and BKV, but also in the long terminal repeats (LTRs) of retroviruses. Using a combination of *in vivo* and *in vitro* assays, we have demonstrated that enhancer sequences show host-cell specificity, and thus may be among the elements involved in controlling the host range of certain viruses as well as the tissue-specific expression of certain eukaryotic genes. Several lines of research have suggested that enhancer sequences are not unique to viruses but are also present within the eukaryotic genome. A major effort in our laboratory will be directed at determining whether or not enhancer sequences play a role in the developmental and tissue-specific regulation of gene expression. In addition, we are interested in mutagenizing regions of enhancer elements to elucidate those sets of nucleotide associated with the general activation phenomenon as well as the cellular specificity. Experiments have been designed in an attempt to elucidate the mechanism by which the activator/enhancer sequences function. A principal interest in our laboratory for the

future will be the definition of biological macromolecules which interact with these regulatory elements.

A major emphasis has been directed toward an understanding of the structure and function of the class I histocompatibility antigens (the classical transplantation antigens), and in particular, the roles of these cell-surface antigens in relation to the neoplastic state. These studies are of singular importance because the ability of the immune system to identify and destroy tumor cells depends upon their presentation by the class I antigens to the cytotoxic T-lymphocytes.

In studies involving the isolation and characterization of cDNA clones derived from different class I genes, it was observed that class I antigens with different primary structures are expressed from the same class I gene by alternate RNA splicing. This novel finding suggests that there exist different functional subsets of these antigens and offers the opportunity to compare the products of these cDNA clones in their role in presenting tumor antigens to the cytotoxic T-cell. Such analyses would lead to a biochemical definition of how class I antigens present "aberrant" cells to the immune system.

Molecular cloning and identification of class I loci have led to the finding of a gene that encodes a secreted class I-related antigen. Because of variations in the level of expression of this gene in different inbred mouse strains and an unusual tissue-restriction in its expression, it is suggested that this serum protein may be a tolerogenic form of the class I antigens, acting as a blocking factor to regulate the function of cytotoxic T-cells in the process of immune surveillance. Studies are in progress to confirm this hypothesis by using this secreted class I antigen to modulate T-lymphocyte recognition of tumor cells.

A considerable effort has been directed towards obtaining nucleotide sequences of murine and human oncogenes. It is hoped that this information will contribute to an understanding of the process of cell transformation and tumorigenesis. Studies in progress are designed to isolate proto-oncogenes and to study their structure and expression in growing and differentiating cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05101-05 LMV

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Molecular Mechanisms for Malignant Transformation of Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Gilbert Jay Head, Cell Physiology Section, LMV, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to investigate the molecular mechanisms underlying the malignant transformation of cells. We have been studying the structure and function of tumor antigens involved in neoplastic transformation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George Khoury Chief LMV, NCI

Objectives:

Studies on the structure and function of the simian virus 40 tumor antigen in infected cells.

Methods Employed:

Proteins were detected by immunoprecipitation using either conventional or monoclonal antibodies.

Major Findings:

The simian virus 40 tumor antigen (SV40 T-antigen) is a multifunctional protein that plays a critical role both in cell transformation and in virus propagation. We have attempted to dissect the T-antigen molecule with the hope of obtaining information on the structure-function relationship which might be helpful for an understanding of the neoplastic process. Using human adenovirus 2-simian virus 40 recombinants, we have previously demonstrated that the carboxy-terminal portion of T-antigen is sufficient to induce SV40-specific tumor rejection. We have now shown, by partial cleavage with proteolytic enzymes, that the amino-terminal portion of T-antigen is sufficient to induce binding to the viral origin of DNA replication. Such experiments involving the molecular dissection of a virally induced transforming protein is particularly pertinent to a clear understanding of the mechanism of cellular transformation by oncogenic viruses.

Significance to Biomedical Research and the Program of the Institute:

The mechanism whereby a virally coded protein induces the uncontrolled proliferation of cells, a process which leads to a transformed state is at present obscure. The fact that SV40 T-antigen can bind to the viral origin of DNA replication, a sequence which has its counterpart in the cellular genome, suggests a mechanism for a viral transforming protein in altering the replication of host DNA. Our attempt to analyze this macromolecular interaction may have direct bearing on the process by which normal cells lose their growth control upon neoplastic transformation.

Proposed Course:

To define the molecular mode of action of this protein during the process of cell transformation.

Publications:

Morrison, B., Kress, M., Khoury, G., and Jay, G.: Simian virus 40 tumor antigen: Isolation of the origin-specific DNA-binding domain. J. Virol. 47: 106-114, 1983.

Prives, C., Barnet, B., Scheller, A., Khoury, G. and Jay, G.: Discrete regions of simian virus 40 large T antigen are required for non-specific and viral origin-specific DNA binding. J. Virol. 43: 73-82, 1982.

Rhim, J. S., Trimmer, R., Huebner, R. J., Papas, T. S. and Jay, G.: Differential susceptibility of human cells to transformation by murine and avian sarcoma viruses. Proc. Soc. Exp. Biol. Med. 170: 350-358, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05214-03 LMV

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic Elements Regulating the Initiation of Transcription

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

George Khoury Chief, LMV, NCI

COOPERATING UNITS *(If any)*

Biochemical Virology Section, Laboratory of Molecular Microbiology, NIAID, NIH

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

An unusual and unique eukaryotic transcriptional control element which markedly increases the transcriptional activity of eukaryotic genes is the activator or enhancer element. We first characterized the enhancer elements in the genomes of SV40 and the Moloney sarcoma virus (MSV) and demonstrated that these sequences act in a host-cell specific fashion. This implies a potential for tissue-specific gene expression. The core activity of the enhancer has been localized to a very short stretch of nucleotides within the SV40 72 bp repeat. In attempting to identify cellular enhancer elements, we have investigated sequences in the putative control regions (the long terminal repeats or LTRs) of endogenous retroviruses. Certain of these cloned sequences appear to manifest low levels of enhancer activity.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

John Brady	Expert	LMV, NCI
Michael Kessel	Guest Researcher	LMV, NCI
Laimonis Laimins	Staff Fellow	LMV, NCI
Nadia Rosenthal	Staff Fellow	LMV, NCI
Arifa Khan	Staff Fellow	LMM, NIAID
Malcolm Martin	Chief	LMM, NIAID
Arnold Rabson	Medical Staff Fellow	LMM, NIAID
Paul Steele	Medical Staff Fellow	LMM, NIAID

Objectives:

This project is directed toward an analysis of the genetic elements required for the initiation of transcription. It should provide information about structure and function of eukaryotic promoters.

Methods Employed:

Construction of deletion mutants and point mutants; mapping of recombinant genomes; RNA analysis; protein analysis; cloning in pBR322 and derivatives thereof; expression of recombinants using transient and long-term assays.

Major Findings:

1. The 72 bp repeat of SV40 is a cis-essential element required for the expression of viral genes.

2. Using point-mutants generated in single-strand bacteriophages in conjunction with deletion mutants, a critical core sequence within the 72 bp repeat has been identified.

3. Enhancer sequences have been detected in the mouse, monkey and human genomes. These elements flank the coding regions of putative endogenous retroviral genes.

Significance to Biomedical Research and the Program of the Institute:

The understanding of the molecular mechanisms leading to the initiation of transcription of eukaryotic genes is important in the understanding of transforming genes and cellular differentiation.

Proposed Course:

These projects will continue with an emphasis on the identification of the mechanism by which enhancer elements control gene expression.

Publications:

Gruss, P., Rosenthal, N., König, M., Ellis, R. W., Shih, T. Y., Scolnick, E. M. and Khoury, G.: The expression of viral and cellular p21 ras genes using SV40 as a vector. In Gluzman, Y. (Ed.): Eukaryotic Viral Vectors. New York, Cold Spring Harbor Laboratory, 1982, pp. 13-17.

Hamer, D.H. and Khoury, G.: Introduction. In Gluzman, Y. (Ed.): Enhancers and Controlling Elements, New York, Cold Spring Harbor Laboratory (In Press).

Khoury, G. and Gruss, P.: Enhancer elements. Cell 33: 313-314, 1983.

Levinson, B., Khoury, G., Vande Woude, G. and Gruss, P.: Activation of SV40 genes by the 72 base pair tandem repeats of Moloney sarcoma virus. Nature 295: 568-572, 1982.

Weiher, H., König, M. and Gruss, P.: Multiple point mutations affecting the simian virus 40 enhancer. Science 219: 626-631, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05216-03 LMV

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
DNA Sequences of Important Tumor Virus Genetic Elements

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Ravi Dhar Visiting Scientist, LMV, NCI

COOPERATING UNITS *(if any)*
Clinical Hematology Branch, NHLBI; Virus & Cell Biology Research, Merck Sharp & Dohme Research Laboratories, West Point, PA

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The transforming proteins of Harvey (Ha) and Kirsten (Ki) murine sarcoma viruses, Ha-ras and Ki-ras, are 21,000 dalton polypeptides. The nucleotide sequence of the Ha murine sarcoma virus gene and its two rat cellular homologues, c-ras I and c-ras II, have been determined. The sequence corresponding to the N-terminal region of the human oncogene from EJ carcinoma cell line and its proto-oncogene (human c-Ha-ras) have also been determined and the crucial difference between these genes has been located within the 12th amino acid codon. A rapid method has been developed to analyze mutations at the 12th codon in different human tumors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Amelia Nieto	Visiting Fellow	LMV, NCI
Martin Ruta	Staff Fellow	CHB, NHLBI

Objectives:

Our major objectives are:

- to determine the structure of p21 ras genes to correlate them with potential function and obtain an evolutionary relationship among ras genes.
- to analyze and correlate structural changes with transforming function.
- to study transcriptional regulation of p21 encoded genes in growing and differentiating cells.

Methods Employed:

Recombinant DNA technology; Maxam and Gilbert DNA sequencing; Southern and Northern blot analysis.

Major Findings:

The comparison of the murine v-Ha-ras and c-Ha-ras I gene sequences indicates only two amino acid differences. The first change is at amino acid 12 from arginine in v-ras to glycine c-Ha-ras I and c-Ha-ras II. The second difference is at the 59th amino acid represented to be a change from threonine in v-ras to alanine in c-Ha-ras I and c-Ha-ras II. The threonine at position 59 in the v-ras phosphorylated, whereas there is no known phosphorylation event for the cellular Ha-ras genes. The gene structure of c-Ha-ras II suggests that it is a pseudogene. This gene has been shown to be transcriptionally active, however, when inserted into the late region of an SV40 vector.

The human homologue of the v-Ha-ras gene has been isolated from the human EJ bladder carcinoma cell line and from normal tissue. The human EJ Ha-ras oncogene transforms NIH 3T3 cells, whereas its proto-oncogene does not. The ability to transform relates to a difference in the 12th amino acid; the proto-oncogene contains a glycine, whereas the tumor cell line has a valine. Comparison of the human Ha-ras sequences with murine v-ras and the murine c-ras sequences suggests that this single base change in the 12th codon is sufficient to confer transforming ability to the Ha-ras gene.

We have developed a rapid technique for analyzing mutations in the 12th amino acid in human tumors. The analysis of 15 primary bladder carcinomas and 13 other tumors showed no mutation at this site, suggesting it may be a very infrequent event in human cancers.

The murine Kirsten sarcoma virus gene has been shown by others to be homologous to the oncogene isolated from human lung and colon carcinomas. We have isolated three different Ki-related genes from human lambda libraries. The restriction endonuclease cleavage maps are different from the Ki-ras genes isolated from lung and colon carcinomas.

We have also analyzed the transcripts of both the Ha and Ki ras gene in various tissues and also during differentiation. The concentration of these transcripts increases three to five fold during pregnancy in the mammary glands of rats and falls during the second day of lactation.

Significance to Biomedical Research and the Program of the Institute:

The Harvey and Kirsten ras sequences have been associated with certain human tumors. Analysis of these genes and their transcripts will allow for a better understanding of their potential role in normal and neoplastic cells.

Proposed Course:

We are currently analyzing the Ha-ras genes isolated from yeast. Preliminary analysis suggests that the N-terminal half of the molecule is conserved, whereas the C-terminal half has diverged. This observation suggests a common function residing within the N-terminal region of this polypeptide between yeast and vertebrates.

Publications:

Dhar, R., Ellis, R. W., Shih, T. Y., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D. and Scolnick, E.: Nucleotide sequence of the p21 transforming protein of Harvey murine sarcoma virus. Science 217: 934-937, 1982.

Shih, T. Y., Stokes, P. E., Smythers, G. W., Dhar, R., Oroszlan, S.: Characterization of the phosphorylation sites and the surrounding amino acid sequences of the p21 transforming proteins coded for by Harvey and Kirsten strains of murine sarcoma viruses. J. Biol. Chem. 257: 11767-11773, 1982.

Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R. and Chang, E. H.: Mechanism of activation of a human oncogene. Nature 300: 143-149, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05217-03 LMV

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Regulation of SV40 Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Shigeko Nomura Microbiologist, LMV, NCI

COOPERATING UNITS *(If any)*

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Cell Physiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have identified a novel gene product, the agnoprotein, encoded by the tumor SV40. In order to study its function, we have generated antibodies against this protein and have investigated its biochemical properties. In addition, we have used site-directed mutagenesis to derive mutant viruses which are defective in the agnoprotein, and have studied the biological properties of these mutants.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Gilbert Jay	Head, Cell Physiology Section	LMV, NCI
George Khoury	Chief	LMV, NCI

Objectives:

The primary objective is to study the function of the SV40 agnoprotein.

Methods Employed:

Recombinant DNA techniques; nucleic acids hybridization; gel electrophoresis; electron microscopy; proteins were analyzed by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

By indirect immunofluorescence and biochemical fractionation followed by indirect immunoprecipitation, we have shown predominant localization of the SV40 agnoprotein to the cytosol and perinuclear regions of the infected cell. The protein exhibits high affinity for DNA in vitro and has a rapid turnover rate in vivo. We have generated a frame-shift mutation within the agnogene, which shows a surprisingly high reversion frequency in vivo as a result of base alterations at the same site as the original mutation. In DNA transfection studies, we were able to demonstrate that while the expression of SV40 T-antigen was not markedly affected by mutations in the agnogene, viral DNA replication and all late viral functions were perturbed.

Significance to Biomedical Research and the Program of the Institute:

These studies have established an essential function(s) for the SV40 agnoprotein in the productive cycle of the virus. The results indicate that T-antigen alone may be insufficient to induce viral DNA replication, and suggest that the agnoprotein may have an intimate role in virus growth.

Proposed Course:

We shall continue to define, at the molecular level, the mechanism of action of the agnogene product.

Publications:

Nomura, S., Khoury, G. and Jay, G.: Subcellular localization of the simian virus 40 agnoprotein. J. Virol. 45: 428-433, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05219-03 LMV

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Construction of a Prokaryotic Vector for the Expression of Mammalian Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Gilbert Jay Head, Cell Physiology Section, LMV, NCI

COOPERATING UNITS *(if any)*

Department of Chemistry, University of New Brunswick, Canada

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to investigate the regulatory elements required for recognition (i) by ribosomes during the process of initiation of protein synthesis and (ii) by RNA polymerases in the initiation of transcription. We have generated a general plasmid vector containing a synthetic ribosome binding site and a synthetic promoter that assures the efficient expression of mammalian proteins in bacteria.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George Khoury Chief LMV, NCI

Objectives:

Use of synthetic promoters and ribosome binding sites for the efficient expression of mammalian proteins in bacteria.

Methods Employed:

Recombinant DNA techniques; nucleic acid hybridization; gel electrophoresis; electron microscopy; proteins analyzed by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

We have constructed plasmid vectors carrying different chemically synthesized "hypothetical" promoter sites in tandem with one of several chemically synthesized hypothetical ribosome binding sites and have compared each of these vectors for the expression of cloned mammalian genes. While the synthetic promoter sequence provided for recognition by the E. coli RNA polymerase (a process that led to transcription of the cloned cDNA sequence), the synthetic ribosome binding site allowed recognition of the resulting mRNA by E. coli ribosomes (an event that resulted in the synthesis of the mammalian protein). From such analyses it was possible to identify an ideal combination of the two prokaryotic regulatory elements, one that would induce the highest level of expression of eukaryotic proteins in bacteria. A general expression vector carrying these two recognition sequences has been constructed and has been used successfully in the expression of biologically active human gamma (immune) interferon at an unusually high level.

Significance to Biomedical Research and the Program of the Institute:

One of the major goals of recombinant DNA research is to produce significant quantities of medically and agriculturally important proteins. If these products are to be used as human therapeutics, they must be "authentic", so as not to induce an immune response in the recipients. These two prerequisites have induced a great deal of investigation over the past several years. We have successfully generated a prokaryotic expression vector which not only can express authentic mammalian proteins in E. coli, but can do so at high levels. Such a vector is important for future production of human products synthesized in bacteria.

Proposed Course:

We shall continue to improve our expression vector with the goal of being able to induce the bacteria to secrete authentic mammalian proteins.

Publications:

Jay, E., Seth, A. K., Rommens, J., Sood, A. and Jay, G.: Gene expression: Chemical synthesis of E. coli ribosome binding sites and their use in directing the expression of mammalian proteins in bacteria. Nucleic Acids Res. 10: 6319-6329, 1982.

Jay, E., Jay, F. and Jay, G.: Comparison of different synthetic ribosome binding sites for the efficient expression of eukaryotic proteins in Escherichia coli. In Papas, T. S., Rosenberg, M. and Chirikjian, J. (Eds.): Expression of Cloned Genes in Prokaryotic and Eukaryotic Vectors. New York, Elsevier/North-Holland, Inc. (In Press).

Jay, E., and Jay, G.: Synthesis of mammalian proteins in bacteria. In Cheremisinoff, P. N. and Ouellette, R. P. (Eds.): Biotechnology Handbook. Ann Arbor, Butterworth (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05220-03 LMV

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Structure and Function of Cell Surface Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

Gilbert Jay Head, Cell Physiology Section, LMV, NCI

COOPERATING UNITS *(if any)*

Virology & Cellular Immunology Section, Laboratory of Viral Diseases, NIAID

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have cloned and analyzed cDNA sequences derived from genes which encode the classical transplantation antigens. Our findings have led to a better understanding of the structure and function of these cell surface antigens, particularly with regard to their role in the presentation of tumor and viral antigens to the immune system.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Michel Kress	Visiting Fellow	LMV, NCI
Wendy Davidson	Visiting Scientist	LVD, NIAID
George Khoury	Chief	LMV, NCI

Objectives:

We wish to study the organization and expression of the genes coding for the H-2 histocompatibility antigens by molecular cloning technologies.

Methods Employed:

Recombinant DNA techniques; nucleic acids hybridization; gel electrophoresis; electron microscopy; proteins were analyzed by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

The genes for the classical transplantation antigens are unique in that they belong to a multigene family of which each member is represented by a large number of alleles. Since all of these genes are highly related in sequence, it has been difficult to study the expression of individual members of this complex gene family. Based upon our suggestion that the 3' noncoding regions of these genes may be useful in identifying mRNA molecules transcribed from different loci, we have compared a large number of sequences from different inbred mouse strains and have been able to assign each of these sequences without ambiguity to distinct allelic series. The availability of specific probes generated from these unique sequences will allow the analysis of individual genes and their specific expression, without interference from other highly homologous sequences in this multigene family.

One of the major roles of the classical transplantation antigens is the presentation of virus-infected cells to the cytoxic T-cells, a process that leads ultimately to the destruction of the cell displaying the viral antigen. In keeping with this function is the finding that these transplantation antigens (encoded by the H-2K, D, and L genes) are cell-surface glycoproteins with amino-termini protruding extracellularly and carboxy-termini located inside the cell. The recent observation that the cytoplasmic domain of the molecule is encoded by three separate DNA exons has suggested a complex role for this portion of the polypeptide chain. We have now obtained evidence for the use of alternate splice acceptor sites in the primary transcript of the H-2K gene, thereby resulting in two RNA molecules that would encode H-2K antigens differing in their carboxy-termini. This observation suggests the existence of different functional subsets of antigens encoded by the same H-2K gene.

Significance to Biomedical Research and the Program of the Institute:

In the process of immune surveillance, the cytotoxic T-cell recognizes tumor cells and virus-infected cells in context with the classical transplantation antigens. The consequence of this dual recognition process is the destruction of cells displaying both the "self" antigen and the "foreign" antigen. The molecular mechanism underlying this interaction, while of particular importance to our understanding of the cancer problem, has remained obscure. With the molecular cloning of genes for the classical transplantation antigens, and the ability to express them in transfected cells, one can begin to dissect this seemingly complex recognition process at the biochemical level.

Proposed Course:

Our observations with the mouse H-2 system are now being extended to the human HLA system, with the goal of obtaining molecular definitions for different HLA-associated diseases.

Publications:

Jay, G., Palladino, M., Khoury, G. and Old, L. J.: Mouse Lyt-2 antigen: Evidence for two heterodimers with a common subunit. Proc. Natl. Acad. Sci. USA 79: 2654-2657, 1982.

Tanaka, K., Ozato, K., Jay, G., Parnes, J. R., Ramanathan, L., Seidman, J. G., Chang, K. S. S. and Appella, E.: Transcriptional control of H-2 antigen and β_2-microglobulin gene expression in mouse trophoblast cell clones. Proc. Natl. Acad. Sci. USA (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05251-02 LMV

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Host-Specific Activation of Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Laimonis Laimins Staff Fellow, LMV, NCI

COOPERATING UNITS *(if any)*
Gene Regulation Section, Laboratory of Molecular Biology, DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have examined the role of activator/enhancer elements in the regulation of eukaryotic viral gene expression. In SV40, a pair of 72 bp tandem repeat sequences, located approximately 100-200 bp from the site of initiation of transcription, are required for transcription. These sequences activate other genes placed in their immediate vicinity. Similar sequences have been identified in the long terminal repeat (LTR) of murine sarcoma virus (MSV) and have been found capable of replacing the 72 bp repeat sequences of SV40 to form a viable virus (SVrMSV). In this study, employing an assay for viral T-antigen expression as well as an in vitro assay for early gene expression involving the prokaryotic gene, chloramphenicol acetyltransferase (CAT), host-specific gene activation was examined. Using identical promoter regions from SV40, the SV40 tandem repeats were found to be more active in monkey kidney cells, whereas the MSV repeats were more active in mouse cells. Therefore, at least some activators appear to function in a host-specific manner. In addition, the MSV enhancer has been shown to work both 5' and 3' to the SV40 promoter in the CAT assay although not at equivalent rates. The two MSV repeats are not identical in sequence and have been found to enhance expression at different rates.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George Khoury	Chief	LMV, NCI
Bruce Howard	Senior Investigator	LMB, NCI
Cornelia Gorman	Staff Fellow	LMB, NCI

Objectives:

In these studies, we wish to examine the role and function of activator sequences in the regulation of eukaryotic gene expression.

Methods Employed:

Immunoprecipitation of T-antigen; Ca^{++}-phosphate transfection of eukaryotic cells; enzymatic assay for chloramphenicol acetyltransferase; cloning and restriction endonuclease analysis of DNA.

Major Findings:

The MSV derived 72 bp repeat sequences enhance gene expression in a host-cell specific manner. These sequences activate gene expression in mouse cells to a level approximately ten times that seen in monkey cells. In monkey cells, however, the MSV repeats enhance expression to levels well above those seen without any enhancer sequences.

The SV40 derived 72 bp tandem repeats are not as host-specific as are the MSV-derived repeats. In monkey kidney and in human HeLa cell lines, the SV40 repeats enhance expression only 1.5 times the level seen in mouse cell lines.

Significance to Biomedical Research and the Program of the Institute:

The finding that some enhancer elements are host-cell specific provides a model for one regulatory element which may play a role in the host range of DNA tumor viruses. Such sequences could also control eukaryotic gene expression in a tissue-specific environment.

Proposed Course:

Experiments will focus on the exact sequences of the MSV repeats required for activation of expression in a tissue-specific manner.

Publications:

Laimins, L. A., Khoury, G., Gorman, C., Howard, B. and Gruss, P.: Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 79: 6453-6457, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05252-02 LMV

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

SV40 Mediated Expression of Cellular Transforming Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Nadia Rosenthal Staff Fellow, LMV, NCI

COOPERATING UNITS *(if any)*

Institute of Microbiology, University of Heidelberg, West Germany; Virus & Cell Biology Research, Merck Sharp & Dohme Research Laboratories, West Point, PA; Laboratory of Molecular Oncology, DCCP, NCI

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The transforming gene of Harvey murine sarcoma virus has apparently evolved from either of two structurally distinct cellular genes represented in a rat genomic library. The relative ability of these genes to direct the synthesis of the transforming gene product was tested by placing them under the control of SV40 late regulatory elements. One of these genes (which contains several introns) produces both messenger RNA and the transforming protein; the other gene, which lacks introns, is transcribed efficiently but is translated in greatly reduced amounts. Since the "intron-less" gene cannot transform NIH 3T3 cells under the control of a retroviral long terminal repeat, the above experiments suggest that a mutation which reduces translational efficiency resides either within or very near the coding region of this gene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George Khoury	Chief	LMV, NCI
Thomas Shih	Chemist	LMO, NCI

Objectives:

This study proposed to investigate the nature of the expression of two rat cellular transforming genes.

Methods Employed:

Recombinant DNA techniques including isolation and cloning of specific fragments; construction and characterization of SV40/pBR322 vectors; growth and isolation of cloned molecules from bacterial and animal cell cultures; mRNA extraction from virus-infected cell cultures; restriction enzyme cleavage of DNA; polyacrylamide and agarose gel electrophoresis of DNA and RNA; transfer of DNA or RNA by blotting technique; hybridization of radioactive DNA probes to DNA or RNA blots; protein extraction from cell cultures and immunoprecipitation; translation of mRNAs.

Major Findings:

This work is based on the previous isolation of a rat cellular gene (c-ras I) which is highly homologous to the transforming gene of Harvey murine sarcoma virus. The viral gene, which encodes a 21 K dalton protein, synthesizes an unspliced message, whereas the first rat cellular gene c-ras I contains several introns. A second rat cellular gene (c-ras II) is also homologous to the viral sequence and appears to be colinear with it, i.e., does not contain introns. When ligated to the HaMuSV long terminal repeat sequence, the cloned c-ras I gene transforms NIH 3T3 cells with high efficiency, whereas the c-ras II gene transforms very poorly (De Feo, et al., PNAS 78, 3328, 1981). This implies a possible regulatory role for introns in the expression of the two c-ras genes. To investigate this possibility, SV40-c-ras hybrids were constructed. The SV40 late region was replaced by either of the two cellular genes in both orientations (sense, antisense) with respect to the SV40 late control region. Using an SV40 tsA mutant as helper, viable virus stocks were generated for all four recombinants. Northern blot analysis revealed that both cellular genes inserted in the sense orientation are transcribed in equivalent amounts, but in the antisense orientation, are not transcribed. In vivo protein analysis demonstrated that the sense orientation of c-ras I synthesizes abundant 21 K protein. Surprisingly, the sense orientation construction of c-ras II produced greatly reduced amounts of a similar 21 K protein. In vitro translation of cytoplasmic poly(A+) mRNAs synthesized by sense and antisense constructions of each gene yielded the same results, suggesting that the reduced level of

c-ras II expression is due to a translational defect rather than the absence of a splicing event. Furthermore, this defect may underlie the low transformation efficiency of the c-ras II gene.

Significance to Biomedical Research and the Program of the Institute:

Endogenous transforming genes are relevant to cancer research in at least two ways: 1) Their structural similarity to viral transforming genes suggests that viruses may have acquired their transforming potential by capture and regulation of a cellular sequence. 2) Mutations associated with endogenous cellular genes may alter their ability to induce the transformed state. In the case of c-ras II, such changes may obliterate their transforming potential. Thus, elucidation of the mechanisms controlling expression of these cellular transforming genes is a primary step towards understanding the transformed state.

Proposed Course:

The differences between the two rat c-Ha-ras genes that accounts for their differential expression will be determined by DNA sequencing. Further studies on gene expression will be based on these data.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05253-02 LMV

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Early Control Region of BKV

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Nadia Rosenthal Staff Fellow, LMV, NCI

COOPERATING UNITS *(if any)*
Cell Biology Section, Laboratory of Molecular Virology, DCCP, NCI; Department of Microbiology, University of Heidelberg, West Germany

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human papovavirus BKV contains a 68 base pair (bp) triplication unrelated in sequence to the 72 bp repeat of its monkey counterpart SV40. We have shown that the BKV repeats function as an enhancer by testing BKV subclones in a transient expression assay. Like SV40, the BKV enhancer activates transcription in human, monkey and mouse cells. A unique locus in the human genome has been isolated by DNA hybridization to the BKV enhancer. The homologous 1.5 kb region contains approximately 75 repeats of 21 bp with sequence similarity to the BKV repeats. In tests to assay enhancer function, the human repeats are active only in human cells, at levels 7-10X lower than the BKV repeats.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George Khoury	Chief	LMV, NCI
Michel Kress	Visiting Fellow	LMV, NCI

Objectives:

To identify a transcriptional enhancer in the BKV genome and to relate the sequence of the BKV repeats to homologous sequences in the human cellular genome.

Methods Employed:

Isolation and cloning of specific fragments; construction and characterization of SV40/pBR322 vectors; growth and isolation of cloned molecules from bacterial and animal cell cultures; mRNA extraction from virus-infected cell cultures; restriction enzyme cleavage of DNA; transfer of DNA or RNA by blotting technique; hybridization of radioactive DNA probes to DNA or RNA blots; protein extraction from cell cultures; immunoprecipitation.

Major Findings

Comparison of the genomes of BKV and SV40 show extensive similarity. However, the region of the repeated sequences which in SV40 has been shown to function as an enhancer of early gene expression, is markedly different in BKV. This control region may play a role in the host range of the two viruses.

We have also characterized a locus from human DNA, isolated from a genomic library by hybridization to a BKV repeat probe. The locus is unique as shown by Southern blot hybridization. The homologous region contain approximately 75 copies of a 21 bp repeat which appears to be evolutionarily related to the BKV sequences, and which contains no open reading frames. Although the human repeats do not activate gene expression to the same high level as do the BKV repeats, the activation by subclones of the human sequences in the CAT assay is species-specific; that is, activation is seen only in HeLa cells and not in monkey kidney cells or mouse L-cells.

Significance to Biomedical Research and the Program of the Institute:

BKV is a human papovavirus which probably causes a subacute infection in humans. It is of particular interest to study this virus and the elements which regulate its expression. Similar elements may function in gene regulation in human cells.

Proposed Course:

Experiments are in progress to screen regions flanking the human repeats for coding sequences, and to test potential tissue specificity of human enhancer sequences.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05254-02 LMV

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Insulin Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Ruth J. Muschel Medical Staff Fellow, LMV, NCI

COOPERATING UNITS *(if any)*
Viral Oncology and Molecular Pathology Section, Laboratory of Pathology, DCBD, NCI; Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We had previously established cell culture conditions in which rat insulinoma cells can be maintained. The total amounts of insulin RNA in high and low glucose were identical. In conditions of low glucose, insulin mRNA was found to be shorter by approximately 100 bases. This difference was due to a significantly shorter poly(A) tract.

To pursue these studies, we have utilized bovine papilloma virus (BPV) as an expression vector. In preliminary studies to optimize the system, we found that the orientation of the insulin insert in the BPV genome significantly influences gene expression. It may be that crucial promoter-enhancer interactions are affected by their relative orientations. Current studies to examine this point are in progress. In addition, flanking upstream sequences may contain inhibitory signals. Present studies will further map and characterize the potential inhibitory region, and define enhancer/promoter interactions.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Laimonis Laimins	Staff Fellow	LMV, NCI
George Khoury	Chief	LMV, NCI
Peter Howley	Head, Viral Oncology & Molecular Pathology Section	LP, NCI
Nava Sarver	Senior Staff Fellow	LP, NCI

Objectives:

The goal of this study is to understand the expression and regulation of insulin genes in fibroblasts and insulinoma cells.

Methods Employed:

RNA analyses by blot hybridization and primer extension; cell culture in serum-free media and on extracellular matrix; recombinant DNA technology; protein gel electrophoresis; insulin RIA; in vitro translation.

Major Findings:

Our major findings include the following observations:

1. Insulin specific mRNA contains different size poly(A) tracts depending upon glucose concentrations.

2. Growth of insulinoma cells on an extracellular matrix in serum-free medium markedly increases levels of insulin mRNA.

3. Orientation of insulin inserts in BPV vectors affects expression of this gene in fibroblasts.

4. Upstream inhibitory segments may be present in sequences flanking the insulin gene.

Significance to Biomedical Research and the Program of the Institute:

Insulin represents a gene of importance in mammalian metabolism and disease states. Upstream flanking sequence polymorphisms in the human insulin genes have been correlated with the onset of diabetes. Systems allowing study of these sequences may indicate the mechanisms of aberrant expression.

Proposed Course:

Studies will be pursued using expression vectors for insulin, with emphasis on defining regulatory signals and comparing these signals in fibroblasts and insulinoma cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05255-02 LMV

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Hormonal Regulation of Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Michael Kessel Guest Researcher, LMV, NCI

COOPERATING UNITS *(if any)*
Viral Immunogenetics Section, Laboratory of Tumor Virus Genetics, DCCP, NCI

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.9	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have studied the regulatory elements in the mouse mammary tumor virus long terminal repeat (MMTV-LTR) by using a transient expression system. Plasmids were constructed that contained the LTR upstream from the prokaryotic gene chloramphenicol acetyltransferase (CAT). After transfection into mouse cells and growth with or without hormone, the CAT activity in cell extracts was measured as a parameter of gene expression. In fibroblasts (LTK-minus cells) as well as in transformed mammary tumor cells (34i) which express MMTV-RNA, the levels of CAT expression were low. The presence of enhancers on the transfected plasmids however, raised the level of CAT-expression considerably. Hormone inducibility could be observed using plasmids with or without enhancers.

Deletion of sequences from the 5' end of the LTR results in a decrease in CAT activity in cells grown in the presence of hormone and an increase in the CAT activity in cells grown in the absence of hormone. A deletion retaining 179 bp upstream of the cap site is constitutively active. This may suggest that a negative regulatory element is involved in hormone regulation.

After substituting the MMTV promoter region with one derived from Moloney sarcoma virus (MSV), we still observed induction of gene expression by steroid hormones. This suggests the presence of a discrete hormone responsive region which can function independently from the MMTV promoter.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George Khoury	Chief	LMV, NCI
Gordon Hager	Head, Viral Immunogenetics Section	LTVG, NCI
Michael Ostrowski	Senior Staff Fellow	LTVG, NCI

Objectives:

These studies are directed at delineation of the hormone target region on the MMTV-LTR.

Methods Employed:

Construction of chimeric genes; deletion mutagenesis; DNA transfection; protein assays.

Major Findings:

Deletion of LTR sequences in the presence of an exogenous enhancer leads to constitutive expression from the MMTV transcriptional unit. Futhermore, our studies indicate that the promoter region of MMTV is not necessary for induction.

Significance to Biomedical Research and the Program of the Institute:

Steroid hormone regulation of gene expression is an important aspect of gene regulation, which concerns basic biological mechanisms and has important implications.

Proposed Course:

Site directed mutagenesis should allow a detailed analysis of the MMTV hormone inducible regulatory site.

Publications:

Kessel, M. and Khoury, G.: Induction of cloned genes after transfer into eukaryotic cells. In Papas, T. S., Rosenberg, M. and Chirikjian, J. G. (Eds.): Expression of Cloned Genes in Prokaryotic and Eukaryotic Cells. New York, Elsevier/North-Holland, Inc. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05354-01 LMV

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Activated Form of the Human Proto-oncogene, c-Ha-ras

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Rudy Pozzatti Guest Researcher, LMV, NCI

COOPERATING UNITS *(if any)*
Gene Regulation Section, Laboratory of Molecular Biology, DCBD, NCI

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The activated form of the human proto-oncogene c-Ha-ras (EJ or T24) that was cloned from a cell line established from a bladder carcinoma is capable of transforming NIH 3T3 cells in culture. The DNA sequences involved in the control of expression of this gene are being defined. The techniques of S1 mapping and primer extension will determine the 5' end of the processed transcript. To analyze the DNA sequences that constitute the promoter element, sequences 5' to the coding region of the gene will be cloned into a recombinant plasmid vector and assayed for their ability to initiate expression of a foreign gene by RNA and protein production in a transient assay system.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George Khoury	Chief	LMV, NCI
Bruce Howard	Senior Investigator	LMB, NCI

Objectives:

This study is directed at an examination of the DNA sequences that control expression of the human proto-oncogene, c-Ha-ras, and the corresponding oncogene isolated from a bladder carcinoma.

Methods Employed:

Isolation and cloning of specific fragments; construction of recombinant vector molecules; extraction of mRNA S1 nuclease analysis; primer extension; Northern and Southern blotting analysis of RNA and DNA; immunoprecipitation.

Major Findings:

A human tumor cell line (T24), that was established from a bladder carcinoma, has been shown to contain a gene that is capable of morphologically transforming NIH 3T3 mouse cells. Analysis of this gene has shown that it is the human cellular homologue (c-Ha-ras) of the transforming gene present in the Harvey murine sarcoma virus. Comparison of the c-Ha-ras oncogene from the tumor cell line with a normal human c-Ha-ras proto-oncogene indicates that the activation of the proto-oncogene is the result of a single base pair change in the amino acid coding region of the gene. However, the normal c-Ha-ras gene is capable of morphologically transforming NIH 3T3 cells if its level of expression is elevated through the use of a retroviral LTR promoter element. It is therefore important to gain an understanding of the nucleic acid signals that are involved in the control of the expression of the c-Ha-ras gene.

DNA sequences 5' to the coding region of the activated c-Ha-ras gene have been cloned into the recombinant plasmid $pA_{10}cat_{3M}$ at a site immediately 5' to the CAT coding sequences. This plasmid contains the coding sequences of the gene chloramphenical acetyltransferase, but does not contain any portion of a known eukaryotic promoter element. Transformation of this plasmid into monkey CV-1 cells followed by transient assay of CAT gene enzymatic activity has shown that a 1.25 kb fragment of c-Ha-ras DNA is capable of inducing levels of CAT activity 10 fold greater than the control plasmid, $pA_{10}CAT_{3M}$ without insert.

Primer extension and S1 nuclease analysis will pinpoint the transcription initiation site of the c-Ha-ras gene in the T24 cell line. With these results to guide us, the c-Ha-ras promoter element will be characterized by deletion mutagenesis using CAT vectors and the transient assay system.

Significance to Biomedical Research and the Program of the Institute:

The activated form of the human proto-oncogene c-Ha-ras has been isolated from a bladder carcinoma. This DNA is capable of morphologically transforming NIH 3T3 cells and therefore is very likely to be intimately involved in the formation of the original tumor. Information regarding the function of this gene or the control of its expression could be important to an understanding of neoplastic transformation.

Proposed Course:

In addition to the studies directed at characterization of the human c-Ha-ras promoter, we have begun a series of experiments, in collaboration with Dr. Bruce Howard, designed to determine if oncogenes act in consort to bring about cellular transformation.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05355-01 LMV

PERIOD COVERED
October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Immune Surveillance Against Tumor Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)
Gilbert Jay Head, Cell Physiology Section, LMV, NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Cell Physiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Analysis of mouse cDNA clones has led to the identification of a novel MHC class I (H-2)-related gene that encodes a truncated transplantation-like antigen. Unlike the products of the class I gene (designated H-2K, D and L) which are synthesized and displayed on the surface of all cells, the product of the class I-related gene is expressed only in liver cells and is secreted. We propose that this serum protein acts as a tolerogenic form of the transplantation antigen and is responsible for regulation of self-nonself recognition.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Michel Kress	Visiting Fellow	LMV, NCI
Yves Barra	Guest Researcher	LMV, NCI
George Khoury	Chief	LMV, NCI

Objectives:

To study the mechanisms involved in regulating immune surveillance.

Methods Employed:

Recombinant DNA techniques; nucleic acids hybridization; gel electrophoresis; electron microscopy; proteins were analyzed by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

Structurally, the major transplantation antigens, designated H-2K, D and L in mice and HLA-A, -B and -C in humans, are classical cell-surface glycoproteins. The presentation of these antigens on the cell-surface is a functional prerequisite both for their role in inducing allograft rejection, as well as for their involvement in the associative recognition of viral and tumor antigens.

Sequence analysis of mouse H-2 cDNA clones has suggested the existence of a novel class of H-2-related antigens which, unlike the classical membrane-associated molecules, retains only the extracellular portion and is likely to be secreted. The expression of this class of H-2 related mRNA is tissue restricted; it is detectable in liver, but not in brain, kidney, testis, thymus or spleen. In the liver, its accumulation represents about one-fourth of all the H-2-specific transcripts. This class of transcripts is present in mice of different inbred strains, but the level of expression differs markedly among them. A model is presented in which such a soluble form of the H-2 antigen would play the role of a blocking factor in maintaining peripheral inhibition of H-2 recognition. This would assure tolerance of the H-2 molecule as a self antigen while permitting it to act as a guidance molecule for the associative recognition of viral and tumor antigens by cytotoxic T-cells.

Significance to Biomedical Research and the Program of the Institute:

The transplantation antigens have been shown to be restricting elements that permit T-cells to detect foreign antigens in the context of self. In the process of immunosurveillance, the T-cell receptor on the cytotoxic T-lymphocyte must recognize both the foreign antigen and a self transplantation antigen. This dual recognition process, however, may invoke a conceptual paradox. As self molecules, present on all cells in the body, the immune system must be

rendered tolerant to the transplantation antigens. Yet, it is with these same self antigens that the immune system recognizes foreign antigens.

It is tempting to speculate that the putative protein product of this H-2-related gene may function as a "blocking" factor. As a "self" antigen, H-2 should have induced a state of immunological unresponsiveness; but as a "guidance" molecule, H-2 has to be recognized in conjunction with the foreign antigen. It is possible that immunocompetent cells with H-2 reactivity are regulated by some form of suppression in the adult ("active tolerance") instead of the complete deletion of H-2 specific immunoreactive cells during prenatal and/or neonatal life ("passive tolerance"). A molecule with H-2 specificity that is constantly secreted into the circulation may well act as a blocking factor to suppress H-2 recognition. The secretion of such H-2 molecules would then be responsible for maintaining the fine balance between self-nonself recognition of the H-2 antigen present on the cell surface. Any perturbation in the level of expression of these secreted H-2 molecules could serve to tip this delicate balance of immune recognition mediated by membrane-associated H-2 molecules and would have significant physiological implications.

Proposed Course:

Attempts are being made to express this H-2-related antigen in transfected cells and to demonstrate that the secreted H-2 product can specifically block the recognition of virus-infected cells by the cytotoxic T-cell.

Publications:

Cosman, D., Kress, M., Khoury, G. and Jay, G.: Tissue-specific expression of a novel H-2 (class I)-related gene. Proc. Natl. Acad. Sci. USA 79: 4947-4951, 1982.

Kress, M., Cosman, D., Jay, E., Khoury, G. and Jay, G.: Molecular cloning and expression of a gene that encodes a novel transplantation-related antigen. In Pearson, M. L. and Sternberg, N. O. (Eds.): Gene Transfer and Cancer. New York, Raven Press (In Press).

Kress, M., Cosman, D., Khoury, G. and Jay, G.: Secretion of a transplantation-related antigen. Cell (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04986-06 LTVG

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Steroid Hormone Action; MMTV Oncogenesis; Viral Oncogenes and Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

G.L. Hager Head, Viral Immunogenetics Section, LTVG, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Viral Immunogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Deletion analysis of molecular chimeras between the MMTV LTR and the v-ras transformation gene from Ha-MuSV, and between the LTR and the chloramphicol acetyl transferase (CAT) of Tn9, has localized the steroid target site in the MMTV LTR. The hormone regulatory sequences are located between 100 and 200 nucleotides upstream from the cap site. A negative as well as positive effect is suggested for the hormone regulatory element. Specific initiation of transcription at the MMTV cap has been demonstrated with MMTV LTR plasmids introduced into Xenopus Oocyte nuclei. The transformation phenotype of NIH 3T3 cells transformed with MMTV v-ras fusions is under the control of glucocorticoids; hormone dependent phenotype switching parallels the affinity curve for dexamethasone binding to the glucocorticoid receptor. This system is being exploited to monitor the interaction of the p21 transforming protein with potential cellular targets. A fourth gene (pLTR) is located at the 3' terminus of mouse mammary tumor virus (MMTV), within the long terminal redundancy (LTR). Sequence analysis has confirmed the maintenance of this reading frame in exogenous C3H-S MMTV, and in two endogenous proviruses of C3H, Mtv-1 (unit V) and unit II. A pLTR message uniquely encoding the information for this gene is expressed in preneoplastic mammary tissue (hyperplastic outgrowth, HOG) induced in Balb/c animals by the chemical carcinogen DMBA, and in lactating mammary tissue. The structure of this message suggests that it can be expressed as a second subgenomic MMTV transcript; in the Balb/c tissues, however, it is probably expressed from a partial endogenous provirus (unit I).

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

D. Wheeler	Chemist (graduate student)	LTVG, NCI
R. Wolford	Microbiologist	LTVG, NCI
D. Berard	Microbiologist	LTVG, NCI
A. Lichtler	Guest Researcher	LTVG, NCI
S. Simmons	Chemist	LC, NIADDK
P. Miller	Staff Fellow	LC, NIADDK
M. Kessel	Guest Researcher	LMV, NCI

Objectives:

Analysis of hormone regulated transcription of MMTV. Localization of hormone regulatory sequences involved in this regulation.

Determination of mechanism of hormone action in the up-regulated MMTV system. Extension of the investigation of glucocorticoid regulation into systems where the expression is down-regulated.

Identification of the cellular locus encoding the glucocorticoid receptor protein, and molecular characterization of this locus.

Functional expression of the glucocorticoid receptor in cells defficient for receptor protein by gene transfer techniques.

Investigation of mechanisms involved in other types of hormone regulation, particularly androgen action.

Application of tools developed for the study of hormone action to the study of other transcriptional regulatory systems important in cell growth, with initial emphasis on cell cycle regulation of histone transcription.

Structural analysis of MMTV genome, utilizing full-length molecular clone of endogenous Unit V Mtv-1 proviral DNA and clones of other MMTV strains.

Function of newly discovered pLTR gene in MMTV. Potential role in MMTV induction of neoplasia. Mechanism of mammary carcinogensis.

Investigation of mechanism of cellular transformation mediated by the ras family of oncogenes. Deveoplment of a conditional mutation system for the complementation analysis of oncogene action utilizing the regulated MMTV promoter.

Methods Employed:

Molecular chimeras between the MMTV LTR and the v-ras gene of HaMuSV will be used in a hormone dependent transfection assay to probe the regulatory

regions involved in hormone induction of MMTV expression. Similar fusions between the LTR and the CAT gene from the bacterial Tn9 transposon will be tested in a transient expression assay. Deletion analyis of molecular chimeras will be performed to localize these regions.

Utilizing the S1 nuclease or mung bean nuclease mapping techniques, probes available from molecular clones of MMTV will be utilized to analyze steroid dependent MMTV regulation.

High resolution mutagenesis of hormone response regulatory sequences will be performed using site-directed chemical mutagenesis at gaps in regions of interest created by heteroduplexing full length and deleted LTR fragments from single-stranded M13 clones.

Initiation of transcription at the regulated MMTV promoter will be carried out in Xenopus Oocytes. An analysis of the role of chromatin organization in hormone action will be carried out by comparing naked DNA templates and minichromosomes containing MMTV promoters as transcription templates in this system.

Transcriptional promoters responsive to other hormone effects (androgens) and other regulatory elements (histone - cell cycle) will be engineered into the v-ras transformation system to test for transfer of regulation.

Insertional mutagenesis with murine retroviruses, and positive-selection vectors derived from retroviruses, will be utilized to establish and tag mutants in the glucocorticoid receptor locus.

Cell lines transformed with MMTV v-ras fusions will be established in serum-free media to facilitate the analysis of steroid regulation. Such lines will be subjected to mutagenesis to generate variants both in transformation response and in the hormone response.

Expression of MMTV gene expression, particularly for the 3' terminal pLTR gene recently discovered in this lab, will be analyzed in MMTV-infected cells, and in MMTV-induced and chemically-induced mammary tumors. Methods will included northern analysis of RNA's present, information content analysis of RNA's with subgenomic probes, and structural characterization by S1 analysis, cDNA cloning, and DNA sequencing.

The biological activity of full-length cloned Mtv-1 (Unit V) proviral DNA will be determined by transfection of DNA into appropriate cell lines and examination of viral expression.

Major Findings:

Regulatory signals involved in the control of MMTV transcription by glucocorticoids have been examined in detail in two independent expression systems. In the first, fusions between the v-ras gene of HaMuSV, the MMTV LTR, and an enhancer element from the HaMuSV LTR have allowed us to monitor steroid

inducible transcription from the MMTV LTR by a rapid transfection assay. Efficient transfection of NIH 3T3 cells to the transformed phenotype occurs only when glucocorticoids are present in the medium. In the second assay system, the chloramphenicol acetyl transferase gene (CAT) from the bacterial Tn9 transposon has been placed under control of the MMTV promoter; in this system, the elaboration of CAT enzyme activity was shown to be inducible by glucocorticoids during transient expression after acute DNA-mediated transfection. Using these assays, deletion analysis by molecular techniques has localized the site conferring hormone sensitivity on the MMTV LTR to within 100 nucleotides of the MMTV cap site.

In both of these assay systems, an increase in the uninduced, constitutive expression from the MMTV LTR was observed after deletion of the hormone responsive sequences, suggesting that the mechanism of hormone action may be more complex than a simple induction effect. In this regard, it is noted that glucocorticoids have been shown to decrease the expression of certain cellular genes.

The v-ras MMTV fusion system has been transfered into the M13 bacteriophage, permitting high-resolution site-directed mutagenesis in gapped molecules created by heteroduplex formation between single-stranded molecules containing complete LTR regions and duplex molecules deleted for selected areas of the regulatory sequence. This system now permits the analysis of sequences involved in regulation at the single nucleotide level.

A competition assay has been developed in which the interaction between glucocorticoid receptor protein and MMTV DNA containing the hormone target region can be detected. LTR sequences implicated in the hormone response by the gene transfer experiments described above have been shown to compete for receptor binding in receptor containing cell-free extracts more efficiently than random DNA sequences. This assay serves as one measure of the interaction of the glucocorticoid receptor and its target. The single strand mutagenesis system described in the previous paragraph has been constructed so that mutants identified by the biological response as containing lesions in critical sequences can be amplified and tested easily in this competition assay.

We have previously been unable to demonstrate specific initiation at the MMTV cap in cell-free extracts. An experimental system has now been established, however, for examining initiation of transcription at the MMTV promoter with defined templates, either naked DNA or more complex minichromosomes. Microinjection of MMTV LTR-containing DNA into the nuclei of Xenopus Oocytes leads to correct initiation of transcription at the normal MMTV cap. This represents an important step in our attempt to identify the critical elements involved in hormone regulation, and to reconstruct this system from its component parts.

Cell lines transformed with the MMTV v-ras fusions had previously been established in serum-free media. Since this medium is completely defined, and in particular is free of glucocorticoids, interaction of steriod receptors

and the MMTV promoter can now be studied at the cellular level in a totally defined culture environment. The concentrations of dexamethasone required to induced the switch from a normal to a transformed phenotype exactly parallels the binding of dexamethasone by its cellular receptor, confirming that induction of the cellular phenotype switch occurs via glucocorticoid receptor mediated regulation of p21 gene expression. Experiments are underway to derive mutants in which this phenotypic switch no longer occurs.

A fourth gene at the 3' terminus of the MMTV genome was previously discovered by DNA sequence analysis. We have shown that the reading frame for this gene is maintained in each of three variants of MMTV; the C3H-S strain, the endogenous Mtv-1 locus, and endogenous unit II. Since this gene (pLTR) is completely encoded within the long terminal repeat of proviral DNA, several possible modes exist for its expression. We have characterized a message that contains only the coding information for this gene from preneoplastic stages of murine mammary cancer, and in lactating mammary tissue. The structure of the transcript indicates that it is expressed as a third spliced MMTV transcript, with the normal viral leader transposed to a position 72 nucleotides from the left end of the 3' LTR. In order to identify the protein encoded by this reading frame, synthetic peptides have been prepared based on the predicted sequence. Antibodies have been raised against these antigens, and experiments are underway to identify the putative gene product utilizing these reagents.

Significance to Biomedical Research and the Program of the Institute:

A major goal of the experimental program directed by the P.I. is to decipher the mechanisms involved in steroid hormone regulation as a model for the general problem of hormone action. Control of cell proliferation is a central issue in neoplastic transformation; hormones are directly implicated in many aspects of growth control. Steroid hormones are directly implicated in the biology of certain human neoplasms, such as breast cancer. A thorough knowledge of the mechanisms of hormone action is therefore necessary to our eventual understanding and control of the neoplastic process.

Proposed Course:

Site-directed molecular mutagenesis will be applied with the M13 MMTV LTR v-ras fusion system to precisely characterize the hormone regulatory sequences at the single nucleotide level.

High-resolution mutants in the hormone response region will be transferred into amplification vectors for study in cell-free binding to the glucocorticoid receptor.

High-resolution mutants in the hormone response region will also be examined in the Xenopus Oocyte transcription system to determine their effect on the basic promoter structure of the MMTV LTR. Efforts will also be made to demonstrate regulation in the Oocyte system by co-injection of extracts enriched in glucocorticoid receptor.

The role of chromatin structure in the hormone response will be investigated in the Oocyte system by comparing the transcription initiation capacity of naked DNA templates and minichromosomes isolated from cells harboring bovine papilloma virus MMTV LTR episomal chimeras.

Attempts will be continued to demonstrate correct initiation in cell free transcription extracts, and to demonstrate regulation with receptor-enriched extracts and DNA and minichromosomal templates.

The transfer of androgen regulation in the v-ras transformation system will be attempted utilizing regulatory sequences from genomic clones of seminal vesicle protein genes. Successful transfer of regulation will permit the experimental approaches pioneered in the MMTV v-ras system to be applied to a new hormone system. This will then allow the identification of the regulatory elements, and a comparison to be made with the glucocorticoid system to examine general features of steroid hormone action.

A further extrapolation of the tools developed for the study of hormone action in the MMTV system will be carrried into the study of cell cycle regulation of histone expression. Histone gene BPV chimeras have been prepared and are being characterized as a prelude to the investigation of of cell cycle regulation at a defined episomal chromatin element.

A program has been initiated to molecularly clone the structural gene for the glucocorticoid receptor. The approach will be to utilize retroviruses and/or retroviral based vectors as insertional mutagens to inactivate the receptor locus in cells hemizygous for the receptor allele. This is a well characterized system, and will permit a rigorous test of the potential of retrovirus vectors as useful insertional mutagens.

Cellular variants of the MMTV LTR v-ras transformed lines resistant to phenotype switching will be derived. Two classes of variants are expected, one in which cellular targets of the transformation protein are altered, and one in which elements of the hormone response pathway are modified. Both classes will be extensively characterized to identify the respective cellular targets.

The potential function of the MMTV pLTR gene product in the life cycle of the virus, or in its unique oncogenic activity will be examined. Utilizing the complete molecular clone of MMTV recently characterized in this lab, mutations will be engineered in the pLTR reading frame by molecular techniques, and the replication and transformation potential of resultant viruses tested. The pLTR gene will also be placed in an MuLV based retrovirus vector; morphological and functional effects of pLTR expression in cultured cells and the whole mouse will be tested with this system.

Publications:

Donehower, L. A., Fleurdelys, B. and Hager, G. L.: Further evidence for the protein coding potential of the mouse mammary tumor virus long terminal repeat: nucleotide sequence of an endogenous proviral LTR. J. Virol. 45: 941-949. 1983.

Hager, G. L.: Expression of a viral oncogene under control of the mouse mammary tumor virus promoter: A new system for the study of glucocorticoid regulation. Prog. Nucl. Acid Res. Mol. Biol. 28: (In Press) 1983.

Hager, G. L., Huang, A. L., Bassin, R. H. and Ostrowski, M. C.: Analysis of glucocorticoid regulation by linkage of the mouse mammary tumor virus promoter to a viral oncogene. In Gluzman, Y. (Eds.): Eukaryotic Viral Vectors. Cold Spring Harbor N.Y., Cold Spring Harbor Laboratory, 1982, pp. 165-169.

Tsichlis, P. T., Donehower, L. D., Hager, G. L., Malavarca, R., Astrin, S. and Skalka, A. M.: Sequence comparison in the crossover region of an oncogenic avian retrovirus recombinant and its non-oncogenic parent: Genetic regions that control growth rate and oncogenic potential. J. Cell. Mol. Biol. 2: 1331-1338. 1982.

Wheeler, D. A., Butel, J. S., Medina, D., Cardiff R. D. and Hager, G. L.: Transcription of mouse mammary tumor virus: Identification of a candidate mRNA for the pLTR gene product. J. Virol. 46: 42-49. 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05118-04 LTVG

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of Transcriptional Regulation of Murine Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

M.C. Ostrowski Staff Fellow, LTVG, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Viral Immunogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	2.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Mouse Mammary Tumor Virus (MMTV) long terminal repeat (LTR) contains the target site for steroid hormone action associated with this virus. Ultimately, in order to directly address the molecular mechanisms by which hormone-receptor complex, RNA polymerase II and other nuclear protein components interact with the MMTV LTR to produce the observed biological phenomenon of elevated rates of transcription, we feel that reconstitution of hormone regulated transcription in vitro will be required. In an attempt to develop a system capable of faithfully mimicing the in vivo situation, we have introduced MMTV LTR into eukaryotic cells as an episome, by using the Bovine Papilloma Virus (BPV) as a vector. The MMTV LTR, present at 200 copies per cell, is still responsive to steroid-specific induction of transcription in this episomal form. The chromatin structure of the MMTV LTR-containing minichromosomes has been examined by nuclease digestion and the minichromosomes can be purified from the bulk of chromosomal information based primarily on their small size. These minichromosomes should serve as useful templates for in vitro transcription studies.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

H. Richard-Foy	Guest Researcher	LTVG, NCI
G.L. Hager	Head, Viral Immunogenetics Section	LTVG, NCI

Objectives:

Levels of MMTV-initiated mRNA contained in cell lines harboring 200 copies per cell of BPV-LTR hybrids will be measured in either the presence or absence of glucocorticoids. This will be accomplished both by S1-mapping and by in vitro transcription run-off experiments. These run-off experiments will be performed on subnuclear extracts enriched for the minichromosomes.

Nuclease sensitivity of the minichromosomes in isolated nuclei will be determined using micrococcal nuclease and pancreatic DNase I.

Episomal chimeric DNA will be isolated from these cells so that nucleoprotein particles are obtained. These particles will be used in nuclease digestion studies as above to ensure that purification does not damage epigenetic structure.

The enriched minichromosomes will serve as templates for in vitro transcription experiments. Various nuclear extracts and purified glucocorticoid receptors will also be used in these transcription experiments. The cloned, naked DNA is used as a control.

Mutant MMTV LTR's will be made and combined in chimeras such as those described above. Comparison of the transcriptional ability of these mutants as compared to the parental LTR should yield additional insights into the mechanism of steroid hormone action.

Methods Employed:

Isolation of steady-state levels of RNA and S1-mapping of this RNA using end-labeled probes will be accomplished using published procedures.

Minichromosomes used for run-off transcription are prepared from nuclei of cells containing LTR episomes by ammoninum sulfate extraction. This procedure relies on the small size of these episomes as compared to chromosomal DNA. These particles are then incubated with radioactive ribonucleotides. The RNA produced is analyzed by hybridization to single-strand probes representing the sequences present in the chimeric BPV-LTR episomes.

Minichromosomes to be used for nuclease digestion and in vitro transcription templates will be purified by low-salt procedures that are less likely to damage chromatin structure. These procedures will depend on the small size of the episomes, but they must also be able to dissociate the higher orders of structure present in the nuclear matrix including so-called nuclear scaf-

folding. Chelaters of specific ions may be useful in this process.

Nuclease digestion of whole nuclei and of purified minichromosomes will be accomplished according to previously published procedures, utilizing indirect end labeling techniques.

Various whole cell and nuclear extracts which contain factors required for transcription will be made according to published techniques.

Mutants in MMTV LTR sequences will be introduced using standard recombinant techniques and procedures previously reported. These mutants will then be introduced into murine cells using BPV as described previously.

Major Findings:

We have identified murine cells that are stably transformed by BPV-MMTV LTR chimeras following calcium-phosphate transfection. These chimeras are found as episomes in the cells and not integrated into host information. The physical map of these episomes is not significantly different from the input recombinant DNA. The copy number of episomes can be as high as 200 copies per cell. More recently, we have seen that single-cell cloning of these lines after interferon treatment can increase the copy number of BPV-LTR episomes 5-10 fold in some of the clones obtained. We have also found by S1 mapping of total cellular RNA that steady-state levels of MMTV-initiated mRNA are increased in these cells when they are grown in the prescence of glucocorticoid hormones. We have demonstrated that this increase in LTR-initiated RNA levels is dependent on transcriptional induction of the LTR promoter by transcription run-off experiments performed in vitro. These run-off studies, which measure the number of RNA polymerase II molecules already in place in vivo, were accomplished using subnuclear extracts 400-fold enriched for BPV-LTR mini-chromosomes. These experiments constitute the first evidence conclusively demonstrating that MMTV LTR contains all the information necessary for the steroid response independent of any contribution from host chromasomal information, and is also the first demonstration of any hormone regulated promoter retaining its activity in a non-integrated, extrachromasomal form. The amount of MMTV-initiated RNA found, as determined by either S1 or run-off experiments, varies significantly in between independently obtained cell lines that have comparable copy numbers of episomes. Because of the episomal nature of the LTR in these cells, which precisely defines the genetic location of these sequences, we believe this variability in expression may reflect a major role for chromatin structure in the hormone response mechanism. More recently, we have directly examined the chromatin structure of the minichromosomes by nuclease digestion studies. Two enzymes, micrococcal nuclease and pancreatic DNase I, have been used in these studies. Limited micrococcal treatment of nuclei from transformed cells indicate that BPV-LTR episomes are particularly sensitive to digestion with this enzyme. Additionally, probing of these digests by indirect end-labeling reveals a specific ordering or "phasing" of the nucleosomes associated with LTR sequences, but not of sequences located immediately upstream or downstream. The phasing of nucleosomes on LTR is found whether glucocorticoids are present or absent in the cell culture medium. In contrast, limited DNase I digestion of the same

nuclei indicate a DNase I hypersensitive site exists within the LTR only when cells are treated with hormone. This hypersensitive region is located within those LTR sequences that our own laboratory, as well as others, have identified to contain the target site for hormone-receptor binding. The hypersensitive site thus may reveal some change in chromatin structure associated with interaction by hormone-receptor complex. Interestingly, both the hypersensitive site and the hormone-receptor binding site are contained within the core region of a phased nucleosome as detected by micrococcol nuclease, and not in the linker region. Studies are currently in progress to relate these features of chromatin structure to varying RNA expression levels indicated above. The suitability of purified minichromosome preparations as in vitro transcription templates is also currently under investigation.

Significance to Biomedical Research and the Program of the Institute:

Genes homologous to the onc genes of retroviruses are present in normal vertebrate cells; in some cases these normal cellular genes can be activated to produce transformation. The mechanisms by which the expression of these genes are regulated becomes of obvious importance to the understanding and ultimate control of oncogenic disease. Our efforts are aimed at the ultimate description in molecular terms of mammalian regulatory mechanisms.

Proposed Course:

Minchromosomes will be purified from cells based on their small size compared to chromosomal information after the disassembly of the normal nuclear matrix.

The structure of purified minichromosomes will be examined by nuclease digestion procedures and compared to the structure found in whole nuclei.

Binding of glucocorticoid receptor present in crude cellular extracts to purified minichromosomes will be tested.

Whole cell and nuclear extracts that contain RNA polymerase II and various nonhistone nuclear proteins will be prepared from rodent cells.

Attempts will be made to reconstitute faithful hormone-regulated transcription in vitro by recombining the nucleoprotein minichromosomes, prepared from cells grown both in the presence and absence of glucocorticoids, with appropriate extracts. S1 mapping will be employed to assay for successful initiation events.

If these attempts are not completely successful, partially purified hormone receptor will be prepared and added to the in vitro extracts.

MMTV LTR mutants will be constructed and tested in the in vitro system.

When attempts at in vitro transcription are successful classic biochemical purification of components from the crude extracts that are required for in vitro reconstitution will be undertaken.

Publications:

Ostrowski, M. C. and Hager, G. L.: Structural and functional analysis of the glucocorticoid-regulated MMTV transcriptional promoter. In Pearson, M. and Sternberg, N. (Eds.): Workshop on Gene Transfer and Cancer. New York, Raven Press, (In Press) 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05181-03 LTVG

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Biology of Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

W. David Hankins Cancer Expert, LTVG, NCI

COOPERATING UNITS *(if any)*

National Heart, Blood and Lung Institute; Armed Forces Radiation Research Inst.

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This approach to the cell biology of carcinogenesis is multi-faceted and attempts to integrate information concerning tumor induction by chemicals, viruses, or radiation. Four related studies on the induction of cancer by carcinogens and the relation of cancer to hormonal control of growth are described.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

Joseph Kaminchik	Visiting Fellow	LTVG, NCI
Christine Eastment	Postdoctoral Fellow	LTVG, NCI

Objectives:

Our overall objective is to answer fundamental questions in normal and cancer cell biology and to explore any possible application of our findings to the erradication of human disease. The specific research goals are:

Study 1. To assess qualitative and quantitative effects of environmental carcinogens (various chemicals and ionizing radiation) on hemopoietic stem cells and their progeny. In contrast to other experimental systems, these studies allow one to approach "tumor induction" and "tumor promotion" at the cellular level rather than at the tissue or organismal level.

Study 2. To apply somatic cell hybridization, chromosomal transfer, and molecular cloning technology to determine the physiologic niche of a putative "new" regulatory protein which we discovered during the past year in this laboratory. Current results will be described and this project will be terminated.

Study 3. To continue our studies toward understanding the molecular mechanism by which retroviruses cause increased hormone sensitivity and increased cell proliferation. These studies will employ our recently developed in vitro hemopoietic transformation system which permits the study of a purified glycoprotein hormone, erythropoietin, with homogeneous populations of erythroid precursors.

Study 4. To test the generality of the "SISTER" hypothesis in human neoplasms, and chemically-induced, radiation-induced, and MMTV-induced tumors in mouse. These studies will include in vitro hormone sensitivity testing, and analysis of the regulatory and differentiation factors in primary cultures.

STUDY I: EFFECTS OF ENVIRONMENTAL CARCINOGENS ON HEMOPOIETIC STEM CELLS.

Methods Employed:

(1) Suspension cultures of hemopoietic cells
(2) Microinjection of cloned genes into fertilized eggs
(3) In vivo and in vitro hemopoietic stem cell assays.
(4) Time lapse photography

Major Findings:

We have initiated a collaborative study with Dr. Makio Ogawa (Univ. S. Carolina) to expose purified multipotential hemopoietic stem cell to oncogenic chemicals and viruses. This provides an opportunity to better define specific effects of known specific carcinogens on specific hemopoietic cells. It is now possible to grow hemopoietic stem cells (HSC) for several months in vitro. However, many details for maximal growth of HSC remain to be worked out. Therefore, phase I of this project has been to identify the HSC, its progeny and to optimize growth conditions so that large numbers of HSC are available.

Dr. Hagan (AFRRI), has continued to modify and improve his stem cell kinetic assay, an in vivo stem cell assay which allows detection of minor qualitative alterations in stem populations. This assay will continue to aid in this project as we attempt to assess the initiation of leukemogenesis by treating HSC with chemicals and radiation.

By microinjection procedures, Dr. Humphries has developed "new" strains of mice which contain new gene markers that can be identified by molecular hybridization procedures. The HSC from these mice are very useful for leukemogenic studies since the origin (host or recipient) of differentiated progeny can be unequivocally established by assessing the molecular genotype of the progeny.

Significance to Biomedical Research and the Program of the Institute:

Cellular hematopoiesis permits in vivo and in vitro study of readily identifiable self-renewing and differentiating cell populations. Since blood cells and their progenitors are amenable to various types of investigations, hematopoiesis has long been at the scientific forefront. Consequently, a high percentage of fundamental concepts of biology (cell regulation, protein synthesis, molecular evolution and molecular disease) have been derived in this model system. It is not surprising, therefore, that hemotopoiesis has been successfully employed for a number of years to elucidate the mechanisms by which viruses bring about cellular transformation and altered growth. It is surprising, however, that although certain chemicals and radiation clearly induce leukemia and other hemopoietic tumors there has been little exploration of the direct effects of these carcinogens on specific hemopoietic cell types. Our recent initiation of the study described here represents an attempt to fill a research void, and establish an integrated program to study the effects of different classes of carcinogens in an effort to develop and test unifying concepts of carcinogenesis.

Proposed Course:

STUDY 1:

First, we will classify hemopoietic stem cells by morphology, growth potential and progeny. In vitro studies will include: (a) definition of conditions for growth of stem cells to allow long-term culture of stem cells, freeze-downs of stem cells for later use, and assay for human stem cells in vitro; (b) isolation of hormones which regulate stem cell growth; and (c) transfection (or infection

with genes in retroviral vectors) of stem cells followed by implantation in vivo as an approach to cures of thalassemias, sickle cell disease, or other hemoglobinopathies. The in vivo studies with mice carrying the human beta globin gene will include: (a). positive identification of progeny by molecular markers and (b). identification of a possible common stem cell for gut, skin and blood.

Also, we will test the effects of carcinogens (radiation and chemicals) on stem cells. These studies will include: in vivo treatment with irradiation and chemicals followed by assay in vitro; and in vitro treatment with irradiation and chemicals followed by assay in vivo.

Finally, we will examine the relative roles of cell-cell interactions, autocrinology and endocrinology during hematopoiesis. Specifically, we will: (a) determine the role of macrophages in erythropoiesis; (b) prepare condition media from different subsets of macrophages and attempt to induce differentiation of leukemia cells by co-cultivation with macrophages; (c) measure the production of hemopoietic regulatory factors by clonally purified cells and cells selected at different stages of differentiation; and (d) assess the role of interferon in hemopoietic regulation.

STUDY 2: CHARACTERIZATION OF A "NEW" PUTATIVE REGULATORY PROTEIN.

Methods Employed:

(1) Molecular hybridization (Southern and Northern blotting procedures)
(2) Immune precipitation with monoclonal antibodies
(3) cDNA cloning techniques
(4) Heteroduplexing and electromicroscopic analysis

Major Findings:

Elsbeth Lee joined our group in January, 1982. To become familiar with molecular biology techniques, she initiated a project with Dr. Kaminchik to look for a cellular counterpart to the SFFV "transforming" protein, gp52. The rationale for this approach was that the growth promotion of hemopoietic cells by SFFV may be due to the presence of a cellular protein in the virus (as a passenger) or to a virus protein which mimics the function of a putative cellular protein with growth regulatory properties.

An SFFV-specific DNA probe was hybridized to RNA from uninfected fibroblasts of DBA and NIH/Swiss mice. An RNA species, with an approximate size of 26S, was detected by this hybridization analysis. The 26S RNA was detected only in DBA cells and not in NIH/SWISS fibroblasts. Previous DNA analysis by Dr. Lowy's group indicated few, if any, differences between the SFFV-specific DNA sequences in these two strains of mice.

Dr. Lee has characterized this RNA by Poly A selection and hybridization to LTR probes from three classes of viruses represented by Moloney, MMTV, and Xenotropic (endogenous) virus. On the basis of her results, she has concluded that the RNA probably represents the messenger encoding the gp70 related to xenotropic viruses that have been detected in various tissues, serum, and

epididymal fluid in uninfected mice.

Significance to Biomedical Research and the Program of the Institute:

Endogenous gp70-like molecules have been implicated in a variety of disorders including autoimmune disease, leukemia, and graft versus host disease. In addition, a physiological role for these proteins has also been proposed (perhaps to interact with hormones such as erythropoietin). Therefore, Dr. Lee's identification of the putative message(s) for these regulatory proteins should contribute to the following research areas:

(A) The mechanism of leukemic transformation and the relation of transforming to normal cellular genes.
(B) The control of hemopoietic cell growth and differentiation.
(C) The identification of receptors for erythropoietin or other glycoprotein hormones.
(D) The mechanism of action of glycoprotein hormones.

Proposed Course:

Dr. Lee and the PI submitted a research proposal requesting postdoctoral salary support from the NIH extramural funding program. This proposal was reviewed by scientists outside the NIH and was recommended for funding. She had been promised back-up support by Dr. Scolnick. Unfortunately, after Dr. Scolnick left the NIH, temporary (during the review) support for Dr. Lee could not be guaranteed and she had to accept another position two months before learning that her grant application was sucessful. Therefore, this project has now been terminated.

STUDY 3: MECHANISM BY WHICH RETROVIRUSES BRING ABOUT INCREASED HORMONE SENSITIVITY

Methods Employed:

(1) XC plaque assay for MuLV
(2) Virus-induced erythroid burst transformation assay
(3) Fibroblast transformation assay
(4) Aggregate culture system
(5) Molecular cloning techniques
(6) Immune precipitation

Major Findings:

We have continued to investigate the biological effects of a number of RNA tumor viruses on a variety of hemopoietic and other cell types. As a result of these studies we have:

1. Continued to develop conditions for routine establishment of hemopoietic cell tumors. We have now several lines from different leukemic animals or multiple lines from the same animal. We have characterized these and other lines (developed elsewhere) according to their hormone requirements or hormone production. We have discovered that some of

these leukemic lines produce substantial quantities of hemopoietic growth factors. These factors are now being purified and may soon be of clinical value in treatment of hemopoietic disorders.

2. Three distinct types of transformation of erythroid precursors by viruses carrying different oncogenic sequences. The transformed cells differ in regard to their origin (i.e. target cells), their proliferative potential, and hormone sensitivity.

3. By constructing the appropriate RNA tumor virus vectors, we have demonstrated that cellular information can be transferred into and expressed in hemopoietic precursor cells. Thus, a cellular onc gene, carried by a virus vector, transformed two types of hemopoietic cells in vitro. In addition to providing a new system for studying the function of putative onc genes, this observation may serve as a model for transferring therapeutic genes into hemopoietic stem cells which can, in turn, be used to reconstitute the hemopoietic system in vivo. This will represent a significant advance toward realizing "gene therapy" for genetic disorders.

4. We have further characterized human skin carcinoma cells (A431) that were transformed in vitro with Harvey and Kirsten sarcoma viruses. These viruses caused a profound morphological transformation of human skin carcinoma cells (A431). This transformation occured without a dramatic reduction in the binding of the epidermal growth factor (EGF) to these cells. Further, the transformed cells remained sensitive to the biological actions of EGF as evidenced by an EGF-dependent increase in tyrosine phosphorylation of the EGF-receptor as well as the EGF-dependent "ruffling" of these cells.

Significance to Biomedical Research and the Program of the Institute:

Induction of cancer by RNA tumor viruses represent the only known examples where a product encoded by a specific (already identified) nucleotide sequence (i.e. an "onc" gene) directly produces leukemia or solid tumors. Since most, if not all, of these cancer genes have counterparts in normal cells, an understanding of their actions at the molecular and cellular levels will contribute equally to an ultimate understanding of the fundamentals of normal as well as cancerous cell growth.

Proposed Course:

First, we will continue to develop more efficient methods for gene transfer into hemopoietic and other cell types.

Second, we will continue to molecularly map the phenotypic determinants of Anemia- or polycythemia inducing variants of Friend virus. This will be done by making (a). recombinants between Anemia- and Polycythemia-inducing strains; (b) recombinants between A or P with, MCF (Friend, AKR, Moloney); (c) deletion mutants or glycosylation mutants of MCF (SFFV-like disease?); (d) a correlation of biological activity with number of glycosylation sites; (e) a correlation with

membrane localization; (f) site-specific mutants, (g) correlation with level of SFFV expression.

Third, we will study the interaction of erythropoietin with virus transformed cells. These studies will include (a). binding studies to determine the number of receptors, the hormone internalization and degradation, and the phosphorylation of receptors; (b) use monoclonal antiserum to gp52, (p21) and gp70 are available and monoclonal antibodies to EPO which are now available to define the relative location of these proteins, or their receptors within the cell or on it's surface; and (c) we will attempt to relate EPO-sensitivity, EPO-binding, gp52 levels and gp52 localization to specific stages of erythroid as defined by erythroid markers (spectrin, glycophorin, and hemoglobin) and cellular morphology.

STUDY 4: TESTING THE GENERALITY OF THE "SISTER" HYPOTHESIS IN HUMAN NEOPLASMS AND CHEMICALLY-, MMTV-, AND RADIATION-INDUCED MOUSE TUMORS: A HORMONAL APPROACH TO CANCER.

Methods Employed:

Several tissue culture systems will be employed in this study.
These include: "aggregate" cultures
Dexter long-term marrow cultures
macroburst cultures (mixed colony assays)
"cannonball" assays

Major Findings:

The major finding in this project was that transfusion of mature erythrocytes into mice which are near death with erythroleukemia led to a rapid and profound remission of the disease, which has not relaped in some cases for at least four months. While other interpretations are possible, our working hypothesis is that the erythroleukemic cells are still dependent (perhaps hypersensitive) on the hormone, erythropoietin and that this hormone is being reduced by the transfusion of erythrocytes (i.e. physiologic feedback inhibition). We are therefore pursuing this model as an approach to anti-hormone therapy of leukemia and other tumors.

Before coming to NIH, we developed a system for the induction of leukemic transformation in vitro by retroviruses and have continued to examine the early events of transformation, target cell and endocrine physiology. The results have dramatically altered my approach to cancer research and has led me to seriously question the general applicability of certain "truisms" i.e., that tumor cells are blocked in differentiation, hormone-independent, and immortal to all tumor cells.

Many of the currently accepted characteristics of tumor cells have been deduced from studies of cell lines which occasionally (but not always) grow out of tumor masses placed into culture. While these studies provided important information relating to the properties of cells at specific stages of differentiation, I

believe that extrapolation of these properties to tumor cells in general may be misleading and counter-productive. Our studies of primary cultures of leukemic cells, soon after transformation by various RNA tumor viruses, have demonstrated that these tumor cells continue to terminally differentiate and are hypersensitive to physiologic levels of their natural regulatory hormones.

Our current thinking is reflected in our working hypothesis (the SISTER hypothesis) which suggest that oncogenic transformation may result from a selective increase in sensitivity to external regulators. Therefore, rather than being hormone-independent, the tumor cells would actually be hypersensitive to specific hormones. It was further postulated that the positive growth stimulation does not block the differentiative potential of transformed cells and therefore, the phenotype of the "tumor" reflects properties of counterpart normal cells. Normal cells, of course, do not share the growth advantage enjoyed by tumor cells.

Several practical predictions were apparent from this hypothesis. For example, tumor cells may serve as producers of regulators or as hypersensitive substrates for identification of new physiologic regulators. Futhermore, implications of this hypothesis for cancer treatment are even more significant. Thus, a better understanding of the hormonal requirements and differentiation of tumor cells will undoubtedly lead to more effacious use of hormone (or anti-hormone) therapy either as the primary treatment or as an adjunct to conventional therapy for the patient with cancer.

Significance to Biomedical Research and the Program of the Institute:

These findings led to the development of new concepts in carcinogenesis which have important implications for both cancer research and therapy. These ideas are embodied in the "SISTER" hypothesis and its implications which are stated below.

"SISTER HYPOTHESIS"

It is proposed that the transforming effects of carcinogenic agents (viruses, chemicals or radiation) may be mediated through a Selective Increase in Sensitivity To External Regulators (SISTER) which does not alter the transformed cell's ability to differentiate. The basic tenets:

1. Transforming effects are positive: stimulating growth rather than blocking differentiation.

2. Positive stimulation results from an increase in sensitivity of a cell for a naturally occurring regulator which has a physiologic role (proliferative and/or differentiative) at a specific stage in a cellular differentiation pathway.

3. Transformation is non-instructive. Therefore, the nature of the transformed cell phenotype reflects the intrinsic characteristics of counterpart normal cells at the particular developmental stage at which the transformation occurred.

The implications of the "SISTER" hypothesis for research are: (1). Tumor cells may provide sensitive targets for and allow discovery of new regulators of growth and differentiation. (2). Transformed cells provide homogeneous populations for differentiation studies. (3). Transformed cells may produce (and respond to) their own regulators and therefore provide an excellent source for the characterization and purification of these molecules.

The implications for cancer therapy are: (1). The hypothesis provides a possible explanation for heterogeneity and "evolution" of tumor populations (e.g. CML and erythroleukemias). (2). In vitro hormone sensitivity testing of biopsy samples may be useful in diagnosis or staging of neoplasms. (3). Study of hormone sensitivity may provide rationale for improved hormone-related therapy, (e.g. monoclonal anti-hormone antibodies).

Proposed Course:

As I continue to examine data from other experimental carcinogenesis systems (chemically-induced or spontaneous tumors in man and rodents) I am increasingly excited that these concepts may, at least in part, have general applicability. The following is an outline of the projects which will test this idea.

First, we will examine cultures of tumor tissues: chronic myelogenous leukemia (erythroleukemias, mammary carcinoma, prostate carcinoma, and skin carcinomas [melanomas]). The basic strategy in this project is to study the physiology of tumor cell populations in primary cultures rather than in established cell lines. We believe that some of the information gained from study of cell lines may be misleading since extensive selection has occurred while the line was being established. We will attempt to answer the following questions for several spontaneous tumors as well as those induced by a variety of agents: (a) Are the tumor cells hypersensitive to known regulators? (b) Can they be used as hypersensitive targets to identify previously undetected regulators? (c) Do tumor cells produce known regulators? (d) Longitudinal studies: Do different cell types grow out at different stages of a disease? (e) Do the cells differentiate in culture? If so, can differentiation be blocked by addition of plasma from same patient? (f) Does growth in vitro allow selection and expansion (and purification) of tumor cell population to permit biochemical studies?: (g) Can hormone sensitivity testing be used for diagnosis, staging of tumor, monitor tumor progression, or implications for therapy?

Second, we will continue to develop better methodology for establishing cell lines from tumor or normal tissues. Note: Although the cell lines derived after many passages may not be representative of the tumor, such lines nevertheless offer several practical advantages. We believe that isolation of several lines (clones) from individual patients may permit a better test for clonality and/or heterogeneity of various tumors.

Third, we will develop cancer therapeutic methods based on hormonal responsiveness of particular tumors.

Publications:

Hankins, W. D.: Increased hormone sensitivity after in vitro infection of hemopoietic precursors with Friend virus complexes. JNCI 70: 725-734, 1983.

Hankins, W. D., Cohen, S., Scolnick, E. M. and Furth, M.: Human epidermoid cells (A431) retain epidermal growth factor (EGF) receptors and hormone sensitivity following transformation by Kirsten sarcoma virus. In Yohn, D. and Biggs, P. (Eds.): Leukemia Reviews. (In Press)

Hankins, W. D. and Luna, J. A.: Influence of Fv-1 and Fv-2 gene systems on in vitro erythroid transformation by Friend leukemia virus. In Yohn, D. and Blakeslee, J. (Eds.): Advances in Comparative Leukemia Research, New York, Elsevier, 1982, pp. 107-111.

Hankins, W. D., Kaminchik, J. and Luna, J. A.: Transformation of adult and fetal hemopoietic tissues with RNA tumor viruses. In Neinhuis, A. and Stammatoyannopoulos, G. (Eds.): Hemoglobin Switching. (In Press)

Kaminchik, J., Hankins, W. D., Ruscetti, S. K., Linemeyer, D. L. and Scolnick, E. M.: Molecular cloning of biologically active proviral DNA of the anemia-inducing strain of spleen focus-forming virus. J. Virol. 44: 922-931, 1982.

Khoury, M. J., Bondurant, M. C., Duncan, D. T., Krantz, S. B., and Hankins, W. D.: Specific differentiation events induced by erythropoietin in cells infected in vitro with the anemia strain of Friend virus. Proc. Natl. Acad. Sci. USA 79: 635-639, 1982.

Langdon, W. Y., Ruscetti, S. K., Silver, J., Hankins, W. D., Buchler, L. E., Morse, H. C.: Cas spleen focus forming virus: II. Further biological and biochemical characterization. J. Virol. (In Press)

Luna, J., Chen, C. and Hankins, W. D.: Three distinct erythroproliferative actions by Kirsten. Abelson and Friend spleen focus-forming viruses. J. Cell. Biochem. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05271-03 LTVG

PERIOD COVERED

June 1, 1982 through August 1, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of the Molecular Basis of Disease Induced by the Spleen Focus-Forming Virus

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

Joseph Kaminchik Visiting Fellow, LTVG, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Molecular Virology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two different variants of the defective Friend spleen focus-forming virus (SFFV) have been isolated in the past. One of the strains (SFFV-P) induces polycythemia in susceptible mice and the other (SFFV-A) induces anemia. Although it was found that the envelope (env) gene of the different strains of SFFV was sufficient for induction of characteristic SFFV-A or SFFV-P disease, the molecular basis for the distinctive biological effects of the two variants is not known.

We have constructed in vitro, env genes which are recombinants between the env genes of SFFV-A and SFFV-P. The env genes were constructed from molecularly cloned env genes of the two variants, using standard genetic engineering methods, and were tested for their biological activities both in vitro and in vivo. In these experiments ANA fragment of approximately 700bp at the 3'end of the SFFV env gene was found to encode the genetic differences between SFFV-A and SFFV-P.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institutes Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

W. D. Hankins Cancer Expert LTVG, NCI

Objectives:

The purpose of this project is to study at the molecular level differences in the env gene of $SFFV_A$ and $SFFV_P$ which are responsible for differences in pothogenicity between the two variants.

Methods Employed:

Molecularly cloned env genes of $SFFV_A$ and $SFFV_P$ were recombined using a common Eco R1 site. The resulting env gene recombinants were cloned in pBR 322 and propagated in E. coli RR1.

Recombinant env gene DNA cloned in pBR322 was used to transfect NIH3T3 cells along with Moloney-MulV molecularly cloned genome.

Virus rescued from transfected NIH3T3 cells was used to determine the biological activity of the recombinant env genes both in vivo and in erythroid stem cells cultured in vitro.

Major Findings:

The pathogenic properties of SFFV are encoded by the env gene since only the env gene sequences of the clone 341 were derived from SFFV. This clone was capable of inducing characteristic SFFV diseases in mice when rescued as an infectious particle from NIH3T3 cells.

700 bp at the 3'end of SFFV env gene encodes for the $SFFV_P$ and $SFFV_A$ phenotypes. Clone 341 in which the 3'end of the env gene is of $SFFV_P$ origin and the rest of the gene is of $SFFV_A$ origin induces erythroleukemia accompanied by polycythemia. Clones 545 and 603 in which the 3'end of the env gene was derived from $SFFV_A$ induced erythroleukemia which was characterized by a mild anemia in the terminal phases of the disease.

In vitro BFU-E burst induction by the recombinant env gene clones was also studied. Virus rescued from NIH3T3 cells cotransfected by clone 341 and Mo-MulV DNA induced erythroid bursts in vitro. Bursts induced by clone 341 are indistinquishible from $SFFV_P$ induced burst, i.e., the cells will fully hemoglobinize without added exogenous erythropoietin. Clones 545 and 603 on the other hand induced bursts which were characteristic for $SFFV_A$. Upon infection of erythroid precursor cells either with 545 or 603 rescued virus the cells proliferated in vitro. However, hemoglobin synthesis in those cells depended upon addition of erythropoietin to their growth medium for full hemoglobinization.

Significance to Biomedical Research and the Program of the Institute:

Spleen focus-forming virus as a model system for viral induced leukomogensis provides an opportunity for a genetic analysis of the viral oncogenic envelope gene. Variants of the same gene are present in SFFVp and $SFFV_A$, and each causes closely related but distinct disease. In vitro recombination between the two molecular cloned genes allowed us to determine which part of the env gene is responsible for the differences in pathogenicity of the two viruses. Experiments of this kind will lead to a better understanding of viral-induced leukomogenesis since they define which sequences, alter the growth potential of specific hemopoietic cells.

Proposed Course:

Generate more recombinants within the 700 bp at the 3'end of the env gene.

Sequence the env gene of $SFFV_A$ and compare it to that of $SFFV_P$.

Analyse biochemically the env gene product (gp52) of the recombinant viruses.

Publications:

Kaminchik, J., Hankins, W. D., Linemeyer, D. L., Ruscetti, S. K. and Scolnick, E. M: Molecular cloning of biologically active proviral DNA of the anemia-inducing strain of spleen focus-forming virus. J. Virol. 44: 922-931, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05283-02 LTVG

PERIOD COVERED

October 1, 1982 through September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transmission of Mammalian Genes with Expression-regulated Retrovirus Vectors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

M. Roussel Fogarty Fellow, LTVG, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Viral Immunogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A series of retrovirus based vectors have been developed for the efficient transmission of genetic information in mammalian cells. The vectors are based on the murine MuLV virus. The v-ras gene of Harvey murine sarcoma virus (HaMuSV) has been utilized as a convenient gene for functional expression. Expression of transmitted information has been rendered conditional by linkage of the p21 coding sequence to the glucocorticoid regulated promoter from mouse mammary tumor virus (MMTV).

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) engaged on this Project:

D. Berard	Microbiologist	LTVG, NCI
P. Tsichlis	Expert	LTVG, NCI
G. Hager	Head, Viral Immunogenetics Section	LTVG, NCI

Objectives:

To develop and characterize mammalian gene transducing vectors based on the unique characteristics of retroviruses for transmission of genetic information.

To introduce regulatory elements into retroviral vectors such that transmitted sequences can be subject to conditional expression.

To construct retroviral vectors for the cloning of full length cDNA transcripts and functional expression of these sequences after transmission in viral particles.

To untilize these vectors for the selection, transmission, and characterization of genetic sequences implicated in neoplastic transformation events.

To untilize these vectors for the selection, transmission, and characterization of genetic elements involved in steroid hormone regulation.

Methods Employed:

Functional elements involved in the replication, transmission and integration of retrovirus based vectors are molecularly cloned using plasmid and bacteriophage vectors.

Ecotropic and amphotropic classes of the MuLV family of murine retroviruses are utilized as the basic viral structure for construction of vectors.

The p21 transforming gene of HaMuSV is utilized as a marker gene for functional expression in mouse cells after vector transmission.

Functional expression of p21 is monitored by morphological transformation of susceptible cells. Quantitative levels of p21 protein are monitored by immunoprecipitation with monoclonal antibody.

Vector DNA intermediates and integrated structures are characterized by restriction endonuclease analysis and southern transfer blotting.

Vector RNA genomes and transcripts are characterized by S1 nuclease mapping and northern transfer blotting.

Major Findings:

A series of retroviral transmission vectors have been constructed utilizing the LTR's of MuLV, the v-ras gene from HaMuSV for functional expression, and the promoter from MMTV for regulated expression. The transcriptional enhancer from the HaMuSV LTR is also included as an accessory element to modulate the strength of the MMTV promoter.

We have addressed two questions in preliminary experimentation. First, can functional expression of a retroviral convected sequence be separated from transmission by encoding the functional information on the opposite strand, providing it with a promoter separate from the transmission LTR's? This mode of information convection would resolve a serious problem in cDNA convection in retroviral vectors, the premature termination of genome transcripts at the cDNA polyadenylation site. We have transmitted the v-ras gene in the opposite orientation, with promoter function provided by the MMTV LTR promoter. The efficiency of expression from the antiparallel configuration with respect to the impact of transcription in opposite directions on levels of functional message and transmittible genomes is under investigation.

The second question concerns conditional expression of convected sequences. A major feature of retroviral transmission vectors is the ability to efficiently mobilize a given sequence, or library of sequences, into a wide variety of cell types. Certain sequences of interest may be detrimental, or lethal, if constitutively expressed in a given cell. It may also be of considerable interest to modulate the intracellular concentration of the gene product of interest. We have found that the v-ras gene can be convected into cells on an MuLV based genome, but under effective expression control of the MMTV promoter. Thus the level of v-ras function can be significantly modulated by glucocorticoid hormone.

Significance to Biomedical Research and the Program of the Institute:

Retroviral based vectors are emerging as an effective tool for the mobilization of genetic information in mammalian cells. When established as a transmittible, defective genome, a given sequence can be introduced into almost any cell type with high efficiency and in a predicted structure. These features are of obvious advantage for the manipulation of sequences with oncogenic potential in a variety of cell systems. Furthermore, as an infectious particle, the information can be introduced into the animal, a further advantage for monitoring neoplastic potential. The development of these reagents will be of considerable value in the manipulation of genetic sequences implicated in neoplasia, as well as the mobilization of elements involved in normal biological regulatory processes.

Proposed Course:

Further development of the vector series will be pursued, with emphasis on the establishment of a generalized vector system that permits efficient transmission, as well as conditional expression of the convected function.

The vector system will be tested for the recovery of oncogenic sequences from neoplastic tissue, particularly tumors that do not score in the standard DNA mediated transfection assay.

Efforts will proceed to incorporate the ability to clone full-length, functional cDNA's into the vector system.

A separate series of vectors will be developed for use as potential insertional mutagens.

Publications:

None.

ANNUAL REPORT OF

THE LABORATORY OF VIRAL CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1982 to September 30, 1983

FUNCTIONAL STATEMENT: The Laboratory of Viral Carcinogenesis (LVC) has been charged with the planning, development, implementation, and coordination of research programs on the etiology and mechanisms of carcinogenesis with special emphasis on delineating the roles, mechanisms and regulation of action of oncogenic viruses, virus-related and virus-associated genetic sequences, and gene products. Research efforts are conducted on virus-host relationships in virus-induced cancers, focusing on the detection and characterization of oncogenic viruses, their mechanisms of genetic integration and expression, and their modes of transmission in animals and man. The host genetic and immune systems are studied to elucidate the mechanisms of natural cancer control, especially in virus-related cancers. Activities of the Laboratory are conducted by in-house research and collaborative agreements with other research organizations.

SCIENTIFIC BACKGROUND AND SIGNIFICANCE: The results described here show that remarkable progress has been made through our multidisciplinary, distributed task approach aimed at the characterization of the carcinogenic process. Malignant transformation in humans is emerging as a progression of coordinated steps from normal cellular control to uncontrolled replication. Apparently, it requires the concerted combination of reversible phenotypic alterations (promotion) and permanent alterations in genetic sequences and pathways (initiation). Formerly unaltered molecules and functions, most probably fulfilling normal funtions at some operational or developmental stage, become permanently altered and genetically direct the "uncontrolled" behavior of the tumor cell. Retroviruses have served to elucidate these relationships and to identify a finite library of viral "oncogenes," genes from normal cells which have become altered and are now implicated in cancer development. Molecular probes have identified and located the oncogene cellular homologs in the mammalian and human chromosomes. This has led to the association of given oncogenes with various animal and human tumors and promises to provide an insight into a finite number of potentially common mechanisms of cell transformation applicable to various species, including man. Some of these mechanisms may be implicated even in chemical carcinogenesis. Studies on the nature and function of growth factors (transforming, modulating, inhibitory) indicate that these factors, at least in some cases, may be the products of an oncogene or its cellular precursor as seen by their closely related nucleotide sequences.

Our activities in the field of oncogene characterization have interdigitated very well with the work of other groups, within and outside the Government. This has been done by formal and informal collaborations, in most cases, and independently in others. Specific examples are the detection and characterization of a new oncogene, v-raf, and the chromosomal localization and possible definition of human disease associations for several of the recognized oncogenes. Our projects on the detection and characterization of "transforming" growth factors and on tumor promotion are converging with the work on oncogenes with the demonstrations that some growth factors are participants in the promotion process and are produced as

a result of transformation by defined oncogenes. These growth factors may serve also as the signals or modulators of defined metabolic pathways involved in unique steps, from mitotic induction to host immunosuppression, and even, tumor cell growth inhibition. Basic findings are being followed by the preparation of a variety of specific assay reagents (monoclonal and polyspecific antibodies, nucleic acid probe libraries, etc.), which will be ready within a few weeks or months for numerous, potential clinical applications. For these reasons, and the number and nature of clinical collaborative associations already in effect, the upcoming months promise to be exciting as progressive steps are made towards the objectives of the LVC and the NCI.

ORGANIZATION OF THIS REPORT: The most significant findings obtained during this year can be clustered into five major areas as follows: (A) the identification, origin, transmission, structure, localization and function of a finite number of "oncogenes" that appear to be implicated in some forms of human malignancy; (B) the characterization of the control mechanisms for the expression of normal and transformed genes; (C) the identification and characterization of the nature and function of hormone-like control and signal factors that mediate the genetic and phenotypic expression of malignancy in mammalian cells; (D) the characterization of the pathways and intermediate products involved in endogenous transformation, tumor promotion and cocarcinogenesis; and (E) the isolation and characterization of novel retroviruses to better define retroviral evolution and cocarcinogenesis. Applications of basic findings to potentially valuable clinical situations are cited with the appropriate findings. The findings within each cluster are arranged in their order of significance to the objectives of the Laboratory and the Institute.

A. The Identification, Origin, Transmission, Structure, Localization and Function of a Finite Number of "Oncogenes" that Appear to be Implicated in Some Forms of Human Malignancy.

A1. IDENTIFICATION OF A NEW ONCOGENE, v-raf.

Methylcholanthrene-transformed C3H/10T1/2 mouse cells were treated with iododeoxyuridine and a new, acute transforming, replication-defective, mouse type C retrovirus, designated 3611-MSV, was induced. It transforms embryo fibroblasts and epithelial cells in culture and induces fibrosarcomas in mice. Analysis of viral protein expression led to the demonstration of 90,000 and 75,000 molecular weight polyproteins (P90 and P75), both containing amino-terminal murine leukemia virus (MuLV) gag gene proteins, p15 and p12. The oncogene v-raf was isolated from this virus, molecularly cloned and compared with previously isolated retrovirus oncogenes, either by direct hybridization or by comparison of restriction fragments of their cellular homologs. It was shown to be unique. Transfection of NIH/3T3 and RAT-2 cells with cloned v-raf DNA leads to efficient transformation and expression of P75 and P90, with NH_2-terminal gag gene-encoded components linked to the acquired sequence (v-raf) translational product. The v-raf-encoded polyproteins lack detectable protein kinase activity, and 3611-MSV-transformed cells do not exhibit elevated levels of phosphotyrosine.

A2. DNA SEQUENCE OF v-raf.

DNA sequence determination of the acquired oncogene (v-raf) and its flanking viral elements has provided essential information pertaining to its acquisition, composition, and mechanism of action. Until the analysis of 3611-MSV was

accomplished, the recombinant parent of the transforming retrovirus was unknown. The parent of 3611-MSV was determined to be an ecotropic murine type C virus; that is, no prerequisite recombinational events need to occur prior to the transduction of this cellular oncogene. 3611-MSV acquired its transforming sequences by the substitution of 2.39 kilobases (kb) of viral structural information with 1.49 nucleotides of cellular oncogene. Interestingly, this oncogene adoption occurred in a way that precluded its expression (it entered into a non-translatable reading frame). Thus, the oncogenic potential of this recombinant was carried cryptically in the virus stock until a nucleotide deletion shifted the transforming sequences into the proper reading frame. Deletion mutants, constructed using BAL-31 exonuclease, indicated that the active portion of the transforming gene was contained within 890 nucleotides, 35 nucleotides from its 5' point of acquisition. The two polyproteins detected in transformed cells represented the translation products of the molecularly fused viral gag gene and the raf oncogene, which terminated at an amber codon within the cellular sequences. The larger protein (90 kilodaltons, P90) contains a leader peptide, is glycosylated, and is intimately associated with cytoskeletal elements. The smaller protein (75 kilodaltons, P75) is uniquely modified at its amino terminus by the post-translational addition of the fatty acid myristate. This is believed to be the active form of the polyprotein.

The amino acid sequence, predicted from the nucleic acid sequence of v-raf, revealed domains homologous to v-src and v-mos and, therefore, a close relationship between the members of the tyrosine kinase-oncogene superfamily and v-raf. The fact that the latter enzymatic activity is not demonstratable in 3611-MSV-transformed cells suggests that tyrosine phosphorylation may not be essential for oncogenicity. Family comparisons now allow for the prediction of which sequences are related to the kinase activity and which are necessary for transformation. Of a unifying interest is the observation that one of these latter sequences is nearly identical to the presumed active site of the ras family of oncogenes. As with many oncogenic sequences, raf-related RNA is found in uninfected cells. The specific role of these sequences in human malignancy is not yet understood. Preliminary experiments indicate that cells derived from human lung carcinomas express large amounts of raf-related RNA (>1000 copies per cell). The qualitative analysis of these RNAs is in progress.

A3. DETECTION AND PRELIMINARY CHARACTERIZATION OF THE HUMAN CELLULAR HOMOLOG OF v-raf.

A survey of human genomes showed the presence of cellular homologs of v-raf in two related loci. Human DNA clones were obtained which covered 27-31 kb from each of two loci. Restriction fragments of these loci accounted for essentially all the bands detected in human nucleic acids using a v-raf probe. The first locus, c-raf-1, has sequences related to the full 1.2 kb of v-raf. These sequences span 7 kb and contain at least 4 introns. The second locus, c-raf-2, has only 0.45 kb of v-raf-related sequence which occurs as a single block lacking two of the introns present in c-raf-1. By taking advantage of an SphI site which is conserved between v-raf and c-raf-1, a DNA was constructed which contained the 5' portion of the transforming virus including the 5' one-third of the v-raf gene spliced to the 3' two-thirds of the human c-raf-1 gene. Upon transfection, this construct transformed NIH/3T3 cells. Since the 5' one-third of the v-raf gene will not transform by itself, the transforming ability of the construct implies the expression of a polyprotein containing Moloney MuLV gag, mouse v-raf and human c-raf components.

A4. LOCALIZATION OF c-raf-1 TO HUMAN CHROMOSOME 3 AND c-raf-2 TO CHROMOSOME 4.

Somatic cell hybrids were used to map c-raf-1 to human chromosome 3 and c-raf-2 to chromosome 4. The presence of c-raf-1 on chromosome 3 suggests that it might be involved in small cell lung carcinomas which characteristically have deletions on the short arm of chromosome 3, usually beginning at band p14 and extending to p23 or beyond. The possibility of rearrangements in the vicinity of c-raf-1 in these tumors is under intense study.

A5. DETECTION OF THE HUMAN v-abl CELLULAR HOMOLOG.

A contiguous region of a cellular DNA sequence, 64 kb in length and representing overlapping inserts from three independent cosmid clones, was isolated from a representative library of human lung cell carcinoma DNA partially digested with MboI. Within this region of the cellular genome, v-abl homologous sequences are dispersed over a total region of approximately 32 kb. These sequences represent the entire v-abl human cellular homolog, are colinear with the viral v-abl transforming gene, and contain a minimum of seven intervening sequences. At least eight regions of highly repetitive DNA sequences have been shown to map in close proximity to c-abl coding sequences. In addition to the major c-abl human locus, three regions of human DNA sequence, corresponding to only portions of the v-abl gene, have been identified. Two of these have been molecularly cloned and shown to be distinct from the primary human c-abl locus. Upon transfection to rat embryo fibroblasts in culture, none of the cosmid DNAs containing v-abl homologous sequences exhibited transforming activity. These findings identify and map a single genetic locus of human DNA, c-abl, representing the complete v-abl homolog, and demonstrate the existence of additional human DNA sequences corresponding to more limited, subgenomic regions of v-abl.

A6. SEQUENCE ANALYSIS OF THE HUMAN c-abl TYROSINE PHOSPHORYLATION ACCEPTOR SITE.

Sequences encoding the tyrosine phosphorylation acceptor region of the human c-abl oncogene have been identified and their nucleic acid sequence determined. Extensive sequence homology between this region of c-abl and the acceptor regions of the v-src, v-yes and v-fes/fps family of viral oncogenes was established, as well as more distant relatedness to the catalytic chain of the mammalian cAMP-dependent protein kinase. These findings argue that, of the homologs of retroviral oncogenes with tyrosine protein kinase activity examined to date, all were probably derived from a common progenitor and may represent members of a diverse family of cellular protein kinases.

A7. A CELLULAR ONCOGENE IS TRANSLOCATED TO THE PHILADELPHIA CHROMOSOME IN CHRONIC MYELOCYTIC LEUKEMIA.

The human cellular homolog (c-abl) of the transforming sequence of Abelson MuLV was localized to human chromosome 9 by analysis of a series of somatic cell hybrids. The long arm of this chromosome is involved in a specific translocation with chromosome 22, the Philadelphia translocation, t(9;22) (q34, q11), occurring in patients with chronic myelocytic leukemia (CML). Investigations were made on whether the c-abl gene was included in such translocations. Using c-abl and v-abl hybridization probes on blots of somatic cell hybrids, positive hybridization was found when the $22q^-$ (the Philadelphia chromosome), and not the $9q^+$ derivative of the translocation, was present in the cell hybrids. From this it was concluded that in CML, c-abl sequences are indeed translocated from

chromosome 9 to chromosome 22q$^-$. Moreover, it has been demonstrated, for the first time, that this translocation is reciprocal, since c-sis, another oncogene, was also found to be translocated simultaneously from chromosome 22 to 9, as well as in CML.

A8. MOLECULAR CLONING OF THE HUMAN c-fms ONCOGENE AND LOCALIZATION TO CHROMOSOME 5.

By the use of a probe corresponding to the long terminal repeat (LTR) U_3 region of the Snyder-Theilen strain of feline sarcoma virus (FeSV), the McDonough FeSV proviral DNA was molecularly cloned from nonproductively transformed rat cells. Molecular probes representing subgenomic regions of the McDonough FeSV transforming gene, v-fms, were prepared and a cosmid library of human lung carcinoma DNA screened for v-fms homologous sequences. Three cosmid clones containing overlapping v-fms homologous cellular DNA inserts, representing a contiguous region of a cellular DNA sequence approximately 64 kb in length, were isolated. Within this region of human genomic DNA, v-fms homologous sequences are dispersed over a total region of approximately 32 kb. These include the entire human v-fms cellular homolog (c-fms), are colinear with the viral v-fms transforming gene, and contain a minimum of four intervening sequences. At least twelve regions of highly repetitive DNA sequences have been mapped in close proximity to c-fms coding sequences. Using a molecular probe corresponding to a 0.9 kb, KpnI, v-fms homologous restriction fragment isolated from one of the cosmid clones, a series of mouse-human somatic cell hybrids containing different complements of human chromosomes was analyzed for human c-fms sequences. c-fms was thereby assigned to chromosome 5. Regional localization of c-fms to band q34 on chromosome 5 was accomplished by analysis of Chinese hamster-human cell hybrids containing terminal and interstitial deleted forms of chromosome 5. The localization of c-fms to human chromosome 5(q34) is of interest in view of reports of a specific, apparently interstitial, deletion involving approximately two-thirds of the q arm of chromosome 5 in acute myelogenous leukemia.

A9. GENETIC ANALYSIS OF THE 15;17 CHROMOSOME TRANSLOCATION ASSOCIATED WITH ACUTE PROMYELOCYTIC LEUKEMIA.

Analysis of a large series of mouse x human somatic cell hybrids permitted the localization of the c-fes oncogene to human chromosome 15 and a more precise mapping of several genetic markers on chromosomes 15 and 17. Somatic cell hybrids were prepared between a thymidine kinase-deficient mouse cell line and blood leukocytes from a patient with acute promyelocytic leukemia (APL) showing the 15q$^+$;17q$^-$ chromosome translocation frequently associated with that disease. One hybrid contained the 15q$^+$ translocation chromosome and very little other human material. The c-fes oncogene was not present in this hybrid and was, therefore, probably translocated to the 17q$^-$ chromosome. Analysis of the genetic markers present in this hybrid allowed a more precise localization of the translocation breakpoints on chromosomes 15 and 17.

A10. TYROSINE PHOSPHORYLATION OF A CELLULAR SERINE-SPECIFIC PROTEIN KINASE BY VIRAL (v-fes AND v-abl) ENCODED PROTEIN KINASES.

A cellular glycoprotein of 150,000 molecular weight (P150) was identified which specifically binds v-fes-encoded proteins and is a substrate for v-abl- and v-fes-encoded protein kinases. Phosphotyrosine was found on P150 isolated from [^{32}P]orthophosphate-labeled cells of several species transformed by these

viruses, but not on P150 isolated from nontransformed control cells. P150 exhibits an associated, serine-specific protein kinase activity which recognizes $P120^{gag-abl}$ and Gardner (GA) $P110^{gag-fes}$, as well as P150 itself, as substrates. This enzymatic activity has a divalent cation preference for Mg^{++} and uses [γ^{32}P]ATP as a phosphate donor. Interspecies antigenic determinants on P150 permitted its isolation from cells of all mammalian species tested, including mouse, rat, cat, dog, mink and a number of human tumor lines. In addition, similar [^{35}S]methionine and [^{32}P]orthophosphate-labeled tryptic peptides were characteristic of P150s isolated from cells of several mammalian species, including human, indicating that P150 is highly conserved. Glycosylation of P150 was demonstrated by incorporation of [^{3}H]mannose, and, by tryptic peptide analysis, the glycosylation site was localized to a single peptide. P150 was shown to be distinct from each of several previously demonstrated substrates for tyrosine-specific protein kinases, including vinculin, growth factor receptors, and several glycolytic enzymes.

A11. TRANSFORMING GENES OF AVIAN (v-fps) AND MAMMALIAN (v-fes) RETROVIRUSES CORRESPOND TO A COMMON CELLULAR LOCUS.

The Gardner and Snyder-Theilen isolates of FeSV represent genetic recombinants between feline leukemia virus (FeLV) and transformation-specific sequences (v-fes gene) of cat cellular origin. A related transforming gene (v-fps), common to the Fujinami, PRC II, and UR 1 strains of avian sarcoma virus was also described. Translational products of each of these recombinant virus isolates are expressed in the form of polyproteins exhibiting protein kinase activities with specificity for tyrosine residues. v-fes and v-fps homologous sequences of GA-FeSV, Snyder-Theilen (ST)-FeSV and Fujinami sarcoma virus were defined. These independently derived transforming genes were shown to correspond to a common cellular genetic locus which has remained highly conserved throughout vertebrate evolution.

A12. THE ALIGNMENT OF THE FELINE AND HUMAN LINKAGE MAPS HAS BEEN ACCOMPLISHED AND IS SERVING VERY SUCCESSFULLY AS A PREDICTIVE MODEL BECAUSE OF THE STRIKING CHROMOSOMAL HOMOLOGY BETWEEN PRIMATE AND FELIDAE FAMILIES.

While the development of the human linkage map was relatively advanced, that of the Felidae was, at best, rudimentary. The feline genetic map has been progressively accomplished in this Laboratory, and this year was published, demonstrating its unique usefulness as a predictor and experimental model for the localization of homologous loci in humans. A genetic map of 35 biochemical loci located on sixteen feline syntenic (linkage) groups was derived. The majority of these linkage groups has been assigned karyologically to one of 19 feline chromosomes. Using high resolution G-trypsin banding (1000 bond level of resolution) it was demonstrated that 20% of the human genome could be aligned band for band to homologous regions. Linkage homologies in other regions were characterized by small intrachromosomal rearrangements. The striking concordance of feline and primate genetic maps has two major aspects of biological significance. First, the evolutionary implications are rather significant since the chromosome organization has maintained some semblance of order despite 80 million years of divergence (between primates and felids). Second, the comparative genetics has a predictive value, since once a gene has been located in the cat, there is a strong suggestion as to the position of a homologous locus in man. This aspect may be especially important for the identification of mammalian genes (like retroviruses or controlling elements) capable of transposition during mammalian evolution. For example, (1) two classes of somatic cell hybrid panels have been derived and

characterized. The first was made with rodent x human lymphocytes and consists of approximately 60 hybrids segregating human chromosomes. The second panel was made with rodent x cat lymphocytes and consists of approximately 60 hybrids segregating cat chromosomes. These hybrid panels allow the precise chromosomal localization for virtually any human or cat gene for which an assay or a molecular clone is available. (2) In order to localize each of the recognized unique oncogenes, molecular clones of each have been obtained from the initiating laboratories. Batch DNA extracted from the genetically characterized hybrids of the feline hybrid panel has been used to assign feline oncogene homologs to cat chromosomes. Preliminary assignments in the cat have been obtained for myc, c-Ha-ras-1, c-Ha-ras-2, c-Ki-ras-1, c-Ki-ras-2, fes, and sis. Evidence for transposition of some, but not all, of these loci during the mammalian radiations has been derived. Four members of the ras family have also been localized to four distinct human chromosomes (X, 6, 11 and 12) and the two members of the raf family to chromosomes 4 and 5. (3) Lysosomal enzyme deficiencies in man lead to a number of heritable, neurological, storage diseases which have no effective treatment (e.g., Tay-Sachs disease, Hurler's syndrome, GM1 gangliosidosis, mannosidosis, Maroteaux-Lamy syndrome). Models for many of these diseases have been described in the cat with included enzyme deficiencies. These genetic defects represent a good opportunity for monitoring gene therapy in an excellent human model. Toward these ends, the genetic mapping of 10 of these enzymes has been initiated using the cat hybrid panel and procedures developed in Dr. John O'Brien's laboratory at University of California, San Diego. Six of these (GALA, GALB, HEXA, MANA, GUSB, GLUA) have already been assigned to specific feline chromosomes.

A13. IDENTIFICATION AND GENETIC ANALYSIS OF A NUMBER OF CANCER-ASSOCIATED LOCI IN THEIR RESPECTIVE SYSTEMS.

As the feline (and human) genetic map was developing, a number of examples of cancer-associated loci have been identified and are at various stages of genetic analysis in their respective systems. These genes include: (1) endogenous cellular DNA sequences homologous to cDNA radioactive probes transcribed from retroviral genomic RNA; (2) chromosomal integration sites for exogenous retroviral insertion and persistence; (3) receptors on cell membranes which interact with virus glycoproteins to determine cell species compatibility and viral host range; (4) restriction genes which delimit virus replication in various animal species; (5) cellular transforming (onc) genes; (6) cellular enzyme structural genes; and (7) cell surface antigens including antigens homologous to the major histocompatibility complex (MHC) of other mammalian species.

A14. A SINGLE LOCUS MAY BE RESPONSIBLE FOR THE PRODUCTION OF THE MAJOR INDUCIBLE FORM OF RD-114 FELINE VIRUS.

A map of restriction endonuclease recognition sites was derived in the genome of RD-114 retrovirus, an inducible, replication-competent member of this gene family. Its structure was compared to restriction maps of nine molecular clones of endogenous RD-114 sequences isolated from a "library" of cat cellular DNA. The endogenous sequences analyzed were similar to each other in that they were colinear with RD-114 proviral DNA, were bounded by long terminal redundancies and conserved many restriction sites in the gag and pol regions. Several sequences have undergone a small deletion, relative to the inducible viral genome, in a region which may be important to encapsidation of viral RNA. The env regions of the endogenous RD-114 sequences examined were substantially deleted or diverged;

a subset of these sequences contained information at the position of the env region which was not homologous to inducible RD-114 by restriction mapping or by hybridization. Analysis of cat cell DNA confirmed the conclusions about conserved restriction sites in endogenous sequences and showed that a single locus may be responsible for the production of the major inducible form of RD-114.

A15. ASSIGNMENT OF BABOON RETROVIRAL ERV1 and ERV2 TO HUMAN CHROMOSOMES 18 AND 15, RESPECTIVELY.

Using the human hybrid panel, two members of a baboon retroviral family, ERV1 and ERV2, were mapped to human chromosomes 18 and 15, respectively. This represents the first endogenous retroviral sequence described in humans.

A16. INVESTIGATION OF THE INTEGRATION SITE OF HTLV IN HUMAN CHROMOSOMES.

The integration position of the human T cell leukemia virus (HTLV) is being investigated in four classes of custom hybrids made from the two original, infected human patients, HUT 102 and MJ. A high resolution cytogenetic analysis of these tumors revealed some specific rearrangements. The cell lines derived from these patients each underwent de novo proviral integrations in addition to the original provirus. Approximately six integrations from each human patient have been chromosomally identified. They appear to disperse non-specifically throughout the human genome.

B. The Characterization of the Control Mechanisms for the Expression of Normal and Transformed Genes.

B1. REGULATION OF VIRAL AND CELLULAR ONCOGENE EXPRESSION BY CYTOSINE METHYLATION.

Mink cells morphologically transformed by either Snyder-Theilen FeSV or Abelson MuLV exhibit relatively high rates of reversion to the nontransformed phenotype. The proviral DNAs are conserved within the revertant lines and have not undergone changes in integration sites due to translocations or other genomic rearrangements. In contrast, expression of well-defined, viral-encoded transforming proteins is blocked and elevated levels of phosphotyrosine, characteristic of the parental transformed cells, are reduced to control levels. Loss of the transformed phenotype is associated with increased cytosine methylation of proviral DNA sequences, while levels of methylation resume control values upon spontaneous retransformation of revertant clones. Following molecular cloning and transfection to Rat-2 cells, ST-FeSV proviral DNAs from revertant and transformed cells induced similar numbers of transformed foci. Cytosine methylation sites involved in regulation of expression of the major ST-FeSV-encoded transforming protein have been localized within the proviral DNA itself, rather than in adjacent cellular flanking sequences. In contrast to the v-fes proviral DNA, c-fes, the cellular homolog of the ST-FeSV-acquired transforming sequences, is highly methylated in cytosine residues in both transformed and revertant clones. These findings demonstrate regulation of viral oncogene-mediated transformation by cytosine methylation and suggest that expression of cellular homologs of viral oncogenes, such as c-fes, are also subject to regulation at this level.

B2. ROLE OF PROMOTERS AND METHYLATION IN THE EXPRESSION OF ENDOGENOUS RETROVIRUSES.

The endogenous parent of CPC-1, a primate retrovirus from Colobus polykomos, exists essentially as one of a multigene family of 50-70 members (a similarity shared by most endogenous retroviruses). The origin and evolution of these sequences is in doubt. The difficulty of CPC-1 isolation has suggested that it is repressed as the result of multiple factors. This is supported by the observations that (1) both of its cloned, endogenous, proviral promoters are transcriptionally inactive; and (2) Southern blot analysis of the endogenous proviruses reveal all of them to be hypermethylated (internally, as well as over their promoter regions). These findings are, nevertheless, inconsistent with the high proviral copy number found which may be the result of superinfections (an RNA intermediate is required); rather, a model of gene conversion would favor the involvement of the provirus as a transposable element.

B3. THE LONG TERMINAL REPEAT (LTR) DERIVED FROM THE REPLICATION-COMPETENT CPC-1 RETROVIRUS HAS BEEN PARTIALLY SEQUENCED TO DETERMINE THE NATURE OF ITS PROMOTER REGION.

The mechanism of transcriptional regulation in eukaryotes involves the molecular architecture of the template DNA, dictated in part by the nucleotide sequence of the transcriptional promoter. The long terminal repeat (LTR) derived from the replication-competent, CPC-1 retrovirus has been partially sequenced to determine the nature of its promoter region. Two overlapping promoter regions were discovered, each consisting of a canonical TATA box preceded by an identical transcriptional modulation sequence (CCAATCATA). Analysis of stable in vivo transcripts revealed their initiation was directed by the farther downstream promoter. The duplicitous nature of the CPC-1 promoter region suggested that its transcriptional efficiency might be quite high. This was substantiated by C_rt analysis of productively infected A549 cells which revealed the presence of an extraordinarily high number of viral transcripts (5-10,000 copies/cell) in these cells. The activity and specificity of in vivo transcription was demonstrated in vitro employing restriction enzyme-truncated, cloned CPC-1 and a cell-free extract. To ascertain the cellular origin of the CPC-1 viral promoter sequences, two endogenous proviral LTRs were cloned from colobus kidney cells. Nucleotide sequence and in vitro transcription comparisons of the three DNAs revealed (1) the CPC-1 promoter originated from colobus proviral sequences; (2) of the 160 nucleotides sequenced, the endogenous promoters differed from CPC-1 and each other by four base changes; and (3) although both binding (CCAAT) and initiation (TATA) sites were unaltered in the endogenous promoters these DNAs were transcriptionally inactive.

C. The Identification and Characterization of the Nature and Function of Hormone-like Control and Signal Factors that Mediate the Genetic and Phenotypic Expression of Malignancy in Mammalian Cells.

This Laboratory has led in the detection and characterization of growth factors which can confer reversible malignant characteristics to normal cells when added to appropriate soft agar cell cultures. They have been referred to as "transforming growth factors (TGFs)." The following are the most recent advances:

C1. DETERMINATION OF THE AMINO-TERMINAL SEQUENCES OF TRANSFORMING GROWTH FACTORS (TGFs).

TGFs were purified from serum-free medium conditioned by retrovirus-transformed Fischer rat embryo fibroblasts, mouse 3T3 cells and two human melanoma cell lines. The purification of each TGF was monitored in a radioreceptor assay based on receptor cross-reactivity with mouse submaxillary gland epidermal growth factor (EGF) and was achieved by gel permeation chromatography of the acid-soluble, TGF-containing activity, followed by reverse phase high pressure liquid chromatography (rpHPLC) using sequentially acetonitrile and 1-propanol in the presence of aqueous trifluoroacetic acid. The amino-terminal sequences of rat, mouse and human TGFs were determined. Extensive (>90%) sequence homology was found among TGF polypeptides from different species and cell types. The complete amino acid sequence of rat TGF also was deduced from microsequence analysis data of reduced and S-carboxyamidomethylated TGF and Lys-c peptides and from results of carboxypeptidase digestion of modified rat TGF. Rat TGF was found to be a single chain polypeptide with a calculated molecular weight of 5,600 which displays 33% sequence homology with murine EGF and 44% sequence homology with human urogastrone.

C2. DETERMINATION OF THE AMINO ACID SEQUENCE OF SARCOMA GROWTH FACTOR (SGF) AND ITS COMPARISON WITH THE SEQUENCES OF KNOWN EGF-LIKE PEPTIDES.

Sarcoma growth factor (SGF) from serum-free media conditioned by murine sarcoma virus (MSV)-transformed 3T3 cells was purified to homogeneity using rpHPLC. The amino acid sequence analysis indicated that SGF is distinctly different from EGF. Comparison of the N-terminal amino acid sequence of SGF with a TGF from a rat cell transformed by a feline sarcoma virus or the TGF from a human melanoma cell line showed greater homology between the members of the TGF class than with either the TGFs and EGFs or even between mouse EGF and human EGF (urogastrone).

C3. DETECTION OF HIGH MOLECULAR WEIGHT TRANSFORMING GROWTH FACTOR-LIKE ACTIVITIES IN THE URINE OF PEDIATRIC CANCER PATIENTS.

Urine specimens from patients with a variety of pediatric malignancies including small cell carcinoma, osteogenic sarcoma and rhabdomyosarcoma contained acid- and heat-stable, high molecular weight factors which competed for binding to EGF and promoted anchorage-independent growth of nontransformed cells in semi-solid media. High levels of this activity were not found in the patients with acute or chronic lymphocytic leukemia or in the majority of healthy control children. This transforming activity was enhanced by a transformation potentiating factor (TPF) which is distinct from and functionally unrelated to EGF. Highest levels of activity were found in lymphoma patients and a patient with undifferentiated small cell carcinoma.

C4. CHARACTERIZATION OF A REQUIREMENT FOR THE SYNERGISTIC ACTIONS OF THE SARCOMA GROWTH FACTOR AND A MODULATOR TO STIMULATE THE EXPRESSION OF THE TRANSFORMED PHENOTYPE.

It has been shown that SGF acts synergistically with a peptide modulator to cause untransformed indicator cells to form large colonies in soft agar. These two factors coelute from an acidic Bio-Gel P-60 columns and, consequently, stimulation of the indicator cells to form the very large colonies, while assaying the fractions from these columns their synergistic effects were seen. Neither the

SGF nor the modulator, when assayed alone, was able to stimulate formation of large colonies of untransformed indicator cells, even if one used very large quantities of the individual purified components. Reconstruction experiments showed that the efficient stimulation of anchorage-independent growth (AIG), seen as large colonies in soft agar, could be regained by adding SGF along with the modulator to the untransformed indicator cells in soft agar. A similar requirement for a "transformation potentiating factor (TPF)" was seen for a urine derived transforming growth factor. The low molecular weight, 8,000 M_r "transforming" activity found in urine specimens of the tumor patients described above was further purified by HPLC on μ Bondapak C_{18} columns. This activity was resolved into two components following elution with acetonitrile, neither of which by itself supported the growth of non-transformed cells in semi-solid media. The EGF-competing activity elutes at 40-42% acetonitrile as a doublet; both species cross-react equivalently in a homologous radioimmunoassay for human EGF (urogastrone). The second component of the low molecular weight transforming activity elutes at 38% acetonitrile and does not compete with EGF for membrane receptors. In the presence of nanogram amounts of EGF this factor is extremely potent in stimulating the growth of both normal rat kidney and normal human foreskin fibroblasts in semi-solid media. This peptide has been purified to homogeneity and has an apparent molecular weight of 8,600. In addition to EGF, the low molecular weight EGF-competing activity derived from the urine of a patient with a well-differentiated gastric adenocarcinoma also contains TGF. This peptide, unlike EGF, elutes by HPLC from μ Bondapak C_{18} columns in the range of 18-20% acetonitrile, is not immunologically related to EGF and, thus, is similar to TGFs produced by transformed cells in culture. Following HPLC purification, the urine-derived TGF also requires the presence of the second factor in order to promote anchorage-independent cell growth.

C5. COMPARISON OF GROWTH FACTORS OBTAINED FROM THE URINE OF ATHYMIC MICE BEARING TRANSPLANTED NORMAL AND MALIGNANT HUMAN TISSUES.

Acid-ethanol-extracted urine from 8- to 12-week-old BALB/c normal, non-inoculated athymic mice contains a major peak of EGF-competing activity which elutes from Bio-Gel P-100 columns in the region of the 6,000 molecular weight insulin marker. This peptide is indistinguishable from mouse submaxillary gland EGF when tested in a homologous radioimmune assay for murine EGF. In addition, this urine also contains a minor peak of EGF-competing activity eluting with an apparent molecular weight of 20,000. The mean concentration of this 20,000 molecular weight growth factor is 0.7 ng of EGF equivalents (ng eg EGF) per mg of urine peptides. A significant increase in the levels of this factor is seen in mice bearing tumors (human rhabdomyosarcoma cell line A673). The increase is 8- to 10-fold higher than in normal animals. A similar, but less exaggerated response was seen with the human melanoma cell line, A375. The 20,000 molecular weight factor was further characterized; that from control mouse urine was resolved into three EGF-competing activities eluting at an acetonitrile concentration of 32%, 30.5% and 35%. The 20,000 molecular weight component found in the urine of tumor-bearing mice gave a similar profile but it also contained an EGF-competing activity eluting at 20% acetonitrile. This latter activity was not found in the control urine and corresponded in elution position to the 20,000 molecular weight TGFs released into conditioned media by A673 cells. Radioreceptor assay of both control and the unique, tumor bearer-derived TGFs did not show any cross-reactivity when tested at similar concentrations.

C6. CELLS TRANSFORMED BY v-abl AND v-fes PRODUCE HIGH TITERS OF TGF WHILE CELLS TRANSFORMED BY v-fms PRODUCE TGF ONLY AT LOW LEVELS.

Fischer rat embryo cells transformed by v-abl, v-fes and v-fms release TGFs into the cell culture medium. These peptides stimulate phosphorylation of the EGF membrane receptors and promote anchorage-independent cell growth. Cells transformed by v-abl and v-fes produce high titers of TGF (60-200 ng eq EGF/liter) while cells transformed by v-fms produce TGF at only low levels (<10 ng eq EGF/liter). Upon purification, TGF progressively loses transforming activity. A second potentiating factor, when added to purified TGF preparations, restores transforming function. This factor has been partially purified and shown to be distinct from previously described growth factors.

C7. TUMOR CELLS PRODUCE TUMOR INHIBITING FACTORS (TIFs) THAT MAY BE USEFUL IN THE STUDY AND POSSIBLE CONTROL OF SOME ASPECTS OF MALIGNANT PROGRESSION.

Serum-free conditioned media from several human tumor cell lines (e.g., epidermoid carcinomas, melanomas, bronchogenic carcinomas, and rhabdomyosarcomas) and from NIH/3T3 cells transfected with DNA from a human lung carcinoma were observed to produce a class of factors which inhibits the growth of human melanoma and carcinoma cells in soft agar and in monolayer cultures. These inhibitors of tumor cell growth have been designated tumor inhibiting factors (TIFs). Normal human fibroblasts and epithelial cells, however, are stimulated to proliferate by these same factors. A rich source of TIFs has been identified which should facilitate their purification and characterization. The major TIF activities were found to fractionate by gel permeation chromatography into three molecular weight classes (5,000, 10,000, and 20,000). Reverse phase HPLC and an organic solvent gradient were used to further resolve the 10,000 molecular weight TIF into three additional distinct activities which eluted at different solvent concentrations. It has not been determined whether any of the three 10,000 molecular weight TIFs are related to each other or to the 5,000 or 20,000 molecular weight TIFs. The 10,000 and 20,000 molecular weight forms of TIF have been the most characterized thus far. They are trypsin sensitive and heat stable at 56°C for 30 minutes. The 10,000 molecular weight TIF is heat stable at 100°C but the 20,000 form is not. Normal mink epithelial cells are very sensitive to inhibition by the 10,000 molecular weight TIF but not by the 20,000. A549 human lung carcinoma cells are inhibited by both. These two forms appear to have different biological and biophysical properties. TIFs produce gross cell surface and internal cytoskeletal changes in tumor cells. Scanning electron microscopy of human lung carcinoma, melanoma, and normal human fibroblasts treated with TIF showed considerable cell surface changes only in the transformed cells. Lung carcinoma cells showed dramatic changes in cell-cell contact and in the distribution of microvilli. Normal human fibroblasts treated with TIF were indistinguishable from the untreated controls. Fluorescent microscopic studies using antisera against actin showed lung carcinoma cells treated with TIF to have microfilaments which were much thicker and more directionally oriented than the untreated control cells.

C8. DETECTION OF TUMOR CELL INHIBITORY FACTORS IN TISSUE.

Extracts from certain normal human tissues were found to provide a rich source of TIF. The yield of TIF from several grams of tissue is greater than that obtained from large quantities of conditioned media. All of the biological and physical properties of the tissue-extracted, 10,000 molecular weight TIF, appear identical

to the TIF derived from the conditioned media. This suggests that the tissue-extracted TIF is related or identical to that previously described and obtained from conditioned media.

C9. ANTAGONISTIC RELATIONSHIP BETWEEN TUMOR GROWTH FACTOR (TGF) AND TUMOR INHIBITING FACTOR (TIF).

TGF and TIF can be isolated from media conditioned by a rhabdomyosarcoma cell line (A673). Growth in soft agar of lung carcinoma cells is enhanced by this TGF and inhibited by the TIF. This growth of lung carcinoma cells can be modulated by the ratio of TGF to TIF so that there is an apparently antagonistic relationship between the two factors isolated from the same source. This leads to speculation on the mechanism(s) of growth regulation in the rhabdomyosarcoma which produces both of these factors simultaneously. Tumors may produce both growth stimulatory and inhibitory factors to regulate their rate of proliferation. Similar endogenous transforming and growth-stimulating factors as well as growth-inhibitory factors have been detected in beef brain. A protein factor of approximately 30,000 molecular weight was isolated from aqueous extracts of beef brain which induces anchorage-independent growth of rat kidney fibroblastic cells in soft agar. It is heat-, alkali- and acid-labile and does not affect the binding of ^{125}I-EGF or ^{3}H-phorbol-12,13-dibutyrate (^{3}H-PDBu) to their respective receptors, and is thus different from SGF and TGF. Aqueous brain extract also contains a factor(s) which inhibits TGF-induced, anchorage-independent growth of normal rat kidney fibroblasts, and murine and human transformed cells in soft agar. This latter factor is a low molecular weight compound(s) (≈1,000 molecular weight) and is heat-, acid-, trypsin- and chymotrypsin-stable. It does not modulate the binding of EGF or PDBu. Yet another anti-growth factor(s) has been extracted from clam digestive glands. Clams and other mollusks contain potent factor(s) which inhibit TGF-induced, anchorage-independent growth of normal cells, and murine and human transformed cells in soft agar.

D. The Characterization of the Pathways and Intermediate Products Involved in Endogenous Transformation, Tumor Promotion and Cocarcinogenesis.

D1. DEVELOPMENT OF THE JB6 MOUSE EPIDERMAL CELL MODEL AS A UNIQUE IN VITRO SYSTEM FOR THE STUDY OF THE MECHANISMS OF TUMOR PROMOTION.

In order to elucidate the critical rate-limiting steps of the long latent period of premalignant progression, especially when mediated by tumor promoters, an appropriate model was critically needed. The JB6 mouse epidermal cell model system was developed to study the mechanism of tumor promotion. These cells are stably nontumorigenic and anchorage-dependent, and, in response to a variety of tumor promoters, become irreversibly tumorigenic and anchorage-independent.

D2. REACTIVE OXYGEN GENERATORS ARE ACTIVE PROMOTERS OF TRANSFORMATION AND PROBABLY MEDIATE PHORBOL ESTER PROMOTION.

Benzoyl peroxide and hydrogen peroxide are active as promoters of neoplastic transformation of JB6 cells. In contrast to promotion by 12-O-tetradecanoyl-phorbol-13-acetate (TPA), benzoyl peroxide promotion is not inhibited by retinoic acid, suggesting at least one difference in their mechanisms of action. TPA promotion is not inhibited by catalase, indicating that if reactive oxygen is a mediator, the species must be a superoxide or another radical, not hydrogen peroxide.

D3. PROMOTION IN THE JB6 CELL MODEL SYSTEM IS MEDIATED BY THE INTERACTION OF PHORBOL ESTERS AND CERTAIN GROWTH FACTORS WITH THE CELL SURFACE RECEPTORS.

All promotable cells tested have shown specific cell surface receptors for phorbol esters. This was, however, not sufficient for either promotion or mitogenesis, since both promotion-resistant and mitogen-resistant variants of JB6 cells have receptors comparable in number and affinity to their sensitive counterparts.

D4. MITOGENIC STIMULATION BY A TUMOR PROMOTER IS NOT AN ESSENTIAL STEP FOR SELECTION FOR THE PREMALIGNANT PROGRESSION OF CELLS OR ITS INDUCTION BY DNA REPLICATION-DEPENDENT EVENTS.

During the selection of JB6 cells for resistance to plateau density, mitogenic stimulation by the phorbol ester TPA yielded several clonal cell lines that remained sensitive to promotion, thus ruling out mitogenesis as a required event. In addition, the liver tumor promoter di-2-ethylhexyl-phthalate (DEHP) is active in promoting anchorage-independent transformation, but is not mitogenic for JB6 cells. Hence TPA and DEHP must induce other required events on the promotion pathway. The TPA mitogenesis pathway, but not the promotion pathway, appears to require an EGF receptor-mediated event and stimulation of hexose uptake.

D5. A SPECIFIC DECREASE IN CELL SURFACE TRISIALOGANGLIOSIDE MAY BE REQUIRED FOR PROMOTION OF TRANSFORMATION.

Tumor-promoting phorbol esters and other promoters produce a 90% decrease in surface trisialoganglioside (G_T) synthesis which occurs consistently in promotable cells but not in nonpromotable variants. This suggests that the G_T switch in response to TPA may be one of a few critical, required events in tumor promotion. Inhibiting the G_T decrease by G_T reconstitution inhibits promotion of transformation by TPA. Selection of JB6 cells for resistance to the G_T response to TPA, coselected for promotion resistance, arguing further for a causal relationship of G_T response to promotion of neoplastic transformation. Evidence suggesting an oxidative mechanism for the loss of G_T has been obtained.

D6. A SPECIFIC INTERFERENCE WITH THE SYNTHESIS OF COLLAGEN IS PRETRANSLATIONALLY ASSOCIATED WITH CELLULAR EXPOSURE TO A VARIETY OF TUMOR PROMOTERS, BUT COLLAGEN LOSS IS NOT ESSENTIAL FOR THE INDUCTION OR MAINTENANCE OF PROMOTION.

In vitro translation experiments have indicated that phorbol esters produce a rapid loss of translatable and hybridizable messages for collagen. This collagen loss occurs consistently in response to a variety of promoters in both promotable and nonpromotable JB6 cells, indicating that the collagen loss is not sufficient for setting in motion the sequence of events leading to promotion. Retinoic acid antagonizes the phorbol ester action on collagen levels, but not at the level of transcription.

D7. CHARACTERIZATION OF THE PHORBOL AND INGENOL RECEPTOR AND ITS ENDOGENOUS LIGAND.

The cell membrane receptor for the phorbol and ingenol tumor promoters was characterized as a protein kinase. The specific receptors for phorbol and ingenol esters were solubilized and purified to homogeneity. Their physicochemical and biochemical properties were characterized. The endogenous ligands for this receptor were also identified, isolated and characterized from cow milk and murine

intestine, pancreas and stomach. They were found to be mono- and diglycerides with special structural features. The interactions between the receptor and its ligands were studied in solution.

D8. ELUCIDATION OF THE MECHANISM OF AFFINITY MODULATION OF EGF RECEPTORS BY BIOLOGICALLY ACTIVE PHORBOL ÁND INGENOL ESTERS.

TPA-elicited inhibition of EGF binding (TIEB) is not affected by phosphorylation or dephosphorylation of EGF receptors. TPA does not alter EGF-stimulated phosphorylation of the EGF receptor in human epidermal carcinoma vulva (A431) cells. The inhibitors of protein and lipid methylation also do not affect TIEB. Tunicamycin (a glycosylation inhibitor), like TPA, reduces the binding of EGF to its receptors, suggesting a role for the carbohydrate component of receptors in EGF binding. The conclusion has been formulated that TPA is topically linked to the EGF receptors through phospholipids. The binding of TPA to specific receptors perturbs these membrane phospholipids; however, EGF binding does not alter them.

D9. DETECTION AND PARTIAL CHARACTERIZATION OF A PROTEIN FROM MURINE SERUM THAT BINDS BIOLOGICALLY ACTIVE PHORBOL AND INGENOL ESTERS.

A protein (~71,000 molecular weight) from murine serum was isolated and purified 104-fold. It was found to bind directly to biologically active phorbol and ingenol esters, and mezerein in a specific, reversible and saturable manner. The binding of labeled PDBu to this protein was rapid and dose-dependent. Those phorbol and ingenol esters which stimulate cell growth in culture and have tumor-promoting activity in vivo inhibit the binding of labeled PDBu, while the biologically inactive derivatives fail to do so. Other nonditerpene tumor promoters, retinoids, steroids and prostaglandins did not interfere with the PDBu-protein interaction. EGF, insulin, bovine serum albumin, hemoglobin, ovalbumin, ferritin, myoglobin, fetuin and lipase did not directly interact with PDBu. The binding protein, which competitively inhibited the binding of PDBu to specific receptors, is nonglycosylated, slightly hydrophobic, and heat- and acid-labile. The protein is present in sera of various mammalian species. Its concentration in murine serum is age-, sex-, and strain-independent.

D10. ISOLATION AND CHARACTERIZATION OF PHORBOL-12,13-DIESTER 12-ESTER HYDROLASE (PDEH) FROM MURINE AND HUMAN LIVERS.

A phorbol-12,13-diester 12-ester hydrolase (PDEH) was purified to electrophoretic homogeneity from murine liver cytosol using ammonium sulfate fractionation, Sephadex G-200 gel filtration, Con A Sepharose and phenyl Sepharose chromatography. The enzyme is a single chain, hydrophobic glycoprotein with a molecular weight of 60,000. It exhibits optimum activity at pH 7.5-8.5, has an isoelectric point (PI) of 5, is heat- and acid-labile, and is inhibited by Zn^{++}, Co^{++} and F^{-}, phenylmethyl sulfonyl fluoride (PMFS) and sarkosyl. The enzyme inactivates biologically active phorbol 12,13-diesters in a dose-, time- and temperature-dependent manner. The inhibition constant has been observed to be 6.6×10^{-8} M for the enzyme-elicited inhibition of phorbol-12,13-dibutyrate binding to its receptor. The enzyme exclusively cleaves the 12-ester of phorbol-12,13-diesters.

D11. PDEH IS A CRITICAL FACTOR IN THE SUSCEPTIBILITY TO THE TUMOR-PROMOTING ACTION OF PHORBOL DIESTERS.

The esterase PDEH, which converts biologically active phorbol-12,13-diesters to the inactive phorbol-13-monoester, is absent from mouse skin but is expressed at high levels in hamster, rat, guinea pig, and rabbit skin. The nonresponsiveness of species other than mouse to TPA and related compounds is directly related to the level of this enzyme found in the skin. It should be expected that TPA could act as a potent tumor promoter in human skin since, like the skin of the mouse, it lacks this esterase activity.

D12. PROMOTABILITY HAS A GENETIC BASIS AND BEHAVES AS A DOMINANT GENETIC TRAIT TRANSFERABLE BY DNA TRANSFECTION.

Fusion of promotable with nonpromotable JB6 cells yielded promotable hybrids indicating dominance of promotability. DNA from promotable cells, when transfected into nonpromotable cells yielded a 5- to 10-fold increase in promotion of anchorage-independence in response to TPA. Genes for promotability are sensitive to the restriction endonucleases EcoRI and HindIII, but not to BglI and BglII, and appear to be in the 10 kilobase size range. These genes, specifying sensitivity to induction of neoplastic transformation, are expected to be different from oncogenes.

E. The Isolation and Characterization of Novel Retroviruses to Better Define Retroviral Evolution and Cocarcinogenesis.

E1. ISOLATION AND CHARACTERIZATION OF A NEW HISTIOCYTOMA/FIBROSARCOMA-INDUCING VIRUS.

An acute transforming virus was isolated from mice inoculated with a virus obtained by iododeoxyuridine induction of methylcholanthrene-transformed C3H/10T1/2 cells. This virus, designated 3611-MSV, transforms embryo fibroblasts and epithelial cells in culture. Mice inoculated with 3611-MSV at birth develop tumors within four weeks; these contain several distinct mesenchymal cell types with fibroblasts as the predominant component. This new virus isolate resembles previously described mammalian acute transforming viruses in that it is replication-defective, requiring a type C helper virus for successful propagation both in vitro and in vivo. Several nonproductively transformed clones were isolated by endpoint transmission of 3611-MSV to mouse or rat cells. Pseudotype virus stocks obtained from such clones transform cells in vitro, are highly oncogenic in vivo, and exhibit host range and serologic properties characteristic of the helper virus. The major 3611-MSV translational product has been identified as a 90,000 molecular weight polyprotein (P90) with amino-terminal MuLV gag gene proteins, p15 and p12, linked to an acquired, sequence-encoded, nonstructural component. In contrast to gene products of many previously described mammalian transforming viruses, the 3611-MSV-encoded polyproteins lack detectable protein kinase activity. Additionally, 3611-MSV-transformed cells resemble those of the chemically transformed cell line, C3H/MCA-5, from which 3611-MSV was originally derived, in that they do not exhibit overall elevated levels of phosphotyrosine. This virus isolation led to the detection and characterization of v-raf, a new oncogene described above in A1 through A4.

E2. GENOME STRUCTURE OF MINK CELL FOCUS-FORMING MURINE LEUKEMIA VIRUS IN EPITHELIAL MINK LUNG CELLS TRANSFORMED IN VITRO BY IODODEOXYURIDINE-INDUCED C3H/MuLV CELLS.

Mink cell focus-forming (MCF) murine leukemia viruses that were isolated from C3H/MCA-5 cells after induction with 5-iododeoxyuridine in culture were characterized. Mink lung epithelial cells malignantly transformed in vitro by induced virus were the source of four molecular clones of MCF virus, CI-1, CI-2, CI-3, and CI-4. Three clones, CI-1, CI-2, and CI-3, had full-length, MCF viral genomes, one of which (CI-3) was infectious. In addition, a defective viral genome (CI-4) was obtained which had a deletion in the envelope gene. A comparison between the envelope genes of CI-4 and those of spleen focus-forming virus (SFFV) by heteroduplex mapping showed close homology in the substitution region and defined the deletion as being similar to the p15E deletion of SFFV. The recombinant MCF genomes are not endogenous in C3H/MCA-5 cells and, therefore, must have been formed in culture after induction by 5-iododeoxyuridine. CI-3, the infectious clone of MCF murine leukemia virus, was dualtropic, and mink cells infected with CI-3 were altered in their response to EGF. In the presence of EGF at 10 ng/ml, uninfected mink cells retained their epithelial morphology in monolayer culture and did not form colonies in soft agar. In contrast, CI-3 virus-infected mink cells grew with fibroblastic morphology in monolayer culture and showed an increased growth rate in soft agar in the presence of EGF.

E3. ANALYSIS OF THE ENV GENE OF MOLECULARLY CLONED MCF/MuLV RECOMBINANTS ISOLATED IN VITRO WHICH ARE CAPABLE OF TRANSFORMING CELLS IN CULTURE.

2408 nucleotides of CI-3 DNA, including the MCF envelope gene, have been sequenced and compared to ecotropic AKV and dualtropic Moloney MCF sequences. The recombination junctions are within the polymerase gene, less than 50 nucleotides upstream from the MCF-specific BamHI site, and 15 nucleotides 5' of the gp70/p15E cleavage site. The nature and location of the recombinant junctions favor a mechanism involving DNA exchange during reverse transcription, as observed by Junghans et al. The 597 nucleotide, Prp15E sequence contains 5 base changes relative to AKV; the U3 sequences are identical to those of AKV (T1 #101 is absent). The MCF-specific glycoprotein region lies within the amino-terminal 216 amino acids. In contrast, the carboxy-terminal 158 amino acids exhibit an overall homology of 87% to ecotropic AKV sequences, conserving the locations of apolar stretches, cysteine and proline residues, and glycosylation sites. This recombinant appears to be a mirror image of those viruses described by Thomas and Coffin which arise early during the development of leukemogenic MCF virus in vivo. A SFFV-like deletion of CI-4 has removed 696 nucleotides which code for 3 of the 6 glycosylation sites of gp70 and the extensive hydrophobic regions surrounding the gp70/p15E junction. The pre-existent reading frame is uncompromised; the carboxy-terminal p15E sequences remain. Interestingly, this deletion is flanked by a direct repeat (TGGTANCGGGA). Mice infected with CI-4 virus develop, within 3-9 months, malignant lymphomas of granulocytic as well as lymphocytic lineage.

E4. CHARACTERIZATION OF A LUNG CARCINOMA TUMOR CELL LINE DERIVED BY INOCULATION OF NEWBORN NSF/N MICE WITH A CARCINOMA-INDUCING RETROVIRUS.

A cell line was established from an alveologenic lung adenocarcinoma that developed in newborn NFS/N mice after intraperitoneal inoculation with a carcinoma-inducing retrovirus. A clonal derivative of this cell line, 3041, forms

adenocarcinomas when injected into syngeneic weanling mice and in culture retains several properties typical of alveologenic type I cells. These include morphological details, such as tight junctions and interdigitating microvilli in the areas of cell-cell contact, as well as the ability to transport fluid in culture. Fluid transport becomes evident in confluent cultures by the formation of domes or hemicysts. The formation of domes, which is considered to reflect a differentiated function of these secretory epithelial cells, can be enhanced more than 50-fold by treatment with dibutyryl cyclic adenosine monophosphate, but only 5- and 3-fold by sodium n-butyrate and dimethyl sulfoxide, respectively. It is anticipated that the secretory alveologenic cell line, 3041, described here will be useful for studies for both its pathological and physiological properties.

E5. A VIRUS-INDUCED OVARIAN TERATOCARCINOMA.

The experiments that led to the isolation of viruses which induced lung carcinomas also yielded viruses associated with induction of ovarian carcinoma (Rapp, U. R. and Todaro, G. J., 1980). Some of these ovarian adenocarcinomas had the characteristics of a teratocarcinoma. Differentiating cell lines from one such tumor were established in culture and, also, epithelial stem cells which form embryonal bodies in vivo were obtained. This is the first time that such a tumor has been induced by a retrovirus. During differentiation of the myogenic and adipogenic clonal lines that were derived, the cells began to produce infectious virus, whereas this virus was repressed in the undifferentiated lines. Presently, studies are in progress involving (1) analysis of the biological properties of the virus recovered from this tumor, including tumorigenicity and in vitro transforming properties; (2) determination of the genome structure of the virus after molecular cloning; and (3) examination of the integrated viral genome in the tumor cells.

OTHER MATTERS: Effective on the first of March of this year, Dr. George J. Todaro, the Chief of the Laboratory resigned to accept a non-government position. Due to the decentralized organization of the Laboratory and the leadership of distinguished scientists in the Sections, the scientific activities of each Section have continued on in a strong fashion, even in the Section formerly headed by Dr. Todaro. That scientific momentum has resulted in the generation of a significant number of excellent publications as seen in this report. It is expected that by the beginning of FY-84, the Laboratory will be reorganized under the leadership of a new Chief(s), as may be recommended by the DCCP and the NCI administrations in coordination with the DCCP Board of Scientific Counselors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04825-10 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of the Nature and Control of Endogenous (RD-114-like) Viruses in Cats

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

D. K. Haapala Microbiologist, LVC, NCI

COOPERATING UNITS *(if any)*

Dept. of Microbiology, Georgetown Univ. School of Medicine and Tropical Diseases, Washington, DC; Dept. of Virology, Institute Pasteur, Paris, France; Laboratory of Molecular Virology and Carcinogenesis, Litton Bionetics, Inc., Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Uffice of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Feline embryonic cell lines have been developed as a model system for endogenous type C virus control. Although no absolute virus replication restriction exists, all embryonic lines tested could be classified into two groups based on the phenotypic expression of endogenous virus pseudotypes of murine sarcoma virus. Intracellular repression of RD-114-like feline endogenous virus (FEV) pseudotypes of both Moloney and Kirsten transforming viruses was seen. Phenotypic expression (transformation) was blocked in restrictive cells even though virus replication occurred. Cloned and subcloned restrictive cells had normal diploid karyotypes and displayed the same phenotypes as the parental cells. A novel "population effect" of multiplicity of infection (MOI) was observed: As the MOI of FEV increased above 0.1 in restrictive feline embryo fibroblast (FEF) cells, the number of virus-producing cells actually decreased, although the same virus stocks titrated normally on permissive FEF cells. An endogenous FEV with a novel coat was isolated and characterized. This virus apparently arose from the recombination of the gag-pol genes of FEV with the envelope-p15E region of a previously undetected virus. An S+L- cell subline with improved rapidity, sensitivity, and quality of foci was developed and has proven useful for the assay of several type C viruses of primates but not, to date, for exogenous human viruses of the HTLV/ATLV/AIDS groups.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

None

Objectives:

To characterize and utilize viral nucleic acids and proteins as diagnostic and analytical tools for probing the nature and mechanism(s) of viral oncogenesis. To study different virus-cell interactions to determine the nature of the control mechanism(s) operant in eukaryotic cells.

Methods Employed:

Standard biochemical, biophysical, cell and virus culture methods were used. These included ultracentrifugal, chromatographic, restriction enzyme, Southern transfer, nucleic acid hybridization, molecular cloning, gel filtration, electrophoretic, immunological and in vitro protein synthesis techniques.

Major Findings:

1. Studies of the intracellular control of an endogenous virus: The use of feline embryonic cells as a model system. As reported last year, we have searched for different phenotypes of feline embryonic cells based on the intracellular restriction of RD-114-like feline endogenous virus (FEV). All lines tested have supported the growth of FEV and productive infection is established with one-hit kinetics in contrast with two-hit, Fv-1 restriction in mice. The feline embryo fibroblast (FEF) cells used by Fischinger and O'Connor, which also supported FEV growth in contrast to previous reports, were used for a restrictive cell model in further studies. These cells, originally started from a pool of two embryos, were cloned on their own mitomycin-arrested feeder layer. The clones and subclones isolated supported FEV growth and also showed the restrictive phenotype of the parental population. All clones and subclones displayed normal diploid karyotypes. Thus, FEV growth and phenotypic expression do not result from a heterogeneous population effect. It is also clear that these traits do not result from a chromosomal "imbalance" of trans-acting promoters or repressors acting on FEV in a manner described for SV-40 control, for example. Although we found no absolute restriction of FEV replication in all embryonic cell lines tested, we were able to define two distinct groups based on the phenotypic expression of FEV pseudotypes of murine sarcoma virus (MSV). Permissive cells allowed transformation by MSV, whereas those classified as restrictive could be transformed only after addition of a heterologous helper virus such as feline leukemia virus (FeLV). S^+L^- cells made from the two groups behaved as predicted; permissive cells showed focus induction when infected with FEV, but restrictive cells did not. We have attempted to study the molecular nature of FEV phenotypic restriction using both normal and S^+L^- restrictive cells. Attempts to mass-infect these cells (the normal cells with MSV/FEV and the S^+L^- cells with FEV per se) have failed. When using an increasing multiplicity of infection (MOI) (from 0.1 to 3), a decreasing number of infected cells is

obtained. This "population effect" is reproducible, novel, and frustrating. We have established that the effect is caused by the restrictive cells per se since the same highly purified virus stocks give linear infection curves on permissive cat and mink cells. We find that a maximum number of restrictive S^+L^- cells (1-3%) become virus producers after infection with FEV at an MOI of 0.1. This, of course, precludes meaningful studies on the expression of MSV RNA and proteins (for which the reagents have been prepared) in the phenotypically repressed cell population. Repeated attempts to isolate a producer cell from these infected populations have failed to date. We have recently introduced the KiSV/FEV into the cells and will attempt to isolate a flat producer cell for further investigation since these studies have shown that KiSV/FEV is, like MSV, phenotypically repressed by restrictive FEFs and can be detected by adding a heterologous helper virus, e.g., FeLV. It is possible that the phenotypic repression of FEV in the presence of virus replication might serve as a natural model for a mutant mouse cell type recently reported by Bassin et al. (Proc. Natl. Acad. Sci., USA, in press) which suppresses phenotypic expression of several oncogenes while allowing virus replication.

2. The development of a rapid biological assay for primate oncornaviruses. The ease and rapidity of S^+L^- cell assays makes them of great utility in the biological detection of a single replicating type C virus. The FEF S^+L^- line, PG-4, is more rapid than other cat or mink lines available. In a general survey, we have found the line to be suitable for assaying primate viruses as well as mink cell focus-forming virus (MCF) and xenotropic mouse viruses. Large, readily visualized foci appear in 4-5 days and can be rapidly counted microscopically or macroscopically after cell fixation and staining. Primate viruses which induce foci include MAC-1, GaLV, and BaEV, as well as RD-114 which is presumably of primate origin. Bovine leukemia virus and reticuloendotheliosis virus (REV) do not induce foci nor do primate type D viruses. Mouse mammary tumor virus (MTV) will replicate in these cells but does not induce foci or rescue MSV. Neither does the Mason-Pfizer type B virus induce foci. Of obvious interest was whether human type C viruses could be detected by the PG-4 assay. So far, induced supernatant fluids from two cell lines producing human T cell leukemia virus (HTLV) do not induce foci. More recently, a third type C virus, perhaps of the HTLV/ATLV group, was detected in Dr. Chermann's laboratory at the Pasteur Institute (Science 220: 868, 1983) from a human with lymphadenopathy. This virus has not induced foci in PG-4 cells to date. Presumably this virus, like HTLV, is a horizontally transmitted virus exogenous to humans. Therefore, the question of whether PG-4 will prove useful in detecting endogenous human retroviruses is moot.

3. Isolation and characterization of a novel recombinant virus. An apparent coat variant of FEV was discovered in the course of a search for FEF host range variants. The genome of the new isolate, which was called L2LL, was compared with the RD-114-like co-isolate, called L1L, by restriction endonuclease mapping and Southern blot hybridization. Solution hybridization studies showed about 70% homology between the two isolates. Our mapping data confirm and extend that finding. A map of 12 restriction endonucleases which cut the genome of both viruses showed complete identity of 19 sites beginning at the 5' end and extending for 6 kilobase pairs (kbp). Within the remaining 3' 2.5-3.0 kbp, however, we found 5 site differences and an additional 500 base-pairs in the novel virus. These variations were confirmed by an almost complete lack of cross-hybridization

of the env-p15E regions between the two viruses. It appears that the long terminal repeats (LTRs) of the two viruses are closely related, although not identical. Additional protein studies showed complete identity of the p28 gag oligopeptide maps between the two isolates and authentic RD-114. The gp70s of L1L and RD-114 are also identical, but the novel coat of L2LL shows no similarity. The p15E of L2LL is also novel, as determined by Dr. Oroszlan using Western blot analyses. Taken together, these results strongly suggest that L2LL is a recombinant virus arising from a crossing over between normal RD-114-like FEV gag-pol genes and a new virus previously undetected. This virus would be expected to have its own gag-pol genes which might be as unlike those of FeLV or RD-114-like proviruses as are its gp70-p15E determinants. These results demonstrate that undetected, diverse, but functional, viral information can exist even in a thoroughly studied model system such as the domestic cat.

Significance to Biomedical Research and the Program of the Institute:

The use of viral proteins and nucleic acids as markers to study oncogenesis is essential. Increasing the number and availability of such markers will increase our ability to understand the oncogenic process. The question of how a cell organizes vast amounts of genetic material continues to be of fundamental importance. Cancer-causing viruses provide a tool which can be used to examine the control of a few specific gene products. Our studies on the intracellular control of endogenous, vertically transmitted, cat viruses in FEF cells could be a unique model for future studies of endogenous human viruses.

Proposed Course:

Studies on the intracellular control of FEV expression in cat cells will be continued. KiSV will be included in these studies to provide the additional capabilities of detecting both early and late viral expression in a clean genetic background. Available monoclonal antibodies directed against the Kirsten p21 transforming protein will aid these studies. Other cat embryo cells and RD-114-like virus isolates will be studied to determine if more subtypes exist. Immunological and biochemical methods will be employed to further characterize and distinguish the subtypes isolated to date. We are presently cloning the L1L and L2LL FEV isolates. Subclones of L2LL will be used to trace the phylogeny of this virus and to search for its "left arm". Because of the apparent recombination within the LTRs of these two viruses, these DNAs and the p15E junction will be sequenced. The possible role of L2LL in "non-viral" leukemias and lymphomas of cats will be examined. The utility of the PG-4 $S^{+}L^{-}$ assay line will be tested with new virus isolates including potential endogenous viruses of humans.

Publications:

Bassin, R. H., Ruscetti, S., Ali, I., Haapala, D. K. and Rein, A.: Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection. Virology 123: 139-151, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04868-08 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Generation of New Transforming Mouse Type C Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

U. R. Rapp · Visiting Scientist, LVC, NCI

COOPERATING UNITS *(if any)* Laboratory of Molecular Virology and Carcinogenesis, Litton Bionetics, Inc., Frederick, MD; Harvard Medical School, Boston, MA; Laboratory of Viral Immunobiology, Litton Bionetics, Inc., Frederick, MD; NCI-Navy Medical Oncology Branch, Bethesda, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	0.7	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

New transforming murine type C viruses were characterized with respect to their genome structure and their ability to transform cells in culture: (1) A lung carcinoma-associated virus (LCAV, CI 1-4) was molecularly cloned and shown to have a mink cell focus-forming (MCF)-type structure. Transformed mink lung cells harboring this virus were sensitized to epidermal growth factor (EGF) suggesting that the mechanism of transformation by LCAV may involve sensitization of alveologenic lung cells to EGF. The nucleic acid sequence of CI-3 virus showed that the entire envelope gp70 was derived from an endogenous dualtropic env gene; (2) A new oncogene, raf, from 3611 virus was molecularly cloned, sequenced, and used for the isolation of its human cellular homologs which were mapped on human chromosomes 3 and 4. The translational products of this virus were P90 and P75 polyproteins which contained viral p15 and p12 structural proteins fused to a tumor gene product lacking tyrosine-specific phosphokinase activity. The two polyproteins differ only by the fact that P90 is a glycosylated form, whereas P75 is myristilated. A suggested link of c-raf to human small cell lung carcinoma and a familial renal carcinoma is being analyzed; and (3) Several differentiating clonal lines were derived from a cell line established from a virus-induced ovarian carcinoma.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George E. Mark	Expert	LVC, NCI
Tom I. Bonner	Guest Worker	LVC, NCI
Pramod Sutrave	Visiting Fellow	LVC, NCI

Objectives:

To isolate and characterize "transforming genes" that have been incorporated into the genome of nontransforming type C viruses and to employ these new viruses for the development of immunological reagents directed against the products of their human homologs.

Methods Employed:

Directly transforming retroviruses generally are characterized by the presence of cell-derived, transformation-specific sequences that have been incorporated into the retroviral genome. We have developed an in vitro system that has allowed, for the first time, the systematic isolation of new and tissue-specific transforming type C viruses. The starting materials were cells chronically infected with murine leukemia virus (MuLV) after induction of endogenous type C virus with iododeoxyuridine (IUDR). Such virus stocks contained minority component viruses with specific toxicity or transforming activity for selected target cells. They could be obtained by cloning virus from the progeny of acute infections of highly permissive cells, such as chemically transformed C3H/10T1/2 cells or SC-1 cells. Tumors that developed upon inoculation of such selected virus stocks into newborn mice were established in culture and used as a source of specific tumor-inducing viruses. Cloned virus stocks which induced specific tumors were further purified and characterized by molecular cloning in phage vectors, the nucleic acids of their acquired oncogenes were sequenced and the human and mouse cellular homologs were isolated.

Major Findings:

1a. Derivation of directly transforming type C virus from cell cultures. Alveologenic lung carcinoma-inducing virus which had a latency period of 6-12 months was isolated in vitro by selection of virus with the ability to transform mink lung cells from IUDR-induced C3H/MuLV. Transformed mink lung cells were cloned in soft agar and a particular clone of productively transformed cells was the source of lung carcinoma virus. These cells produce a replicating, recombinant mink cell focus-forming (MCF) class of MuLV and contain, in addition, persistent, unintegrated viral genomes in circular as well as linear forms. Subcloning of these cells in soft agar showed continued segregation of producer and nonproducer, transformed as well as revertant, cells. We have cloned the unintegrated circular provirus from productively transformed mink lung cells. The recombinant genome of the CI-3 virus, described below, was preexistent as a minority component in the IUDR-induced population of C3H/MuLV since an identical genome was isolated by molecular cloning from this stock.

1b. Genome structure of mink cell focus-forming murine leukemia virus in epithelial mink lung cells transformed in vitro by IUDR-induced C3H/MuLV cells. We characterized mink cell focus-forming murine leukemia viruses that were isolated from C3H/MCA-5 cells after induction with 5-IUDR in culture. Mink lung epithelial cells malignantly transformed in vitro by induced virus were the source of four molecular clones of mink cell focus-forming virus, CI-1, CI-2, CI-3, and CI-4. Three clones, CI-1, CI-2, and CI-3, had full-length mink cell focus-forming viral genomes, one of which (CI-3) was infectious. In addition, we obtained a defective viral genome (CI-4) which had a deletion in the envelope gene. A comparison between the envelope genes of CI-4 and those of spleen focus-forming virus (SFFV) by heteroduplex mapping showed close homology in the substitution region and defined the deletion as being similar to the p15E deletion of spleen focus-forming virus. The recombinant mink cell focus-forming genomes are not endogenous in C3H/MCA-5 cells and, therefore, must have been formed in culture after induction by 5-IUDR. CI-3, the infectious clone of mink cell focus-forming MuLV, was dualtropic, and mink cells infected with CI-3 were altered in their response to epidermal growth factor (EGF). In the presence of EGF at 10 ng/ml, uninfected mink cells retained their epithelial morphology in monolayer culture and did not form colonies in soft agar. In contrast, CI-3 virus-infected mink cells grew with fibroblastic morphology in monolayer culture and showed an increased growth rate in soft agar in the presence of EGF. In attempts to understand the mechanism of transformation of this new virus, we then determined the growth requirement of transformed mink epithelial lung cells. Cells harboring CI-2,3 and 4 viruses were found to be sensitized towards the action of EGF, both in terms of morphological alterations as well as in regard to their growth in soft agar. These findings suggest that the mechanism of transformation by the lung carcinoma virus may involve the sensitization of alveologenic mink lung cells to the action of EGF.

1c. Analysis of the env gene of molecularly cloned MCF/MuLV recombinants isolated in vitro which are capable of transforming cells in culture. 2408 nucleotides of CI-3 DNA, including the MCF envelope gene, have been sequenced and compared to ecotropic AKV and dualtropic Moloney MCF sequences. The recombination junctions are within the polymerase gene, less than 50 nucleotides upstream from the MCF-specific BamHI site, and 15 nucleotides to the 5' terminal of the gp70/p15E cleavage site. The nature and location of the recombinant junctions favor a mechanism involving DNA exchange during reverse transcription, as observed by Junghans et al. The 597-nucleotide, Prp15E sequence contains 5 base changes relative to AKV; the U3 sequences are identical to those of AKV (T1 #101 is absent). The MCF-specific glycoprotein region lies within the amino-terminal 216 amino acids. In contrast, the carboxy-terminal 158 amino acids exhibit an overall homology of 87% to ecotropic AKV sequences, conserving the locations of apolar stretches, cysteine and proline residues, and glycosylation sites. This recombinant appears to be a mirror-image of those viruses described by Thomas and Coffin which arise early during the development of leukemogenic MCF in vivo.

The SFFV-like deletion of CI-4 has removed 696 nucleotides which code for 3 of the 6 glycosylation sites of gp70 and the extensive hydrophobic regions surrounding the gp70/p15E junction. The preexistent reading frame is uncompromised; the carboxy-terminal p15E sequences remain. Interestingly, this deletion is flanked by a direct repeat (TGGTANCGGGA). Mice infected with CI-4 virus

develop, within 3-9 months, malignant lymphomas of granulocytic as well as lymphocytic lineage.

2a. A new histiocytoma/fibrosarcoma-inducing virus. An acute transforming virus was isolated from mice inoculated with a virus obtained by IUDR induction of methylcholanthrene-transformed C3H/10T1/2 cells. This virus, designated 3611-MSV, transforms embryo fibroblasts and epithelial cells in culture. Mice inoculated with 3611-MSV at birth develop tumors within four weeks which contain several distinct mesenchymal cell types with fibroblasts as the predominant component. This new virus isolate resembles previously described mammalian acute transforming viruses in that it is replication-defective, requiring a type C helper virus for successful propagation both in vitro and in vivo. Several nonproductively transformed clones have been isolated by endpoint transmission of 3611-MSV to mouse or rat cells. Pseudotype virus stocks obtained from such clones transform cells in vitro, are highly oncogenic in vivo, and exhibit host range and serologic properties characteristic of the helper virus. The major 3611-MSV translational product has been identified as a 90,000 (P90) molecular weight (M_r) polyprotein with amino-terminal MuLV gag gene proteins, p15 and p12, linked to an acquired, sequence-encoded, nonstructural component. In contrast to gene products of many previously described mammalian transforming viruses, the 3611-MSV-encoded polyproteins lack detectable protein kinase activity. Additionally, 3611-MSV-transformed cells resemble those of the chemically transformed cell line, C3H/MCA-5, from which 3611-MSV was originally derived, in that they do not exhibit overall elevated levels of phosphotyrosine.

2b. DNA sequence of v-raf. The nucleotide sequence of 1.5 kilobases (kb) encompassing the transforming gene (v-raf) of 3611-MSV has been determined. v-raf sequences were found to have been inserted into the p30 region of an ecotropic MuLV, with the concomitant deletion of the 2.4 kb extending to the middle of the polymerase gene. A 5 nucleotide direct repeat exists at each end of the v-raf sequences. A single nucleotide deletion, 10 bases upstream from the acquired sequences, places the oncogene in an open reading frame terminated by an amber triplet approximately 180 nucleotides from the 3' onc/MuLV junction. Sequences typical of eukaryotic promoters are not found as part of the inserted oncogene. From the deduced amino acid sequence a hybrid gag-raf polyprotein would have a molecular weight of approximately 75 kilodaltons. Consistent with the gag-x structure, we find that only the P75 polyprotein is modified by the fatty acid myristate, whereas only the P90 polyprotein is glycosylated. Comparison of the deduced v-raf amino acid sequence with other oncogenes revealed domains homologous to v-src and v-mos.

2c. Deletion mapping of v-raf. Subclones of 3611-MSV containing deletions from either the 5' or 3' ends of v-raf were tested for transforming activity in transfection assays. A subclone of the viral genome containing the 5' long terminal repeat (LTR), gag region, and the entire oncogene was treated with Bal-31 exonuclease to generate the deleted clones. In clones containing deletions from the 3' end it was determined, by sequence analysis, that 220 nucleotides could be removed from v-raf while still maintaining transforming ability; a clone missing 260 nucleotides did not transform. The normal termination codon for v-raf is located 180 nucleotides in from the 3' end. The studies on the 5' end showed that no more than 35 nucleotides could be removed before a loss in transforming ability was observed. Protein data on the 3'

deleted clones show that both the P75 and P90 are being synthesized, indicating that the carboxy end is not responsible for the size difference in the proteins.

2d. Human cellular homologs of v-raf. We obtained human DNA clones covering 27-31 kb from each of two v-raf related loci in the human genome. The restriction fragments of these two loci account for essentially all of the bands detected in human DNA using a v-raf probe. The first locus, c-raf-1, has sequences related to the full 1.2 kb of v-raf. These sequences span 7 kb and contain at least 4 introns. The second locus, c-raf-2, has only 0.45 kb of v-raf related sequence which occurs as a single block lacking two of the introns present in c-raf-1. By taking advantage of an SphI site which is conserved between v-raf and c-raf-1, we constructed a DNA which contains the 5' portion of the transforming virus including the 5' one-third of the v-raf gene spliced to the 3' two-thirds of the human c-raf-1 gene. Upon transfection this construct transforms NIH/3T3 cells. Since the 5' one-third of the v-raf gene will not transform by itself, the transforming ability of the construct implies the expression of a polyprotein-containing Moloney MuLV gag, mouse v-raf and human c-raf components.

2e. Chromosomal mapping of c-raf-1 and -2 in man. We used somatic cell hybrids to map c-raf-1 to human chromosome 3. c-raf-2 resides on chromosome 4. The presence of c-raf-1 on chromosome 3 suggests that it might be involved in small cell lung carcinomas which characteristically have deletions on the short arm of chromosome 3, usually beginning at band p14 and extending to p23 or beyond. The possibility of rearrangements in the vicinity of c-raf-1 in these tumors is under study.

3. Characterization of lung carcinoma tumor cells. A cell line was established from an alveologenic lung adenocarcinoma that develops in newborn NFS/N mice after intraperitoneal inoculation with a carcinoma-inducing retrovirus. A clonal derivative of this cell line, 3041, forms adenocarcinomas when injected into syngeneic weanling mice and in culture retains several properties typical of alveologenic type I cells. These include morphological details, such as tight junctions and interdigitating microvilli in the areas of cell-cell contact, as well as the ability to transport fluid in culture. Fluid transport becomes evident in confluent cultures by the formation of domes or hemicysts. The formation of domes, which is considered to reflect a differentiated function of these secretory epithelial cells, can be enhanced more than 50-fold by treatment with dibutyryl cyclic adenosine monophosphate but only 5- and 3-fold by sodium n-butyrate and dimethyl sulfoxide, respectively. It is anticipated that the secretory alveologenic cell line, 3041, described here will be useful for studies of both its pathological and physiological properties.

4. A virus-induced ovarian teratocarcinoma. The experiments that led to the isolation of the virus which induced lung carcinomas also yielded a virus associated with induction of ovarian carcinoma (Rapp, U. R. and Todaro, G. J., 1980). Some of these ovarian adenocarcinomas had the characteristics of a teratocarcinoma. We succeeded in establishing differentiating cell lines from one such tumor in culture and also obtained epithelial stem cells which form embryonal bodies in vivo. This is the first time that such a tumor was induced with a retrovirus. During differentiation of the myogenic and adipogenic clonal lines that we derived, the cells began to produce infectious virus, whereas this

virus was repressed in the undifferentiated lines. We are presently involved in: (1) analysis of the biological properties of virus recovered from this tumor, including tumorigenicity and in vitro transforming properties; (2) determination of the genome structure of the virus after molecular cloning; and (3) examination of the integrated viral genome in the tumor cells.

Significance to Biomedical Research and the Program of the Institute:

The goal of this research is the isolation of new cell-derived transforming functions from mouse and human cells. Research from several laboratories over the past several years has demonstrated that spontaneous and chemically induced tumors from mouse and man may have switched on oncogenes identical to those of directly transforming retroviruses. These important findings emphasize the relevance of retroviral oncogenes for an understanding of human malignancy. Another recent advance made in the study of mouse and primate retroviruses was the finding of endogenous human type C virus genes. The previous demonstration of exogenous human T cell leukemia virus (HTLV), therefore, also emphasizes the importance of another class of transforming mouse retroviruses, the MCF class, which are recombinants between exogenous and endogenous MuLV and includes SFFV, as well as our lung carcinoma virus. The new human oncogenes c-raf-1 and c-raf-2 are now being studied for a potential role in human malignancy.

Proposed Course:

The present emphasis will continue on the detailed characterization of viral genomes of nonproducer, transformed mink lung epithelial cells as well as on the virus-induced lung adenocarcinoma cells. Although the viral genome which persists as an unintegrated provirus in productively transformed mink cells has been purified by molecular cloning, it is still possible that nonproducer, transformed mink cell subclones which have been isolated may yet be found to contain an integrated viral genome which may be either a virus cell recombinant or could act by a "promoter-insertion" mechanism. Since the yield of virus rescue by superinfection with helper virus is extremely low, conventional approaches (e.g., the preparation of transforming virus-specific DNA probes or isolation of subgenomic size proviral DNA from Hirt extracts of acutely infected cells) are not readily applicable. Attempts are in progress to select transformants which are more susceptible to rescue. In addition, the recently obtained subclones of lung carcinoma-associated virus will be used to isolate integrated provirus of mouse origin by molecular cloning.

1. Confirmation of the etiological relationship of virus to murine lung tumors. In order to definitively establish whether the viral genome (molecularly cloned from in vitro transformed mink lung cells) is indeed the causative agent in the induction of lung tumors, we will pursue the following lines of research: (1) tumor induction in NFS/N mice with transfected molecularly cloned virus in the presence and absence of various helper MuLVs; and (2) transfection and selection of transformed foci with DNA from (a) the transformed mink lung cells that were the source of the MCF class viruses CI-2,3 and CI-4 described above and (b) cellular DNA from the induced lung carcinoma as well as lung carcinoma cell lines.

2. Completion of c-raf characterization. Both mouse and human c-raf genes will be analyzed. The 5' end of the cellular gene will be determined with the help of c-raf mRNA from cells in which this gene is expressed. We have already identified cell lines with a high level of expression. The transforming activity of c-raf from mouse and man will be tested by itself and in conjunction with viral LTRs. In the mouse, activity of the gene from MCA-5 cells will be compared with that from untransformed cells. Expression of human c-raf will be used for the production of antibodies directed to its product(s). We have already achieved expression of the 3' two-thirds of human c-raf in mouse cells which restore transforming activity to the partially deleted v-raf gene.

The sequence of c-raf, minimally including all exons will be determined in order to better understand its evolutionary relationship to other cellular oncogenes as well as for the production of synthetic peptides needed as immunogens.

The precise chromosomal location of c-raf will be obtained by an extension of our previous somatic hybrid analysis to include in situ hybridization (collaboration with Dr. Phillip Leder) as well as use of recombinant inbred mouse strains to detect their linkage to other cellular genes.

The potential involvement of c-raf in certain human tumors will continue to be tested in two ways: (1) Its position relative to chromosomal rearrangements involving human chromosome 3 is being determined in human small cell lung carcinoma cell lines that have specific deletions on chromosome 3 and in a familial renal carcinoma (collaboration with Dr. Phillip Leder) which is specifically associated with a translocation involving chromosome 3. (2) The search for human tumors with a high level of c-raf expression.

A potential physiological role for c-raf as well as the genetics of its regulation will be studied in mice. We will first screen inbred mouse strains for expression-polymorphism by determining levels of c-raf transcription in various tissues of fetal and adult animals. Strains with different expression levels will then be used in genetic crosses to examine number, dominance or recessiveness and chromosomal map position(s) of the regulatory gene(s) involved.

3. Completion of v-raf characterization. v-raf will be further characterized in respect to its origin, mode of acquisition, potential involvement in MCA-5 transformation, location of the gene product of its cellular homolog and in regard to functional domains by site-specific mutagenesis.

The origin of v-raf will be investigated by a search for a polymorphic marker that may distinguish the gene in different mouse strains. In the absence of such a marker the virus stock that went into the NSF/N mice in which the original tumor developed will have to be examined. We may also decide to determine the frequency of v-raf repair in various differentiated cell types in order to pinpoint a probable cell type in which the original transduction may have occurred.

The question of raf involvement in MCA-5 transformation will be examined first by analysis of its expression in these cells. Preliminary experiments (dot blot hybridization) show it to be expressed, perhaps at elevated levels, relative to

the untransformed C3H/10T1/2 parent cell line. Another line of experiments, employing mainly Southern blot analysis, will determine whether or not c-raf is amplified, rearranged or structurally altered in MCA-5 cells. Preliminary experiments indicated some differences in the size of fragments hybridizing to v-raf DNA between MCA-5 and normal mouse cell DNAs. We will also determine, by transfection of MCA-5 DNA on to untransformed C3H/10T1/2 cells, whether transforming DNA has a restriction pattern compatible with c-raf.

4. Localization of c-raf and v-raf products within the cell. Localization of c-raf and v-raf products within the cell will be determined first by use of indirect immunofluorescence employing raf-specific antibodies that are being prepared against synthetic peptides synthesized on the basis of v-raf sequence data. This work will be performed in collaboration with Drs. L. Henderson, S. Oroszlan and M. Gonda at the Frederick Cancer Research Facility. Intracellular association of raf peptides will be followed by cell fractionation and reconstruction experiments using purified raf peptide(s) from expression vectors containing v-raf DNA after growth in E. coli. Part of this work will be performed in collaboration with Dr. K. Moelling at the Max Planck Institute, Berlin, who first described the DNA binding properties of some onc gene products and developed specific in vitro binding assays for this purpose.

A functional analysis of v-raf will include site-specific mutagenesis of v-raf DNA. We currently plan to use as a strategy for mutagenesis a technique developed in Dr. Bruce Wallace's laboratory which uses synthetic oligonucletide-directed mutagenesis for producing specific point mutations in cloned DNA.

5. Characterization of additional viral isolates. Finally, we would like to begin work on some of the other acutely transforming, perhaps tumor gene transducing, virus isolates that we have previously isolated and which are currently held in reserve. It is particularly important to be able to analyze these additional isolates for several reasons: (1) they might contain additional, unique oncogenes; and (2) their structure might give clues to their origin and also may help determine the reproducibility of our isolation procedure for new oncogenic retroviruses. It is hoped that some of this information will also help in our understanding of the mechanisms of oncogene transduction which, in turn, may allow us to improve the frequency of future in vivo and in vitro isolations.

Publications:

Bonner, T. I., O'Brien, S. J ., Nash, W. G., and Rapp, U. R.: A new oncogene is located on human chromosome 3. Science (In Press)

Bonner, T. I., O'Connell, C. and Cohen, M.: Cloned endogenous retroviral sequences from human DNA. Proc. Natl. Acad. Sci. USA 79: 4707-4713, 1982.

Bonner, T. I., and Rapp, U. R.: The human cellular homologue c-raf of a new mammalian oncogene. Nature (In Press)

Mark, G. E., Goldsborough, M. D., Borchert, P. A. and Rapp, U. R.: Localization of the transforming sequences of the oncogene v-raf by transfection with BAL-31 generated subgenomic fragments. J. Virol. (In Press)

Mark, G. E., Goldsborough, M. D. and Rapp, U. R.: Nucletoide sequence of the transforming gene of 3611-MSV: v-raf. Cell (In Press)

Mark, G. E. and Rapp, U. R.: Sequence analysis of the env gene from a molecularly cloned MCF class MuLV isolated from C3H/MCA 5 cells and determination of the location of the deletion in a SFFV-like mutant of this genome. J. Virol. (In Press)

Keski-Oja, J., Rapp, U. R. and Vaheri, A.: Transformation of mouse epithelial cells by acute murine leukemia virus 3611: Inhibition of collagen synthesis and induction of novel polypeptides. J. Cell. Biochem. 20: 139-148, 1982.

Kozak, K., Gunnell, M. A. and Rapp, U. R.: A new oncogene, c-raf, is located on chromosome 6 in the mouse. J. Virol. (In Press)

O'Brien, S. J., Bonner, T. I., Cohen, M., O'Connell, C. and Nash, W. G.: An endogenous retroviral sequence in normal human DNA is located on chromosome 18. Nature (In Press)

Rapp, U. R.: Kinetics of expression of Eco, Xeno and MCF-MuLV after BdUrd induction of cells from high and low leukemia mouse strains. J. Virol. 45: 755-765, 1983.

Rapp, U. R. and Barbacid, M.: Activation of an endogenous type C-virus in cells from the inbred mouse strain 129/J: Antigenic relationship with the horizontally transmitted type-C viruses of primates. Arch. Virol. (In Press)

Rapp, U. R., Birkenmeier, E., Bonner, T. I. and Gonda, M. A.: Genome structure of MCF/MuLV in Mv-1Lu epithelial mink lung cells transformed in vitro by IdUrd induced C3H/MuLV. J. Virol. 45: 740-754, 1983.

Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr. and Stephenson, J. R.: Structure and biological activity of v-raf, a new oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA (In Press)

Rapp, U. R., Gunnell, M. and Marquardt, H.: Normal mouse serum contains peptides which induce fibroblasts to grow in soft agar. In Fox, F. (Ed.): Proceedings of the ICN-UCLA Symposia. New York, Alan R. Liss (In Press)

Rapp, U. R. and Keski-Oja, J.: Murine leukemia virus-mediated transformation of a mouse epithelial cell line MMC-E. Cancer Res. 42: 2407-2411, 1982.

Rapp, U. R., Reynolds, F. H., Jr. and Stephenson, J. R.: Isolation of new mammalian type C transforming viruses. In Sternberg, N.L. and Pearson, M.L. (Eds.): Gene Transfer and Cancer. New York, Raven Press, 4th Technology Transfer Workshop Series (In Press)

Rapp, U. R., Reynolds, F. H. and Stephenson, J. R.: New mammalian transforming retrovirus: Demonstration of a polyprotein gene product. J. Virol. 45: 914-924, 1983

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04896-11 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of Gene Controlled Events in Neoplastic Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

S. J. O'Brien Geneticist, LVC, NCI

COOPERATING UNITS *(if any)* Laboratory of Molecular Virology and Carcinogenesis, Program Resources, Inc., Frederick, MD; University of California, San Diego, La Jolla, CA; National Zoological Park, Washington, DC; U.S.C., Los Angeles, CA; FDA, Bethesda, MD; Uniformed Services University of the Health Sciences, Bethesda, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	1.4	5.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Sexual and parasexual genetic analyses have permitted the detection, characterization and genetic localization of a number of mammalian cellular genes which participate in neoplastic transformation and retroviral expression. Seven classes of genes are under study: (1) endogenous cellular DNA sequences homologous to retroviral RNA; (2) integration sites of retroviruses in mammalian chromosomes; (3) growth factor receptors; (4) restriction genes which delimit viral replication; (5) enzyme structural genes; (6) cell surface antigens including major histocompatibility (MHC); and (7) cellular transforming genes (onc) rescued by defective retroviruses. Examples of each of these gene classes have been detected and mapped in human and feline model systems. Highlights include the genetic analysis of the ras gene family in man and cat; the genetic analysis of two families of endogenous retroviral loci in man (ERV1+), genetic mapping of the human homologous loci of the raf-1 and raf-2 murine oncogenes; genetic analysis of the RD-114 family in the cat; and genetic localization of 10 independent integration sites of HTLV in human T cell leukemias.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Charles J. Sherr	Senior Surgeon	LTVG, NCI
Malcolm Martin	Medical Officer	LMM, NIAID
Douglas R. Lowy	Deputy Chief	DB, NCI
Ulf R. Rapp	Visiting Scientist	LVC, NCI
Tom I. Bonner	Guest Worker	LVC, NCI
David E. Wildt	Senior Staff Fellow	LVC, NCI
Robert C. Gallo	Chief	LTCB, NCI

Objectives:

1. The construction of a biochemical genetic map of the domestic cat (Felis catus) with emphasis on genes which relate to the viral etiology of leukemia and lymphoma in the species. The specific classes of genes under study fall into seven general groups. The genes include: (1) endogenous cellular DNA sequences homologous to cDNA radioactive probes transcribed from retroviral genomic RNA; (2) chromosomal integration sites for exogenous retroviral insertion and persistence; (3) receptors on cell membranes which interact with virus glycoproteins to determine cell-species compatibility and viral host range; (4) restriction genes which delimit virus replication in various animal species; (5) cellular transforming (onc) genes; (6) cellular enzyme structural genes; and (7) cell surface antigens including antigens homologous to the major histocompatibility complex (MHC) of other mammalian species.

2. The combined application of somatic cell genetics and electrophoretic resolution of cellular DNA sequences following digestion with specific restriction endonucleases to the biology of retroviral integration, excision and transposition in human and feline cells.

3. Genetic analysis of cooperative and sequential gene action in the neoplastic processes and in virogene expression. This consideration involves application of the principles and techniques of microbial genetics to cultured mammalian cells.

4. The development of new approaches to the understanding of genetic control of carcinogenesis. These considerations involve the identification and characterization of genetic targets (cellular genes) of carcinogenesis.

5. Use of inherent genetic variation in human and mouse cells to provide genetic signatures of markers for cells studied in cell culture laboratories. By using allelic isozyme (allozyme) variation in different isolates of human or mouse cells, an allozyme genetic signature of cells can be derived for cell contamination monitors and for approaches to the genetics of development.

6. The genetic analysis of natural populations of man, mice and cats with specific emphasis on populations and cellular genes relating to the epidemiology of certain neoplasias with familial, virological or environmental etiology.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3) cytological chromosome banding procedures (G and Q) for specific chromosome identification; (4) virological procedures including radioimmune assay, reverse transcriptase and viral cloning; (5) immunological assays including radioimmune assay (RIA), cytotoxicity, fluorescent antibody procedures, surgical allograft, and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques including cDNA transcription in vitro, solution hybridization, HAP chromatography, visualization of restricted DNA by the techniques of Southern following agarose electrophoresis and molecular cloning of eukaryotic genes; and (7) in situ hybridization to metaphase chromosomes.

Major Findings:

1. Alignment of the feline and human linkage maps predicted and later proved a striking cytogenetic homology between the Primate and Felidae families. Development of the feline genetic map in our laboratory revealed that 35 biochemical loci assigned to 16 of the 19 cat chromosomes were particularly homologous in linkage to the human map using the same loci. Using high resolution G-trypsin banding (1000 bond level of resolution) we were able to demonstrate that 20% of the human genome could be aligned band for band to homologous regions. Linkage homologies in other regions were characterized by small intrachromosomal rearrangements. The striking concordance of feline and primate genetic maps has two major aspects of biological significance: first, the evolutionary implications are rather significant since the chromosome organization has maintained some semblance of order despite 80 million years of divergence (between primates and felids); and second, the comparative genetics has a predictive value, since once a gene has been located in the cat, a strong suggestion as to the position of a homologous locus in man can be made. This aspect may be especially important in identifying mammalian genes (like retroviruses or controlling elements) capable of transposition during mammalian evolution.

Two classes of somatic cell hybrid panels have been derived and characterized. The first was made with rodent x human lymphocytes and consists of approximately 60 hybrids segregating human chromosomes. The second panel was made with rodent x cat lymphocytes and consists of approximately 60 hybrids segregating cat chromosomes. Each hybrid was expanded, karyotyped using both G-11 banding and G-trypsin banding, and isozyme typed for 30 + isozymes previously assigned to the human or cat chromosomes, respectively. At the same passage, high molecular weight DNA was extracted for Southern analysis. DNA and isozyme extracts from these panels were used in several of the genetic assignments described below. These hybrid panels allow the precise chromosomal localization for virtually any human or cat gene for which an assay or molecular clone is available.

2. Adding to the feline gene map: lysosomal enzymes, rRNA, oncogenes, and the major histocompatibility complex. Lysosomal enzyme deficiencies in man lead to a number of heritable, neurological, storage diseases which have no effective treatment (e.g., Tay-Sachs disease, Hurler's syndrome, GM1 gangliosidosis,

mannosidosis, Maroteaux-Lamy syndrome). Models for many of these diseases have been described in the cat with included enzyme deficiencies. These genetic defects represent a good opportunity for monitoring gene therapy in an excellent human model. Toward these ends, the genetic mapping of 10 of these enzymes has been initiated using the cat hybrid panel and procedures developed in John O'Brien's laboratory, UCSD. Six of these (GALA, GALB, HEXA, MANA, GUSB, GLUA) have been assigned to specific feline chromosomes. In addition, hormone protocols for collecting viable cat embryos have been developed for embryo transplant and for gene delivery protocols.

Using ^{3}H-labeled RNA probes, rRNA genes have been localized to feline chromosome E1 by in situ hybridization. Procedures for single copy detection using in situ hybridization are in progress.

Molecular clones of each of 17 unique oncogenes have been obtained from initiating laboratories. Batch DNA extracted from the genetically characterized hybrids of the feline hybrid panel has been used to assign feline oncogene homologs to cat chromosomes. Preliminary assignments have been obtained for myc, c-Ha-ras-1, c-Ha-ras-2, c-Ki-ras-1, c-Ki-ras-2, fes, sis. Evidence for transposition of some, but not all, of these loci during the mammalian radiations has been derived.

Reciprocal skin grafts between 32 domestic cats were performed (3x each over a 9-week period) and the timing of autograft rejection was determined and correlated with the production of allogenic antibodies. A custom somatic cell hybrid panel positive for an allele which is particularly immunogenetic was derived and analyzed to genetically map the feline MHC locus. Inbred lines of specific MHC alleles are under development in Poolesville (NIH Animal Center).

3. Detection and chromosome mapping of mammalian genes which participate in transformation. As the feline (and human) genetic map was developing, a number of examples of cancer-associated loci have been identified and are at various stages of genetic analysis in their respective systems. Examples of these include: (1) retrovirus integration site: BEVI is a gene located on human chromosome six which is a high affinity site for integration of baboon endogenous virus; (2) receptors: the feline receptor for epidermal growth factor is modulated in transformed cells and has been shown to be controlled by two cellular genes both required for expression of epidermal growth factor receptor (EGFR). The two genes map to feline chromosomes A3 and C2, respectively; (3) restriction genes: BVR1 is a feline X-linked gene which restricts murine B-ecotropic virus in mouse x cat somatic cell hybrids. Akvr-1 is a murine restriction gene polymorphic in the feral, Lake Casitas, California mouse population which dominantly restricts viremia and associated leukemia in the AKR mouse. Akvr-1 has been shown to be allelic to the Fv-4 locus previously described in Mus musculus molossinus and has been mapped to murine chromosome 12; (4) oncogenes, cat, see above; man, see below; (5) endogenous viral sequences, RD-114, see below; and (6) major histocompatibility complex, see above.

4. Endogenous retroviral sequences in man. Using the human hybrid panel we have mapped two members of a baboon retroviral family, ERV1 and ERV2, to human chromosomes 18 and 15, respectively. This represents the first endogenous retroviral sequence described in humans. A second, larger family of Moloney

murine leukemia virus-related sequences consisting of approximately 30 members in the human genome has been found to be dispersed on several human chromosomes as well. Their relationship to unscheduled expression of linked oncogenes is under investigation.

5. Genetic analysis of human oncogenes. Nearly all of the human oncogene homologs have been mapped in a variety of laboratories in the last year. We have localized the four members of the ras family to four distinct human chromosomes (X, 6, 11, and 12) and the two members of the raf family to chromosomes 4 and 5. The association of these loci with specific chromosomal rearrangements of cancers is being examined.

6. The integration position of HTLV. The integration position of HTLV is being investigated in 4 classes of custom hybrids made from the 2 original, infected human patients HUT 102 and MJ. A high resolution cytogenetic analysis of these tumors revealed some specific rearrangements. The cell lines derived from these patients each underwent de novo proviral integrations in addition to the original provirus. Approximately 6 integrations from each human patient have been chromosomally identified and appear to disperse non-specifically throughout the human genome.

7. Molecular and genetic characterization of RD-114, a family of endogenous retroviral segments in the cat. We have derived a map of restriction endonuclease recognition sites in the genome of RD-114 retrovirus, an inducible, replication-competent member of this gene family. We compared its structure to restriction maps of nine molecular clones of endogenous RD-114 sequences isolated from a "library" of cat cellular DNA. The endogenous sequences analyzed are similar to each other in that they are colinear with RD-114 proviral DNA, are bounded by long terminal redundancies and conserve many restriction sites in the gag and pol regions. Several sequences have undergone a small deletion, relative to the inducible viral genome, in a region which may be important to encapsidation of viral RNA. The env regions of the endogenous RD-114 sequences examined are substantially deleted or diverged; a subset of these sequences contains information at the position of the env region which is not homologous to inducible RD-114 by restriction mapping or by hybridization. Analysis of cat cell DNA confirmed the conclusions about conserved restriction sites in endogenous sequences and indicated that a single locus may be responsible for the production of the major inducible form of RD-114.

Significance to Biomedical Research and the Program of the Institute:

The characterization of identified loci which participate in cell transformation has two important applications: (1) as the raw material for the dissection of developmental genetic analysis of the cellular events which lead to neoplastic transformation; and (2) as possible targets for carcinogens in screening protocols. The specific understanding of the developmental genetic sequence which characterizes the neoplastic event is necessary for any meaningful attempt to correct and to destroy cancerous tissues. A comprehensive genetics program, from the molecular to the biological species level, holds promise in the ultimate resolution of the neoplastic process in man.

Proposed Course:

The vigorous pursuit of genetic mapping of cellular transforming genes in cats and humans is anticipated. Genetically characterized panels of cell hybrids will be used as sources of DNA for restriction analysis using a variety of cloned probes of transforming genes. The same hybrid panel of cat will be used to extend the feline genetic map to include the major histocompatibility complex, endogenous RD-114 sequences, lysosomal enzymes and a number of new marker loci. The molecular biology of integration of baboon endogenous virus will be vigorously pursued by chromosome "walking" about the integration of several clones.

Publications:

Nash, W. G. and O'Brien, S. J.: Conserved subregions of homologous G-banded chromosomes between orders in mammlian evolution: The cat and man. Proc. Natl. Acad. Sci. USA 79: 6631-6635, 1982.

O'Brien, S. J. (Ed.): Genetic Maps. Bethesda, Frank Gumpert Printing, 1982, Vol. II, 315 pp.

O'Brien, S. J. and Barile, M. F.: Isozyme resolution in mycoplasmas. In Razin, S. and Tully, J. G. (Eds.): Methods of Mycoplasmology. New York, Academic Press, 1982, pp. 391-396.

O'Brien, S. J., Bonner, T. I., Cohen, M., O'Connell, C. and Nash, W. G.: Mapping of an endogenous retroviral sequence to human chromosome 18. Nature 303: 74-77, 1982.

O'Brien, S. J., Goldman, D., Merril, C. R., Bush, M. and Wildt, D. E.: The cheetah is depauperate in biochemical genetic variation. Science (In Press)

O'Brien, S. J., Moore, J. L., Martin, M. A. and Womack, J. E.: Evidence for the horizontal acquisition of murine AKR virogenes by recent horizontal infection of the germ line. J. Exp. Med. 155: 1120-1132, 1982.

O'Brien, S. J., Nash, W. G., Goodman, J. L., Lowy, D. R. and Chang, E. H.: Dispersion of the ras family of transforming genes to four different chromosomes in man. Nature 302: .839-842, 1982.

O'Brien, S. J., Nash, W. G., Winkler, C. A. and Reeves, R. H.: Genetic analysis in the domestic cat as an animal model for inborn errors, cancer and evolution. In Migaki, G., Desnick, R. J. and Patterson, D. F. (Eds.): Animal Models of Inborn Errors of Metabolism. New York, Alan R. Liss, 1982, pp. 67-90.

Pearson, P. L., Roderick, T. H., Davisson, M. T., Lalley, P. A. and O'Brien, S. J.: Comparative genetic mapping in mammals: Report of the international committee. Cytogenet. Cell Genet. 32: 208-220, 1982.

Seibert, K., Shafie, S. M., Tricke, T. J., Whang-Peng, J. J., O'Brien, S. J., Toney, J. H., Huff, K. K. and Lippman, M. E.: Clonic variation of MCF-7 breast cancer cells in vivo and in vitro. Cancer Res. (In Press)

Surti, U., Szulman, A. E. and O'Brien, S. J.: Dispermic origin and clinical outcome of three complete hydatidiform moles with 46, XY karyotype. J. Obstet. Gynecol. 144: 84-87, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04942-13 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Cellular Gene Products with Transforming Function

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

J. R. Stephenson Visiting Scientist, LVC, NCI

COOPERATING UNITS *(if any)*

Imperial Cancer Research Fund, London, England; Dept. Biol. Chem., Univ. Calif., Berkley, CA; Dept. Cell Biol. and Genetics, Erasmus University, Rotterdam, The Netherlands; Div. Biol., California Institute of Technology, Pasadena, CA

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.4	1.2	2.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human cellular homologs of several representative viral oncogenes including v-abl, v-fms and v-fes have been molecularly cloned by use of a cosmid vector system. Sequences representing the complete cellular homologs of these viral genes are dispersed over regions of from 12 to 40 kilobases (kb), are colinear with their viral counterparts, and contain variable numbers of intervening sequences. Sequences encoding the tyrosine phosphorylation acceptor region of the human c-abl oncogene have been identified, and by nucleic acid sequence analysis, shown to exhibit homology with acceptor regions of the v-src, v-yes, and v-fes/v-fps families of viral oncogenes. By somatic cell hybridization, c-fes has been localized to a region of human chromosome 15 that is translocated to chromosome 17 in acute promyelocytic leukemia, while c-fms has been mapped within the q24 band of chromosome 5. Using a similar approach, c-abl has been localized to the long (q) arm of chromosome 9. The further demonstration of the translocation of c-abl from chromosome 9 to chromosome 22 (the Philadelphia chromosome) in chronic myelogenous leukemia (CML) sublocalizes c-abl to the q34 terminal band of chromosome 9, establishes the Philadelphia translocation to be reciprocal, and raises the possibility that c-abl may be involved in CML. These findings, in combination with recent studies in the c-myc system, raise the possibility that the association of chromosomal rearrangements with specific human cancers may involve a direct or indirect influence on expression of cellular oncogenes. In other studies, we have identified and molecularly cloned a new mammalian oncogene, v-raf, established the role of cytosine methylation in regulating viral and cellular oncogene expression, and identified a 150,000 Mr, highly phosphorylated cellular glycoprotein that specifically binds to, and is phosphorylated by, viral oncogene-encoded protein kinases. Finally, a transforming growth factor produced by Snyder-Theilen feline sarcoma virus (FeSV) transformed rat cells has been purified to homogeneity and its amino acid sequence determined.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Fred H. Reynolds, Jr.	Expert	LVC, NCI
John Groffen	Visiting Fellow	LVC, NCI
Nora Heisterkamp	Visiting Fellow	LVC, NCI
Ulf R. Rapp	Visiting Scientist	LVC, NCI
George J. Todaro	Medical Officer	LVC, NCI
Daniel R. Twardzik	Research Chemist	LVC, NCI
George E. Mark	Expert	LVC, NCI

Objectives:

The primary objective of this project involves a determination of the potential role of the human homologs of prototype viral oncogenes, including v-fes, v-fms and v-abl, in naturally occurring tumors of man.

Methods Employed:

Cell culture techniques including microtiter procedures, isolation of transformation-defective viral and cellular mutants, and development of hybridoma cell lines. Biochemical and immunological procedures include gel electrophoresis, isoelectric focusing, immunoprecipitation, tryptic peptide mapping and phosphoamino acid determinations. Molecular cloning of viral and cellular genes in plasmid, phage and cosmid vector systems, application of expression plasmids to identification of gene products and nucleic acid sequencing techniques for analysis of cloned genes were also employed.

Major Findings:

1. The human v-abl cellular homolog. A contiguous region of a cellular DNA sequence, 64 kilobases (kb) in length and representing overlapping inserts from three independent cosmid clones, has been isolated from a representative library of human lung cell carcinoma DNA partially digested with MboI. Within this region of the cellular genome, v-abl homologous sequences are dispersed over a total region of approximately 32 kb. These sequences represent the entire v-abl human cellular homolog, are colinear with the viral v-abl transforming gene, and contain a minimum of seven intervening sequences. At least eight regions of highly repetitive DNA sequences have been shown to map in close proximity to c-abl coding sequences. In addition to the major c-abl human locus, three regions of a human DNA sequence, corresponding to only portions of the v-abl gene, have been identified. Two of these have been molecularly cloned and shown to be distinct from the primary human c-abl locus. Upon transfection to rat embryo fibroblasts in culture, none of the cosmid DNAs containing v-abl homologous sequences exhibited transforming activity. These findings identify and map a single genetic locus of human DNA, c-abl, representing the complete v-abl homolog, and demonstrate the existence of additional human DNA sequences corresponding to more limited, subgenomic regions of v-abl.

2. Sequence analysis of the human c-abl tyrosine phosphorylation acceptor site. Sequences encoding the tyrosine phosphorylation acceptor region of the human c-abl oncogene have been identified and their nucleic acid sequences determined. Our findings establish extensive sequence homology between this region of c-abl and the acceptor regions of the v-src, v-yes and v-fes/fps family of viral oncogenes, as well as more distant relatedness to the catalytic chain of the mammalian cAMP-dependent protein kinase. These findings argue that of the homologs of retroviral oncogenes with tyrosine protein kinase activity examined to date, all were probably derived from a common progenitor and may represent members of a diverse family of cellular protein kinases.

3. A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukemia. The human cellular homolog (c-abl) of the transforming sequence of Abelson murine leukemia virus (A-MuLV) was localized to chromosome 9 by analysis of a series of somatic cell hybrids. The long arm of this chromosome is involved in a specific translocation with chromosome 22, the Philadelphia translocation, t(9;22) (q34, q11), occurring in patients with chronic myelocytic leukemia (CML). We have investigated whether the c-abl gene is included in this translocation. Using c-abl and v-abl hybridization probes on blots of somatic cell hybrids, positive hybridization is found when the $22q^-$ (the Philadelphia chromosome), and not the $9q^+$ derivative of the translocation, is present in the cell hybrids. From this we conclude that, in CML, c-abl sequences are translocated from chromosome 9 to chromosome $22q^-$. Moreover, our findings provide a direct demonstration of a reciprocal exchange between the two chromosomes and suggest a role for the c-abl gene in CML.

4. Molecular cloning and chromosomal localization of the human c-fms oncogene. By use of a probe corresponding to the long terminal repeat (LTR) U_3 region of the Snyder-Theilen (ST) strain of feline sarcoma virus (FeSV), the McDonough FeSV proviral DNA was molecularly cloned from nonproductively transformed rat cells. Molecular probes representing subgenomic regions of the McDonough FeSV transforming gene, v-fms, were prepared and a cosmid library of human lung carcinoma DNA screened for v-fms homologous sequences. Three cosmid clones containing overlapping v-fms homologous cellular DNA inserts, representing a contiguous region of a cellular DNA sequence approximately 64 kb in length, were isolated. Within this region of human genomic DNA, v-fms homologous sequences are dispersed over a total region of approximately 32 kb. These include the entire human v-fms cellular homolog (c-fms), are colinear with the viral v-fms transforming gene, and contain a minimum of four intervening sequences. At least twelve regions of highly repetitive DNA sequences have been mapped in close proximity to c-fms coding sequences. Using a molecular probe corresponding to a 0.9 kb KpnI v-fms homologous restriction fragment isolated from one of the cosmid clones, a series of mouse-human somatic cell hybrids containing different complements of human chromosomes was analyzed for human c-fms sequences, and c-fms thereby assigned to chromosome 5. Regional localization of c-fms to band q34 on chromosome 5 was accomplished by analysis of Chinese hamster-human cell hybrids containing terminal and interstitial deleted forms of chromosome 5. The localization of c-fms to human chromosome 5(q34) is of interest in view of reports of a specific, apparently interstitial, deletion involving approximately two-thirds of the q arm of chromosome 5 in acute myelogenous leukemia (AML).

5. Genetic analysis of the 15;17 chromosome translocation associated with acute promyelocytic leukemia. By analysis of a large series of mouse x human somatic cell hybrids, we have localized the c-fes oncogene to human chromosome 15. Subsequently we prepared somatic cell hybrids between a thymidine kinase-deficient mouse cell line and blood leukocytes from a patient with acute promyelocytic leukemia (APL) showing the $15q^{+};17q^{-}$ chromosome translocation frequently associated with this disease. One hybrid contains the $15q^{+}$ translocation chromosome and very little other human material. The c-fes oncogene is not present in this hybrid and is, therefore, probably translocated to the $17q^{-}$ chromosome. Analysis of the genetic markers present in this hybrid has enabled a more precise localization of the translocation breakpoints on chromosomes 15 and 17. Our experiments have also enabled an ordering and more precise mapping of several genetic markers on chromosomes 15 and 17.

6. Tyrosine phosphorylation of a cellular serine specific protein kinase by viral (v-fes and v-abl) encoded protein kinases. A cellular glycoprotein of 150,000 molecular weight (P150) has been identified which specifically binds v-fes-encoded proteins and is a substrate for v-abl- and v-fes-encoded protein kinases. Phosphotyrosine was found on P150 isolated from [^{32}P]orthophosphate-labeled cells of several species transformed by these viruses, but not on P150 isolated from nontransformed control cells. P150 exhibits an associated serine-specific protein kinase activity which recognizes $P120^{gag-abl}$ and Gardner-FeSV (GA) $P110^{gag-fes}$ as well as P150 itself as substrates. This enzymatic activity has a divalent cation preference for Mg^{++} and uses [γ^{32}P] ATP as a phosphate donor. Interspecies antigenic determinants on P150 permitted its isolation from cells of all mammalian species tested, including mouse, rat, cat, dog, mink and a number of human tumor lines. In addition, similar [^{35}S]methionine- and [^{32}P]orthophosphate-labeled tryptic peptides were characteristic of P150s isolated from cells of several mammalian species, including human, indicating that P150 is highly conserved. Glycosylation of P150 was demonstrated by incorporation of [^{3}H]mannose, and by tryptic peptide analysis the glycosylation site was localized to a single peptide. P150 was shown to be distinct from each of several previously demonstrated substrates for tyrosine-specific protein kinases, including vinculin, growth factor receptors, and several glycolytic enzymes.

7. Transforming genes of avian (v-fps) and mammalian (v-fes) retroviruses correspond to a common cellular locus. The Gardner and Snyder-Theilen isolates of FeSV represent genetic recombinants between feline leukemia virus (FeLV) and transformation-specific sequences (v-fes gene) of cat cellular origin. A related transforming gene (v-fps), common to the Fujinami, PRC II, and UR 1 strains of avian sarcoma virus has also been described. Translational products of each of these recombinant virus isolates are expressed in the form of polyproteins exhibiting protein kinase activities with specificity for tyrosine residues. v-fes and v-fps homologous sequences of GA-FeSV, ST-FeSV and Fujinami sarcoma virus (FSV) have been defined and these independently derived transforming genes shown to correspond to a common cellular genetic locus which has remained highly conserved throughout vertebrate evolution.

8. Regulation of viral and cellular oncogene expression by cytosine methylation. Mink cells morphologically transformed by either Snyder-Theilen FeSV or Abelson MuLV exhibit relatively high rates of reversion to the nontransformed phenotype.

The proviral DNAs are conserved within the revertant lines and have not undergone changes in integration sites due to translocations or other genomic rearrangements. In contrast, expression of well-defined, viral-encoded transforming proteins is blocked and elevated levels of phosphotyrosine characteristic of the parental transformed cells are reduced to control levels. Loss of the transformed phenotype is associated with increased cytosine methylation of proviral DNA sequences while levels of methylation resume control values upon spontaneous retransformation of revertant clones. Following molecular cloning and transfection to Rat-2 cells, ST-FeSV proviral DNAs from revertant and transformed cells induced similar numbers of transformed foci. Cytosine methylation sites involved in regulation of expression of the major ST-FeSV-encoded transforming protein have been localized within the proviral DNA itself rather than in adjacent cellular flanking sequences. In contrast to the v-fes proviral DNA, c-fes, the cellular homolog of the ST-FeSV acquired transforming sequences, is highly methylated in cytosine residues in both transformed and revertant clones. These findings demonstrate regulation of viral oncogene-mediated transformation by cytosine methylation and suggest that expression of cellular homologs of viral oncogenes, such as c-fes, is also subject to regulation at this level.

9. Similar transforming growth factors (TGFs) produced by cells transformed by different isolates of feline sarcoma virus. Fisher rat embryo cells transformed by each of three independent isolates of FeSV release transforming growth factors (TGFs) into cell culture medium. These acid- and heat-stable peptides compete for binding to, and stimulate phosphorylation of, epidermal growth factor (EGF) membrane receptors and promote anchorage-independent cell growth. Cells transformed by the Gardner and Snyder-Theilen strains of FeSV produce high titers of TGF (60-200 nanogram equivalents [ng eq] EGF/liter) while cells transformed by McDonough FeSV produce TGF at only low levels (<10 ng eq EGF/liter). Growth factors produced by cells transformed by each of the three FeSV isolates functionally and biochemically resemble each other, mouse sarcoma growth factor (SGF), and TGFs produced by human tumor cells.

10. Sequence analysis of TGFs produced by retrovirus-transformed rodent fibroblasts and human melanoma cells. Serum-free media conditioned by retrovirus-transformed Fischer rat embryo fibroblasts, mouse 3T3 cells and two human melanoma cell lines contain TGFs. A large-scale isolation procedure for a low molecular weight rat, mouse and human TGF has been developed. The purification of each TGF was monitored in a radioreceptor assay based on receptor cross-reactivity with mouse submaxillary gland epidermal growth factor (mEGF) and was achieved by gel permeation chromatography of the acid-soluble, TGF-containing activity, followed by reverse phase high pressure liquid chromatography using sequentially acetonitrile and 1-propanol in the presence of aqueous trifluoroacetic acid. The amino-terminal sequences of rat, mouse and human TGFs were determined. Extensive sequence homology was found among TGF polypeptides from different species and cell types of different origin. Alignment of the amino acid sequences of rat TGF, mEGF, and human urogastrone (hEGF) reveals statistically significant sequence homology. These results suggest that TGFs which compete for binding to the cellular EGF receptor and EGF may have evolved from a common progenitor.

11. Structure and biological activity of v-raf, a new oncogene transduced by a retrovirus. We have molecularly cloned a new, acutely transforming replication-defective, mouse type C virus (3611-MSV) and characterized its acquired oncogene.

The viral genome closely resembles the Moloney strain of murine leukemia virus (MuLV), except for a substitution in Moloney MuLV in the middle of p30 and the middle of the polymerase gene (pol). Heteroduplex analysis revealed that 2.5 kb of Moloney MuLV DNA was replaced by 1.2 kb of cellular DNA. The junctions between viral and cellular sequences were determined by DNA sequencing to be 517 nucleotides into the p30 sequence and 1920 nucleotides into the polymerase sequence. Comparison of the transforming gene from 3611-MSV, designated v-raf, with previously isolated retrovirus oncogenes, either by direct hybridization or by comparison of restriction fragments of their cellular homologs, show it to be unique. Transfection of NIH/3T3 cells with cloned 3611-MSV proviral DNA leads to highly efficient transformation and the recovered virus elicits tumors in mice typical of the 3611-MSV virus. Transfected NIH/3T3 cells express two 3611-MSV-specific polyproteins (P75 and P90), both of which contain NH_2-terminal gag gene-encoded components linked to the acquired sequence (v-raf) translational product. The cellular homolog, c-raf, is present in 1-2 copies per haploid genome in mouse and human DNAs.

Significance to Biomedical Research and the Program of the Institute:

A determination of the role of cellular homologs of prototype viral oncogenes in naturally occurring human tumors should further our understanding of the molecular basis of malignant transformation and lead to the development of new approaches to cancer detection and control.

Proposed Course:

Future studies will be directed towards fine structure analysis of the human c-fes and c-abl genes and the identification of their transcriptional and translational products. An effort will be made to determine whether sequences flanking c-abl and c-fes are rearranged as a result of the chromosomal translocation associated with CML and APL, respectively. In addition, experiments will be designed to determine whether, following appropriate modification, such sequences can be converted from an inactive to an active transforming state. Studies will also be performed to establish whether these genes are present in an inactive form in a subset of naturally occurring human tumors, and if so, to determine the role of their associated tyrosine-specific protein kinase activities in transformation. For this purpose, hybridomas will be developed which produce monoclonal antibodies directed against human c-fes- and c-abl-encoded proteins and synthetic peptides will be prepared based on nucleic acid sequence data. These will be utilized for initial screening of a diverse range of human tumors. The c-fes and c-abl genes will be molecularly cloned from tumors in which their gene products are expressed at levels as high active protein kinases and subsequently analyzed for transforming activity.

Publications:

de Klein, A., van Kessel, A. G., Grosveld, G., Bartram, C. R., Hagemeijer, A., Bootsma, D., Spurr, N. K., Heisterkamp, N., Groffen, J. and Stephenson, J. R.: A cellular oncogene (c-abl) is translocated to the Philadelphia chromosome in chronic myelocytic leukemia. Nature 300: 765-767, 1982.

Groffen, J., Heisterkamp, N., Blennerhassett, G. and Stephenson, J. R.: Regulation of viral and cellular oncogene expression by cytosine methylation. Virology 126: 213-227, 1983.

Groffen, J., Heisterkamp, N., Shibuya, M., Hanafusa, H. and Stephenson, J. R.: Transforming genes of avian (v-fps) and mammalian (v-fes) retroviruses correspond to a common cellular locus. Virology 125: 480-486, 1983.

Groffen, J., Heisterkamp, N. and Stephenson, J. R.: Isolation of v-fes/v-fps homologous sequences from a human lung carcinoma cosmid library. In Pearson, M. L. and Sternberg, N. L. (Eds.): Proc. Gene Transfer and Cancer Workshop. New York, Raven Press (In Press)

Groffen, J., Heisterkamp, N., Stephenson, J. R., Geurts van Kessel, A., de Klein, A., Grosveld, G. and Bootsma, D.: c-sis is translocated from chromosome 22 to chromosome 9 in chronic myelocytic leukemia. J. Exp. Med. (In Press)

Heisterkamp, N., Groffen, J. and Stephenson, J. R.: The human v-abl cellular homologue. J. Mol. Appl. Genet. 2: 57-68, 1983.

Heisterkamp, N., Groffen, J. and Stephenson, J. R.: Isolation of v-fms and its human cellular homologue. Virology 125: 248-258, 1983.

Heisterkamp, N., Groffen, J., Stephenson, J. R., Spurr, N. K., Goodfellow, P. N., Solomon, E., Carritt, B. and Bodmer, W. F.: Chromosomal localization of human cellular homologs of two viral oncogenes. Nature 299: 747-749, 1982.

Marquardt, H., Hunkapiller, M. W., Hood, L. E., Twardzik, D. R., De Larco, J. E., Stephenson, J. R. and Todaro, G. J.: Transforming growth factors (TGFs) produced by retrovirus-transformed rodent fibroblasts and human melanoma cells: Sequence homology with epidermal growth factor (EGF). Proc. Natl. Acad. Sci. USA (In Press)

Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr. and Stephenson, J. R.: Structure and biological activity of v-raf, a new oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA (In Press)

Rapp, U. R., Reynolds, F. H., Jr. and Stephenson, J. R.: Isolation of a new mammalian type C transforming virus. In Pearson, M. L. and Sternberg, N. L. (Eds.): Proc. Gene Transfer and Cancer Workshop. New York, Raven Press (In Press)

Rapp, U. R., Reynolds, F. H., Jr. and Stephenson, J. R.: New mammalian transforming retrovirus: Demonstration of a polyprotein gene product. J. Virol. 45: 914-924, 1983.

Reynolds, F. H., Jr., Oroszlan, S., Blomberg, J. and Stephenson, J. R.: Tyrosine phosphorylation sites common to transforming proteins encoded by Gardner and Snyder-Theilen FeSV. Virology 122: 134-146, 1982.

Reynolds, F. H., Jr., Oroszlan, S. and Stephenson, J. R.: Abelson murine leukemia virus P120: Identification and characterization of tyrosine phosphorylation sites. J. Virol. 44: 1097-1101, 1982.

Sheer, D., Hiorns, L. R., Stanley, K. F., Goodfellow, P. N., Swallow, D. M., Povey, S., Heisterkamp, N., Groffen, J., Stephenson, J. R. and Solomon, E.: Genetic analysis of the 15; 17 chromosome translocation associated with acute promyelocytic leukemia. Proc. Natl. Acad. Sci. USA (In Press)

Sheer, D., Hiorns, L. R., Swallow, D. M., Povey, S., Goodfellow, P. N., Heisterkamp, N., Groffen, J., Stephenson, J. R. and Solomon, E.: Genetic mapping of the 15; 17 chromosome translocation associated with acute promyelocytic leukemia. In Rowley, J. (Ed.): Proc. Bristol-Myers Symp. on Chromosomes and Cancer. Chicago, Academic Press (In Press)

Stephenson, J. R. and Todaro, G. J.: Viral encoded transforming proteins and associated growth factors. In Klein, G. (Ed.): Advances in Viral Oncology. New York, Raven Press, 1982, Vol. 1, pp. 205-224.

Twardzik, D. R., Todaro, G. J., Reynolds, F. H., Jr. and Stephenson, J. R.: Similar transforming growth factors (TGFs) produced by cells transformed by different isolates of feline sarcoma virus. Virology 124: 201-207, 1983.

Veronese, F., Kelloff, G. J., Reynolds, F. H., Jr., Hill, R. W. and Stephenson, J. R.: Monoclonal antibodies to feline sarcoma virus gag and fes gene translational products. J. Cell. Biochem. 21: 9-18, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05108-05 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Purification and Characterization of Sarcoma Growth Factor

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. E. De Larco Research Chemist, LVC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Chemoprevention, NCI, Bethesda, MD; Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN; Biological Products Laboratory, Program Resources, Inc., Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.9	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Sarcoma growth factor (SGF) is an epidermal growth factor (EGF)-like peptide released by murine sarcoma virus (MSV)-transformed cells. It is one of several well-conserved, EGF-like growth factors (GFs) that are released by transformed cells, including a human melanoma line. Any member of this class of GFs, including EGF, can act synergistically with a modulator molecule released by these transformed cells to reversibly induce the transformed phenotype in untransformed indicator cells. This phenotypic transformation is measured as the stimulation of anchorage-independent growth (AIG) of an untransformed cell line; stimulation of AIG is determined using a soft agar colony formation assay. The GF and the modulator have been purified from a Bio-Gel P-60 preparation of crude SGF. The growth factor is EGF-like and is defined as SGF, while the modulator, which has little or no measurable activity when added to these cells in the absence of one of the GFs, appears to be closely related to the TGF-beta reported in the literature. In the AIG assay, EGF can substitute for the GFs released by transformed cells. Antibodies to the EGF receptor block the mitogenic effect of EGF as well as that of SGF. The kinetics of the inhibition are similar for EGF and SGF using antisera from three separate rabbits. The same antisera are able to block the AIG response of a mixture of SGF and modulator, indicating that the occupancy of the EGF receptor by an EGF-like peptide is a requirement for AIG in this system. Synthetic peptides containing portions of the primary structure analogous to SGF have been obtained for the purpose of producing antibodies to the SGF molecule.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Dennis Pigott Visiting Fellow LVC, NCI

Objectives:

One of the primary objectives at this time is the production of immunological reagents against sarcoma growth factor (SGF) and its modulator released by murine sarcoma virus (MSV)-transformed cells. Taking advantage of the known amino acid sequence of SGF, synthetic peptides of specified sequences will be used for the production of antibodies that will cross-react with the native peptide, and thereby yield reagents for radioimmune assays. These reagents will allow the detection of SGF expression during development as well as in the normal and malignant states (i.e., different tissues and body fluids from tumored and normal individuals). These antibodies will be further examined to determine if they may be used as diagnostic tools for qualitative or quantitative differences in SGF expression in patients having certain malignancies. The possibility of using these antibodies as therapeutic agents will also be explored. Along parallel lines, oligonucleotides will be synthesized for the cloning of these growth factor genes. The expression of these factors can be determined both in vivo and in vitro with these clones. The in vivo data will allow the examination of when and where these factors are expressed under normal developmental or physiological conditions. The in vitro systems will be used to determine if there is a correlation between the expression of these genes, or closely related genes, and the transformed phenotype. This system will be further used to determine if any drugs can affect the expression of these sequences (e.g., Are there drugs that can turn on or increase the level of growth factor expression in normal cells, and are there substances which will decrease or eliminate the expression of these factors in transformed cells?).

Cell clones have been selected to examine both the extracellular components required for transformation and the intracellular events required for the expression of the transformed phenotype.

Methods Employed:

Tissue culture methods were used for both the production of cellular factors and assay of activities present in the serum-free conditioned media. For the production of SGF, transformed cells were grown to confluency in serum-containing media; they were washed twice with serum-free media and 48-hour harvests of conditioned serum-free media were collected. These conditioned media were the sources of the "ectopic" factors released by the transformed cells. The conditioned media were clarified using centrifugation and concentrated 25-fold using a hollow fiber concentrator. The concentrate was desalted by dialyzing against 1% acetic acid and further concentrated by lyophilization. The acid-soluble peptides were extracted with one molar acetic acid. The initial step in the purification was performed using Bio-Gel P-60 gel filtration chromatography. The activities present in these columns were monitored using in vitro assay methods.

Mitogenic activities were measured by either thymidine incorporation or increase in cell number. Phenotypic transformation was measured by morphological changes in monolayers or anchorage-independent growth in soft agar. Ligand binding and competition were measured on tissue culture cells using trace amounts of radiolabeled ligands. The amount of epidermal growth factor (EGF)-competing activity in a sample was determined using a radioreceptor assay and comparing the competition produced by the sample with a standard curve generated by adding increasing amounts of a known preparation of unlabeled EGF to compete with the ^{125}I-EGF. The final three steps in the purification of SGF were performed using reverse phase high pressure liquid chromatography (rpHPLC). To produce specific antibodies against the SGF family of peptides, synthetic peptides will be coupled to both hemocyanin and to bovine serum albumin (BSA). The hemocyanin conjugate will be used for immunization and the BSA conjugate will be radiolabeled for the detection of antibodies specifically directed against the synthetic peptides. A radioimmune assay set up in this manner will not detect the antigenic determinants present on the proteins to which the peptides were conjugated, but will be specific for the synthetic peptide sequences common to the conjugates and also to the native sequence that is located in the SGF molecule.

Major Findings:

1. Requirement for the synergistic actions of the growth factor and modulator to stimulate the expression of the transformed phenotype. It has been shown that SGF acts synergistically with a peptide modulator to cause untransformed indicator cells to form large colonies in soft agar. These two factors coelute from an acidic Bio-Gel P-60 column and, consequently, their synergistic effects stimulated the indicator cells to form the very large colonies seen while assaying the fractions from Bio-Gel P-60 columns. Neither the SGF nor the modulator, when assayed alone, were able to stimulate large colony formation of the untransformed indicator cells even if one used very large quantities of the individual purified components. Reconstruction experiments showed the efficient stimulation of anchorage-independent growth (AIG), seen as large colonies in soft agar, could be regained by adding SGF along with the modulator to the untransformed indicator cells in soft agar.

2. Antibodies for EGF receptors. Rabbit antisera, raised against purified EGF receptors from a cell line obtained from human epidermoid carcinoma (A431), block the binding of radiolabeled EGF to the EGF receptors present on cells derived from humans and several other species. The antisera are able to block the mitogenic effect of EGF. These same antisera also block the mitogenic effect of purified SGF when tested on human skin fibroblasts. The kinetics of inhibition of the mitogenic response, using three different antisera, are similar for EGF and SGF. The same antisera were able to totally block the AIG of normal rat kidney (NRK) cells in response to a mixture of SGF and its modulator. This suggests the SGF is acting through the EGF receptor system.

3. The amino acid sequence of SGF and its comparisons to the sequences of known EGF-like peptides. SGF from serum-free media conditioned by MSV-transformed 3T3 cells was purified to homogeneity using rpHPLC. The amino acid sequence analysis indicated that SGF is distinctly different from EGF. Comparing the N-terminal amino acid sequence of SGF with a transforming growth factor (TGF) from a rat cell transformed by a feline sarcoma virus or the TGF from a human melanoma cell

line shows greater homology between the members of the TGF class than there is between either the TGFs and EGFs or even between mouse EGF and human EGF (urogastrone).

Significance to Biomedical Research and the Program of the Institute:

This work is both significant and of high priority since it has resulted in the purification and characterization of peptides released by transformed cells that contribute to the transformed phenotype of untransformed cells maintained in their presence. These studies will hopefully help in the understanding of the mechanisms and factors responsible for the uncontrolled proliferation and altered social behavior expressed by malignant cells. The data collected from the murine system appear to be directly applicable to certain human malignancies (i.e., the human melanoma cells produce both an EGF-like growth factor and a synergistic modulating activity). The in vitro systems developed will allow for rational evaluation of methods for early detection and screening of possible therapeutic agents for the treatment of these malignancies. Two classes of therapeutic agents can readily be screened using these systems. The first consists of those that suppress the production or release of the factor(s) responsible for the expression of the transformed phenotype. The second includes those which are immunological in origin and would be designed to destroy the cells producing these "ectopic" peptides. These goals are consistent with the main goals and mission of the LVC, the Division and the NCI. It is hoped that they will add to our basic knowledge of growth control and differentiation and, in so doing, will make available methods and rationale that can be applied to patient care, both for early diagnosis and treatment.

Proposed Course:

The knowledge accumulated from this project will afford the opportunity to prepare immunological reagents against the "ectopically" released peptides. These reagents will be used to study the control mechanisms for the expression of these factors during normal growth and differentiation, as well as during malignant transformation. Cellular clones will be selected to determine the extracellular requirements for phenotypic transformation. Another main area of concentration will be the preparation of reagents to determine the intracellular events required for the expression of the transformed phenotype.

Publications:

Carpenter, G., Stoscheck, C. M., Preston, Y. A. and De Larco, J. E.: Antibodies to the EGF receptor-kinase block the biological activities of sarcoma growth factor. Proc. Natl. Acad. Sci. USA (In Press)

De Larco, J. E.: Ectopic production of regulatory factors by tumor cells and their role in the expression of the transformed phenotype. Cell Biol. (In Press)

De Larco, J. E.: Sarcoma growth factors and transforming growth factors. In Guroff, G. (Ed.): Growth and Maturation Factors. New York, John Wiley and Sons, 1983, Vol. 1, pp. 193-208.

De Larco, J. E., Preston, Y. A., Marquardt, H. and Todaro, G. J.: Characterization of purified SGF and its relationship to EGF. In Galeotti, T., Cittadini, A., Neri, G. and Papa, S. (Eds.): Membranes in Tumor Growth, Development in Cancer Research. Amsterdam, Elsevier Biomedical Press, 1982, Vol. 7, pp. 431-436.

De Larco, J. E. and Todaro, G. J.: Properties of sarcoma growth factors produced by sarcoma virus transformed cells. In Rich, M. A. and Furmanski, P. (Eds.): Biological Carcinogenesis. New York, Marcel Dekker, 1982, pp. 19-41.

Marquardt, H., Hunkapiller, M. W., Hood, L. E., Twardzik, D. R., De Larco, J. E., Stephenson, J. R. and Todaro, G. J.: Transforming growth factors produced by retrovirus-transformed fibroblasts and human melanoma cells: Amino acid sequence homology with epidermal growth factor. Proc. Natl. Acad. Sci. USA (In Press)

Massague, J., Czech, M. P., Iwata, K., De Larco, J. E. and Todaro, G. J.: Affinity labeling of a transforming growth factor receptor that does not interact with epidermal growth factor. Proc. Natl. Acad. Sci. USA 79: 6822-6826, 1982.

Rizzino, A., Orme, L. S. and De Larco, J. E.: Embryonal carcinoma cell growth and differentiation: Production of a response to molecules with transforming growth factor activity. Possible role of transforming growth factors during early mammalian development. Exp. Cell Res. 143: 143-152, 1983.

Todaro, G. J., De Larco, J. E. and Fryling, C. M.: Sarcoma growth factor and other transforming peptides produced by human cells: Interactions with membrane receptors. Fed. Proc. 41: 2996-3003, 1982.

Todaro, G. J., De Larco, J. E. and Shoyab, M.: Epidermal growth factor (EGF) receptors interact with transforming growth factors (TGFs) produced by certain human tumor cells and are distinct from specific membrane receptors for phorbol and ingenol esters. In Hecker, E. (Ed.): Carcinogenesis, A Comprehensive Survey. New York, Raven Press, 1982, pp. 443-462.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05124-06 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Retrovirus Expression in Primate Placenta

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

K. J. Stromberg Medical Director, LVC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Placental Pathophysiology, State University of Liege, Liege, Belgium; Department of Medicine, University of Texas School of Medicine, San Antonio, TX

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	0.2

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Primary cells derived from first-trimester rhesus monkey (Macaca mulatta) placenta were transformed with a DNA fragment consisting of the early region of the SV40 genome to become a T-antigen-positive cell strain with numerous characteristics of trophoblast epithelium. The cell strain, termed RheTro, appears to be a useful in vitro analog to normal rhesus trophoblast cells.

An examination of various rhesus monkey (Macaca mulatta) organs has shown type C viral antigen expression preferentially in the placenta (Stromberg, K. and Huot, R., Virology 122: 365-369, 1981). Separate cocultivations of isolated primary trophoblasts from ten rhesus monkey placentas with cell lines from heterologous mammalian species led to rapid isolation of type C rhesus retroviruses in four of ten cases. These four retrovirus isolates have been designated MMC-2 through MMC-5. Distinction of these viral isolates from the initial rhesus isolate (MMC-1) and the previous isolate from the stumptail monkey, Macaca arctoides, (MAC-1) could be made by host range studies and liquid DNA hybridization, but not by limited restriction endonuclease digestion. Specifically, the cellular DNA from rhesus isolates MMC-2 through MMC-5 melted 0.7°C to 1.0°C lower than either MAC-1 or MMC-1. Using our hybridization conditions, it was not possible to distinguish between MAC-1 and the originally reported isolate from rhesus, MMC-1. Both MAC-1 and MMC-1 were obtained in single, long-term cocultivation experiments (over seven months). The present isolates, MMC-2 through MMC-5, were detected in two to five weeks. The prompt detection of type C particle expression following cocultivation of rhesus trophoblast cells with heterologous cell lines demonstrates that trophoblast, as a differentiated cell type, is relaxed for complete retroviral expression. It remains to be seen if other primate species, including higher apes and man, will yield infectious retrovirus using this approach.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Raoul E. Benveniste Medical Officer LVC, NCI

Objectives:

First, to study type C viral expression in rhesus placenta to establish conditions whereby endogenous retroviruses might be more readily isolated from primate species. The aim has been to establish that there is a tissue-specific preference for expression of endogenous type C virus in primate placenta and to apply principles established in the rhesus system to primate species from which retroviruses have not been isolated, including man. Second, to study the expression of human placental hormones (such as chorionic gonadotropin, hCG) or other placental polypeptides (such as growth factors or placental laminin) in normal and malignant trophoblasts in vitro.

Methods Employed:

Rhesus retroviral expression was scored by radioimmunoassay (RIA) of the p26 antigen from Macaca arctoides type C virus (MAC-1). Clarified pellets (100,000 x g) of cell and organ culture supernatants were screened for DNA polymerase activity using rAdT synthetic templates. Cellular DNA was isolated from cell lines infected with the various isolates and hybridized to a ^{3}H-DNA transcript prepared from the MAC-1 isolate, and melting curves were performed. An RIA for alpha and beta subunit secretion of hCG was an additional assay procedure.

Major Findings:

1. In vitro growth of primate placental cells. Because of the short-term nature of trophoblast growth in monolayer culture, a primary rhesus trophoblast strain was transfected with purified DNA from the early region of the SV40 genome. Specifically, primary cells derived from first-trimester rhesus monkey (Macaca mulatta) placenta were transformed with a DNA fragment consisting of the early region of SV40 to become a T-antigen (T-ag)-positive cell strain with several characteristics of trophoblast epithelium. Morphologically, these included: (1) a major population of polygonal cells with growth in culture in a pavement-like manner, as well as a minor population of large, flat, multinucleated cells; and (2) the frequent presence by transmission electron microscopy of intermediate and tight junctions as well as a dense cytoplasm with abundant free polyribosomes, mitochondria and Golgi. By immunofluorescence, the monolayer cultures exhibited, at the cell periphery basement membrane, collagen (type IV), laminin, fibronectin, and type III collagen, but no interstitial tissue type I collagen. Biochemically, the cells had heat-labile alkaline phosphatase activity which was subject to modulation with sodium butyrate and iododeoxyuridine similar to first trimester rhesus placenta in organ culture. Conditioned culture medium did not contain (both alpha and beta subunits) rhesus chorionic gonadotropin by radioimmunoassay. Karyotype analysis showed a rhesus female karyotype ranging from a diploid to principally tetraploid number with numerous structural abnormalities

in all chromosomes. The cell strain, termed RheTro, appears to be an in vitro analog to normal rhesus trophoblasts. Although not able to cause unlimited growth, transformation by the SV40 early region DNA preserves numerous differentiated functions of the original trophoblast cells.

2. Biochemistry and modulation of hCG and other placental polypeptides in normal and malignant trophoblasts. A concern in hCG biochemistry has been the kinetics of carbohydrate synthesis and processing using tunicamycin and endoglycosidase H (endo H). Following labeling of placental organ culture specimens with ^{3}H-mannose, chasing for various time periods, and immunoprecipitation, the oligosaccharides were prepared by endo H digestion and the carbohydrate size and composition were determined. The data suggest that, as in choriocarcinoma cell lines, the 18,000 M.W. form of alpha hCG contains one high mannose core, and that the 12,000 M.W. form is the apoprotein of the alpha subunit, whereas the 15,000 M.W. represents the beta apoprotein. Both the alpha and beta hCG precursors appear to contain the mannose core but not the terminal carbohydrate sequences. Fully processed alpha and beta subunits do not accumulate intracellularly, indicating that further processing of precursors is followed by rapid secretion.

Modulation of hCG expression was evaluated during and after exposure to methotrexate, iododeoxyuridine, hydroxyurea, sodium butyrate, dibutyryl cyclic AMP, and epidermal growth factor. These compounds were chosen because they induce hCG in transformed trophoblastic cells (choriocarcinoma cell lines). In the JAr and BeWo lines of choriocarcinoma, inhibition of DNA synthesis by methotrexate markedly increased secretion of hCG. Of those modulators, only dibutyryl cyclic AMP enhanced hCG expression in normal trophoblasts. Dibutyryl cyclic AMP at 1 mM in placental organ culture resulted in a severalfold stimulation of alpha, but not the beta, subunit secretion. Treatment of choriocarcinoma cells (JAr line) for 24 hours with cyclic AMP (1 mM) or dibutyryl cyclic AMP (1 mM) or theophylline (1 mM) for 24 to 72 hours, increased the synthesis of hCG 3- to 17-fold, whereas treatment with sodium butyrate (1-2 mM), adenosine (1 mM), adenosine 5'-monophosphate (1 mM) or adenosine 5'-triphosphate (1 mM) had no effect. RNA synthesis is required during, but not after, the exposure to dibutyryl cyclic AMP in order to stimulate hCG synthesis. Induction of hCG by theophylline was much slower. Prevention of protein synthesis for the first 12 of a 24-hour theophylline treatment enhanced the hCG stimulation by theophylline. In contrast, theophylline stimulation of hCG synthesis is inhibited if protein synthesis is blocked during the second 12 hours. Thus, early transcription is needed for the induction of hCG by cyclic AMP; and protein synthesis is required after, rather than during, the induction period.

In collaboration with J.-M. Foidart, an immunological study of human placental laminin, fibronectin and several collagens was completed. Using immunofluorescence, types I and III collagens and their precursors, p N I and p N III collagens, were localized in the interstitium of placental villi. Laminin and type IV collagen, but not bullous pemphigoid or Goodpasture antigens, were demonstrated in trophoblast and capillary basement membranes (BM). Fibronectin was localized in the interstitium as well as in the trophoblast BM. By immunoelectron microscopy laminin was found in the lamina lucida and type IV collagen in the basal lamina of this BM. In young villi, capillaries were scarce, centrally placed and surrounded by a matrix of fibronectin and types I-III collagens. Vascular endothelial cells were limited by granular discontinuous

deposits of laminin and type IV collagen which did not form a structured BM. In mature placentas, dilated capillaries were peripherally located, limited by a multilayered BM containing concentric layers of type IV collagen and laminin. In some areas, the trophoblast and vascular BM appeared in close apposition or even fused. Types I-III collagens accumulated in the central core but were only scarcely distributed in the apex of these villi. The redistribution of these connective tissue antigens, during placental aging, results in a villous architecture that is best adapted to rapid and extensive exchanges between mother and fetus.

3. Retroviral isolation from primate trophoblasts. The preferential expression of Macaca arctoides type C retrovirus (MAC-1) p26 antigen in rhesus trophoblast has been documented (Stromberg, K. and Huot, R., Virology 122: 365-369, 1981). Antigen expression was detected in 16 out of 16 placental specimens, but not in 10 other different fetal organs from each of 8 selected animals. The levels of antigen detected in placenta ranged between 2 and 218 ng/mg protein with a correlation between lower antigen expression and term gestation or parity greater than ten. A ten-fold higher level of antigen expression was detected at the external surface of the placenta near the decidua than in the remainder of the placenta towards the amniotic surface. Thus, even within the placenta, there was a preferred site for endogenous retroviral antigen expression. Separate cocultivations of isolated trophoblasts from ten rhesus placentas using three indicator cell lines (A549, FEC and CF2Th) led to rapid isolation in feline embryo cells (FEC) of type C rhesus retroviruses in four of ten cases. These four retrovirus isolates have been designated MMC-2 through MMC-5. Five of the remaining six sets of cocultivations grew simian foamy virus and were discontinued. With all four retroviral isolates, p26 expression was detected in the cell monolayers between two and five weeks, and Mn^{++}-dependent DNA polymerase activity was evident in the culture supernatants between five and nine weeks after initiation of cocultivation. Distinction of these viral isolates from the initial rhesus isolate (MMC-1) and the previous isolate from the stumptail monkey, Macaca arctoides (MAC-1), could be made by liquid DNA hybridization, although not by limited restriction endonuclease digestion. Both MAC-1 and MMC-1 were obtained in single, long-term cocultivation experiments (over seven months). The present isolates, MMC-2 through MMC-5, were detected in two to five weeks. Consequently, primary trophoblast cells represent a useful differentiated cell type for isolation of infectious retrovirus from this primate species.

The buoyant density of all the purified new Macaca mulatta isolates is 1.14 cm^3 and all have a type C morphology by electron microscopy. The host ranges of MAC-1 virus isolated from Macaca arctoides and two of the new retroviral isolates from Macaca mulatta are different. In agreement with previous data, MAC-1 replicates well in cells of dog, cat, or human origin. MMC-2 grew only in the feline cell line, while MMC-3 grew in both the feline and canine cell lines. Neither isolate replicated in the human cell line, A549, under the conditions employed. Thus, these new rhesus isolates can be distinguished from the previously isolated stumptail virus on the basis of host range differences. Radioimmunoprecipitation assay over a 100-fold concentration range of competing p26 antigen revealed no distinction in p26 among MAC-1 and MMC-1 or MMC-2 through MMC-5. Furthermore, homologous radioimmunoassays, kindly performed by Dr. Charles Benton, formerly at the Frederick Cancer Research Facility, with monospecific antisera to MMC-1 p26 and purified MMC-1 p26 antigen, when compared with purified virus extracts of

MMC-1 through MMC-5, again showed parallel competition curves. These results are not unexpected, inasmuch as previous radioimmunoassays with the major internal structural protein of endogenous primate viruses have shown that closely related species cannot be readily distinguished.

In contrast, nucleic acid hybridization studies have been shown to detect differences among the virogenes of closely related species of Old World monkeys. Type C viruses isolated from three species of baboons (Papio cynocephalus, P. hamadryas, P. papio) have been shown to differ in the thermal stabilities of their nucleic acid sequences by 2-4°C, whereas several independent isolates from one species of baboon have nearly identical thermal stabilities (<0.5°C difference). It is a property of genetically transmitted retroviruses that related nucleic acid sequences can be found in evolutionarily related species. The differences in thermal stability among the baboon isolates correlate well with the results expected based on phylogenetic differences between these primate species as determined by the DNA homology of their non-repetitive cellular genes.

Consequently, cellular DNA from cell lines infected with MAC-1, MMC-1, and MMC-2 through MMC-5 was hybridized to a complementary DNA probe prepared from MAC-1 virus RNA. The cellular DNA from rhesus isolates MMC-2 through MMC-5 melted 0.7°C to 1.0°C lower than the homologous hybrid. The lower melting temperatures obtained are consistent with the base-pair mismatching of the viral genomes. The data, therefore, suggest that these M. mulatta viruses can be distinguished from the previously described M. mulatta isolates. Using our hybridization conditions, it is not possible to distinguish between the viral isolate from stumptail macaque (MAC-1) and the originally reported isolate from rhesus (MMC-1). DNA reassociation studies of macaque cellular DNA show that this genera of primates is more closely related phylogenetically than are the various species of the baboon genus Papio. The closer degree of nucleic acid sequence homology among the endogenous, genetically transmitted stumptail and rhesus viral isolates as compared to the baboon viral isolates is thus expected based on the overall relationships among these primate species.

A restriction endonuclease cleavage map of the MMC-1 proviral genome integrated in canine cell line DNA has been reported. We compared the restriction maps of MAC-1, MMC-1, and MMC-2 through MMC-5 genomes integrated in human, canine, and feline cells, respectively. The nick-translated probe used was prepared from cloned DNA representing the internal EcoRI 4.7 kilobase (kb) fragment from colobus virus, a closely related type C virus. Restriction enzyme digests with EcoRI, BamHI, HindIII and SacI did not reveal any differences between the stumptail or macaque viral genomes. Thus, even though the MMC-2 through MMC-5 viral genomes possess a 0.7°C to 1.0°C nucleic acid base-pair mismatch with respect to MAC-1, those sites are not represented by the loci defined by these four restriction enzymes.

Each of the cocultivations of rhesus trophoblasts with feline cells led immediately to cell fusion with formation of large aggregates of multinucleated cells. Since human trophoblast in organ culture, as opposed to monolayer culture, produces a 10^4 increase in chorionic gonadotropin production, considerable effort was made to isolate rhesus retrovirus by seeding a monolayer of FEC cells with rhesus placental tissue in organ culture. This procedure did not induce fusion of cells within the FEC monolayer, or result in expression of viral p26 antigen

or reverse transcriptase activity. Consequently, the intimate juxtaposition and resultant fusion of permissive indicator cell lines with rhesus trophoblast seem to be essential for isolation of infectious retrovirus. Trophoblasts cocultivated with either the human line, A549, or the canine line, CF2Th (from which no virus was recovered), did not result in multinucleations or fusion of the two cell types. Paradoxically, the syncytial formation between trophoblast and indicator cell lines may hasten the appearance of contaminating simian syncytium forming (foamy) viruses, as was the case in 5 of 10 placentas in this study, and is reported also to be 50% in baboon placentas. In any event, the prompt detection of type C particle expression following cocultivation of rhesus trophoblast cells with heterologous cell lines demonstrates that trophoblast, as a differentiated cell type, is relaxed for retroviral expression. Using these techniques, we have isolated several endogenous rhesus retroviruses that can be distinguished by host range and nucleic acid hybridization criteria from the previously isolated viruses from stumptail and rhesus macaques. It remains to be seen if other primate species, including higher apes and man, will yield infectious retrovirus using these approaches.

Significance to Biomedical Research and the Program of the Institute:

The retroviral part of this project has been directed toward use of the rhesus model system to determine factors which influence expression and which might enhance isolation of retroviruses from higher primates. Clearly, rhesus trophoblast is a preferential tissue source for isolation of endogenous retroviruses, and these techniques can be extended to other primates, including man, from which endogenous retroviruses have not yet been isolated. The relevance of retroviruses to carcinogenesis in higher primates might be clearer if endogenous primate retroviruses could be more readily isolated from them.

Knowledge of the mechanism(s) of modulation of expression of biochemical markers of malignancy, such as hCG, is essential for the markers to be useful clinically. These markers can be easily studied in normal and malignant human placental tissues in cell and organ cultures.

Proposed Course:

Because of limited technical help and resources, the approach to primate retroviral isolation using cocultivation with trophoblast cells will be discontinued. Relevant stocks remain frozen down for future use.

Publications:

Foidart, J.-M., Yaar, M. D., Gullino, M., Huot, R., Stromberg, K. and Katz, S.: Immunological studies of human placenta: Identification and immunolocalization of laminin, fibronectin and several collagens in human placental villi. Gynecol. Obstet. Invest. (In Press)

Stromberg, K. J. and Benveniste, R. E.: Efficient isolation of endogenous rhesus retrovirus from trophoblast. Virology (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05141-04 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

A Cell Culture Model System for Studying Late-Stage Promotion of Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

N. H. Colburn Expert, LVC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.2	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this work has been to develop and characterize a mouse epidermal cell culture model system for studying promoter-dependent preneoplastic progression and its prevention. Sensitivity of JB6 cells to promotion of anchorage-independence extends to a variety of classes of tumor promoters including growth factors, benzoyl peroxide, vinyl products, like di-2-ethylhexyl-phthalate, as well as phorbol esters. Retinoic acid inhibits promotion of anchorage-independence by 12-O-tetradecanoylphorbol-13-acetate (TPA), but not by benzoyl peroxide or epidermal growth factor (EGF). A new inhibitor, trisialoganglioside (GT), blocks promotion by TPA and benzoyl peroxide. Thus, retinoic acid and GT may block different required events in promotion. Commitment to anchorage-independent transformation requires at least four days exposure to TPA. New efforts are now directed toward the development of a human model system for studying preneoplastic progression using cells from individuals genetically predisposed to cancer, with much the same emphasis on elucidating genetic and membrane determinants.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

None

Objectives:

To determine the rate-limiting steps which occur during the long latent period of premalignant progression following exposure to carcinogens and/or tumor promoters. To characterize and modify the JB6 mouse epidermal cell model system which has been previously described, for studying promotion of transformation as measured by anchorage-independent growth tumorigenicity. Specifically: (1) to obtain clonal cell lines which are resistant to promotion of transformation by the phorbol diester 12-O-tetradecanoylphorbol-13-acetate (TPA) from promotable cell lines and to ascertain the degree of cross-resistance (and cross-sensitivity in the TPA-sensitive lines) to other classes of promoters; (2) to determine, in this system, the activity of various known or postulated antipromoters; (3) to continually characterize the clonal cell lines and monitor them for stability; and (4) to extend the model system development to a new human model utilizing epithelial cells from individuals genetically predisposed to cancer.

Methods Employed:

Characterization and cloning of mouse epidermal and human epithelial cell lines. Determination of anchorage-independent growth (colony formation in 0.33% agar) in response to tumor promoters and antipromoters. Establishment of promoter-induced transformant lines cloned from agar.

Major Findings:

1. Promotion resistance (or sensitivity) in JB6 cell lines extends to phorbol esters and several other classes of promoters. Clonal heterogeneity has been previously reported for promotion of anchorage-independent growth of clonal derivatives of the promotable JB6 mouse epidermal cell line. The clonal lines that are most responsive to phorbol ester promoters are also responsive to other classes of promoters, including ingenols, detergents, polycyclic hydrocarbon derivatives in cigarette smoke, and epidermal growth factor (EGF). Recent work has extended this observation of cross-sensitivity to other classes of tumor promoters, including benzoyl peroxide and di-2-ethylhexyl-phthalate, to human and rodent transforming growth factors, and to an agent that oxidizes cell surface sialic acid, sodium metaperiodate.

2. Retinoids inhibit promotion of anchorage-independent growth of JB6 cells by TPA, but not by EGF or benzoyl peroxide. Promotion of tumor cell phenotype by the phorbol diester TPA is inhibited by retinoids which also act as antipromoters in mouse skin tumor promotion. Retinoic acid enhances promotion of transformation by EGF. Modulation of receptor binding by retinoic acid may explain the EGF promotion enhancement, but does not explain the retinoid inhibition of phorbol ester promotion. Some transforming growth factors are, and some are not,

sensitive to retinoic acid inhibition. Benzoyl peroxide, when applied to cells two to three times at two-day intervals, induces anchorage-independence at concentrations from 10^{-9} to 10^{-5} M. This process is not inhibited by retinoic acid. Retinoic acid does inhibit sodium metaperiodate ($NaIO_4$) promotion.

3. Trisialoganglioside (G_T) addition blocks promotion of anchorage-independence by TPA. G_T blocks promotion by TPA and benzoyl peroxide. (See Project #Z01CP-05225-03 LVC, entitled "Membrane Biochemical Determinants of Tumor Promotion").

4. Commitment to anchorage-independent transformation requires at least four days of exposure to TPA. Using a TPA hydrolase preparation obtained by one-step purification of a bovine liver catalase, we can achieve rapid removal of TPA from cell culture. Adding TPA hydrolase at various time intervals to JB6 promotion-sensitive cells in agar after TPA treatment, then scoring for colonies at two weeks, has yielded the observation that the TPA exposure time required for determining anchorage-independent transformation in this system is at least four days. The average induced colony requires 7-8 days of TPA exposure (Dion, L. D., Gindhardt, T. D., and Colburn, N. H., submitted for publication). This defines the time intervals during which one needs to study events relevant to inducing neoplastic transformation in JB6 cells.

Significance to Biomedical Research and the Program of the Institute:

The availability of an in vitro model system for studying promotion of transformation by a variety of classes of tumor promoters and its inhibition by various antipromoters is useful both for promoter and antipromoter detection and for mechanism studies. The availability of paired promoter-sensitive and -resistant cell lines is useful for ascertaining the changes which determine promotion of neoplastic transformation vs the correlative ones.

Proposed Course:

(1) To extend this work to study new nonphorbol promoters and other antipromoters. (2) To ascertain whether various endogenous hormones or growth factors can function to mediate or block promotion of transformation in mouse JB6 cells. (3) To determine, through cotreatment studies, whether two different classes of promoters act through a common pathway. (4) To develop new JB6 variants, each resistant to one biochemical or cellular response to phorbol esters seen in the parent cell line(s), and then to ascertain whether coordinate production of resistance to promotion of anchorage-independent cell growth occurs. Variants will be sought that are resistant to induction by phorbol esters of ganglioside shifts, stimulated hexose uptake, hormone-receptor modulation, and changes in protein kinase activity. (5) To develop a human cell model system to study promotion of transformation using cells from patients with familial disorders showing a high cancer risk. (6) To determine whether these cells are promotable to malignancy (tumorigenicity in nude mice) by phorbol esters or other tumor promoters. If not promotable, develop promotable cell lines by appropriate carcinogen exposure at subcarcinogenic concentrations.

Publications:

Colburn, N. H., Wendel, E., Hegamyer, G. A., Talmadge, C. B., Dion, L. D., Srinivas, L. and Gindhart, T. D.: The use of promoter-resistant variants to

elucidate the role of specific membrane and gene-level changes in process of tumor promotion. In Borzsonyi, M. and Yamasaki, H. (Eds.): The Role of Cocarcinogens and Promoters in Human and Experimental Carcinogenesis. Lyon, France, IARC Scientific Publications (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05149-04 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanisms of Tumor Promotion

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

M. E. Shoyab Expert, LVC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.0	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The process of tumor induction has been broadly divided into two stages: initiation and promotion. Initiation apparently involves irreversible alteration in the genetic material. Promotion appears to be epigenetic and reversible at least in the early stages. Hence, interruption of the process should be feasible at the promotion stage. This project aims to isolate and characterize putative, endogenous tumor promoters and their antagonists and to develop rapid and economical assays for their identification; specifically the detection, isolation, characterization, and study of: (1) the mechanism of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inhibition of epidermal growth factor (EGF) binding (TIEB); (2) a compound(s) which can reverse and modulate TIEB at non-toxic doses; (3) membrane receptors of phorbol esters; (4) endogenous ligands (agonists and antagonists) for phorbol receptors; (5) growth factor(s) induced by biologically active phorbol esters; (6) reversal of anchorage-independent growth of transformed cells by differentiation-inducing agents; (7) the metabolism of phorbol diesters; (8) phorbol diester binding protein(s); (9) TPA induction or enhancement of the expression of endogenous oncogenic cellular information; (10) enhancement of carcinogenesis by EGF and other growth factors; and (11) action of retroviruses as promoters in carcinogenesis initiated by chemical or physical agents. Recent results include the following: (1) Specific receptors for phorbol and ingenol esters were solubilized and purified to homogeneity. Their physicochemical and biochemical properties were characterized. It was found that the receptor is a protein kinase. (2) Endogenous ligands from cow milk and murine intestine, pancreas and stomach were purified, identified and characterized. These ligands were found to be mono- and diglycerides with special structural features. (3) Endogenous oncopromotine(s) and oncoretardine(s) have been detected in brain. Their purification and characterization are in progress. (4) A very potent oncostatin (antitumor promoter) is currently being isolated and characterized from clams and other mollusks.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

None

Objectives:

The process of tumor induction has been broadly divided into two stages, i.e., initiation and tumor promotion. The initiation process apparently involves irreversible alteration in the genetic material, whereas tumor promotion appears to be epigenetic in nature and is reversible at least in the early stages. Hence, the interruption of the carcinogenic process should be feasible at the tumor promotion stage. This project aims to isolate and characterize putative endogenous tumor promoters and their antagonists and to develop a rapid and economical assay for their identification. The specific aims are: (1) the elucidation of the mechanism of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inhibition of epidermal growth factor (EGF) binding (TIEB), and to search for a compound(s) which can reverse and modulate TIEB at nontoxic doses; (2) the isolation and characterization of membrane receptors of phorbol esters; (3) to search for endogenous ligands (agonists and antagonists) for phorbol receptors, and their isolation and characterization; (4) isolation and characterization of growth factor(s) induced by biologically active phorbol esters; (5) to test whether EGF and other growth factors enhance carcinogenesis in vivo and in vitro; (6) to investigate whether TPA induces or enhances the expression of endogenous oncogenic cellular information; (7) to study whether retroviruses act as promoters in carcinogenesis initiated by chemical or physical agents; (8) reversal of anchorage-independent growth of transformed cells by differentiation-inducing agents; (9) to study the metabolism of phorbol diesters in vitro and in vivo; and (10) the isolation and characterization of phorbol diester binding protein(s).

Methods Employed:

Standard cell culture, molecular biological, cellular biological and biochemical technologies were employed. Gel permeation chromatography, ion exchange chromatography, silicic acid chromatography, high pressure liquid chromatography (HPLC), thin layer chromatography (TLC), isoelectric focusing, gas liquid chromatography (GLC), mass spectroscopy, nuclear magnetic resonance (NMR), isochromatofocusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used to purify and analyze various enzymes, receptors, factors and putative endogenous ligands. Radioreceptor assays were used to determine the competing activities. Soft agar assays using indicator cells, growth factors and growth inhibitors were used to test for anchorage-independent cell growth.

Major Findings:

1. Mechanism of affinity modulation of EGF receptors by biologically active phorbol and ingenol esters. It was found that TIEB is not affected by phosphorylation or dephosphorylation of EGF receptors. TPA does not alter EGF-stimulated phosphorylation of the EGF receptor in human epidermal carcinoma

vulva (A431) cells. The inhibitors of protein and lipid methylations also do not affect TIEB. Tunicamycin (a glycosylation inhibitor), like TPA, reduces the binding of EGF to its receptors, suggesting a role for the carbohydrate component of receptors in EGF binding. It was concluded that TPA and EGF receptors are topically linked through phospholipids. The binding of TPA to specific receptors perturbs these membrane phospholipids, leading to a reduction in the affinity of EGF receptors. However, EGF binding to its receptors does not alter these phospholipids and does not affect TPA binding to its receptors.

2. Partial purification and characterization of a binding protein for biologically active phorbol and ingenol esters from murine serum. A protein (~M_r 71,000) from murine serum was purified 104-fold. It binds directly to biologically active phorbol and ingenol esters, and mezerein in a specific, reversible and saturable manner. The binding of labeled phorbol-12,13-dibutyrate (PDBu) to protein is rapid and dose-dependent. Those phorbol and ingenol esters which stimulate cell growth in culture and have tumor-promoting activity in vivo inhibit the binding of labeled PDBu, while the biologically inactive derivatives fail to do so. Other nonditerpene tumor promoters, retinoids, steroids and prostaglandins do not interfere with the PDBu-protein interaction. EGF, insulin, bovine serum albumin, hemoglobin, ovalbumin, ferritin, myoglobin, fetuin and lipase do not directly interact with PDBu. Binding protein competitively inhibits the binding of PDBu to specific receptors. It is a nonglycosylated, slightly hydrophobic protein which is heat- and acid-labile. The protein is present in sera of various mammalian species. The concentration of protein in murine serum is age-, sex-, and strain-independent.

3. Isolation and characterization of phorbol-12,13-diester 12-ester hydrolase (PDEH) from murine and human livers. A phorbol-12,13-diester 12-ester hydrolase (PDEH) has been purified to electrophoretic homogeneity from murine liver cytosol using ammonium sulfate fractionation, Sephadex G-200 gel filtration, Con A Sepharose chromatography and phenyl Sepharose chromatography. The enzyme is a single chain hydrophobic glycoprotein and it has a molecular weight of 60,000. The enzyme exhibits optimum activity at pH 7.5-8.5. PDEH has an isoelectric point (PI) of 5. The enzyme is heat- and acid-labile. Zn^{++}, Co^{++} and Fl^{-} inhibit the enzyme. Phenylmethyl sulfonyl fluoride (PMFS) is a potent inhibitor of PDEH. Sarkosyl also inhibits the enzyme at mM concentrations. The enzyme inactivates biologically active phorbol 12,13-diesters in a dose- time- and temperature-dependent manner. The inhibition constant has been observed to be 6.6×10^{-8} M for the enzyme-elicited inhibition of phorbol-12,13-dibutyrate binding to its receptor. The enzyme exclusively cleaves the 12-ester of phorbol-12,13-diesters.

4. PDEH as the critical factor in the susceptibility of skin to the tumor-promoting action of phorbol diesters. The esterase PDEH, which converts biologically active phorbol-12,13-diesters to the inactive phorbol-13-monoester, is absent from mouse skin but is expressed at high levels in hamster, rat, guinea pig, and rabbit skin. The nonresponsiveness of species other than mouse to TPA and related compounds is directly related to the level of this enzyme found in the skin. One would expect that TPA could act as a potent tumor promoter for human skin since, like mouse skin, it lacks this esterase activity.

5. Certain neuroleptic and antipsychotic tricyclic drugs competitively inhibit interaction between tumor-promoting phorbol esters and their specific receptors. Certain antipsychotic drugs such as fluphenazine, chlorpromazine, chlopenthioxal, 2-chloroimpiramine and impiramine competitively decrease the binding of ^{3}H-PDBu to its specific receptors. We find a good correlation between the PDBu binding-inhibiting activity of phenothiazines and impiramine and their biological potency. These results suggest that these widely used drugs might be tumor promoters.

6. Enhancement of dimethyl benzanthracene (DMBA)-induced mammary tumorigenesis in Sprague-Dawley rats by fluphenazine. Sprague-Dawley rats were treated with a suboptimal dose of DMBA (5 mg; one treatment) and after two weeks secondary treatment was started with either 0 µg, 100 µg or 200 µg of fluphenazine intragastrically or intraperitoneally three times a week. Fluphenazine itself did not induce any mammary tumors. However, the rate and number of mammary tumors in DMBA-treated rats was significantly enhanced by fluphenazine.

7. Isolation and characterization of a specific receptor for biologically active phorbol and ingenol esters. A high affinity, specific receptor for biologically active phorbol and ingenol esters was purified to electrophoretic homogeneity from murine brain. The receptor is a single chain hydrophobic protein with a M_r of 81,500, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The receptor has a sedimentation coefficient of 5.2S and stokes radius of 30.3A. It has an isoelectric point (pI) of 5.5. The receptor is heat- and acid-labile. The receptor absolutely depends upon phospatidylserine or phosphatidylinositol (optimum concentration ~4-8 µg/ml) for its activity. A variety of divalent cations stimulates the binding activity of the receptor. A molecule of receptor binds 1-2 molecules of PDBu with a Kd value of 4.2 nm. Those phorbol and ingenol esters which stimulate cell growth in culture and have tumor-promoting activity in vivo inhibit the binding of labeled PDBu to the homogeneous receptor, while the biologically inactive derivatives fail to do so.

8. A homogeneous receptor for biologically active phorbol and ingenol esters is a calcium-independent protein kinase. A homogeneous receptor protein (M_r ~81,500) for biologically active phorbol and ingenol esters contained a serine protein kinase activity. The receptor protein kinase exhibited substantial activity in the absence of Ca^{++} and/or acidic phospholipid, however, phosphatidyl serine enhanced the kinase activity. A divalent cation such as Mg^{++}, Mn^{++}, Fe^{++} or Co^{++} was absolutely required for the activity. The optimum pH was 6.5-7. The apparent Km for ATP and histone H1 were 0.5 µm and 55.5 µg/ml, respectively. Biologically active phorbol and ingenol congeners stimulated the kinase activity only in the presence of phosphatidyl serine, whereas an inactive derivative failed to do so. The ligand binding and kinase activity of the purified receptor were coinactivated by heat, acid, freezing and thawing, and other treatment.

9. Isolation and characterization of an endogenous ligand(s) for the active phorbol diester receptor. Neutral lipids which competitively inhibit the binding of PDBu to its receptor were purified from defatted cow milk and from murine intestine, pancreas and stomach. They have been termed endogenous ligands (EL). EL from murine tissues, but not from milk, elicit certain biological responses as initiated by active phorbol diesters. EL are present in pancreas, intestine, stomach, testicles, skin, brain, kidney, liver, muscle, spleen, lung and heart in

decreasing quantities. EL were found to be glycerides. Free hydroxyl groups and unsaturated double bonds are essential for the biological activity of EL.

10. Endogenous transforming growth factor(s) (oncopromotine) and growth-inhibitory factor(s) (oncoretardine) from beef brain. A protein factor (≈30 K) from aqueous extracts of beef brain was partially purified which induces anchorage-independent growth of normal rat kidney fibroblastic cells in soft agar. The brain oncopromotine is heat-, alkali- and acid-labile. The protein does not affect the binding of ^{125}I-EGF or ^{3}H-PDBu to their respective receptors. Thus, this brain growth factor is different from sarcoma growth factor (SGF) and transforming growth factor (TGF) which do inhibit EGF-receptor interaction. Aqueous brain extract also contains a factor(s) which inhibits transforming growth factor-induced, anchorage-independent growth of normal rat kidney fibroblastic, and murine and human transformed cells in soft agar. It is a low molecular weight compound(s) ($\approx M_r$ 1,000), and is heat- and acid-stable. Trypsin and chymotrypsin do not inactivate the growth inhibitory activity. It does not modulate the binding of EGF or PDBu to their respective receptors. The absorption maximum of the compound has been found to be 264 nm. These endogenous growth-stimulatory and growth-inhibitory factors probably play a role in brain homeostasis and may regulate the growth and differentiation of cells.

11. Partial purification of anti-growth factor(s) (oncostatin) from clam digestive glands. We have found that clams and other mollusks contain a potent factor(s) which inhibits TGF-induced, anchorage-independent growth of normal cells, and murine and human transformed cells in soft agar. We have partially purified the factor from an aqueous extract of clam digestive glands, the organ containing the highest activity, using ammonium sulfate fractionation, Sephadex G-200 chromatography and DEAE-cellulose chromatography. The factor is a protein (≈22 K). It is heat- and acid-labile. It does not interact with EGF or PDBu receptors. Transformed cells are more sensitive to the factor than normal cells.

Significance to Biomedical Research and the Program of the Institute:

The EGF-competing activity of the phorbol esters parallels tumor-promoting activity in vivo. The phorbol derivatives lacking tumor-promoting activity also lack EGF-competing activity. TPA treatment seems to modulate EGF binding by decreasing the affinity of the receptors on the treated cells for EGF, rather than by decreasing the number of receptors per cell. This affinity modulation is reversible and dependent on time, temperature and TPA concentration. The effect appears to be specific for the EGF receptor system, as four receptor-ligand systems tested in the same TPA-treated cells and three receptor-ligand systems in the other cells did not show any alterations in receptor affinity. TPA modulation of EGF binding is observed with doses of promoter comparable to those required to elicit biological response in vivo as well as in vitro. The above data suggest that TPA-mediated alterations in growth factor(s)-receptor interaction might be related to the underlying mechanism by which tumor-promoting agents initiate a chain of events causing alteration in cellular growth and function. Interestingly, EGF has been reported to enhance tumorigenesis induced by chemical carcinogens. SGF produced by mouse sarcoma virus-transformed cells also interacts with EGF receptors, stimulates cell growth and anchorage-independent growth in soft agar. The putative endogenous growth factor(s) produced in response to the exposure of cells to tumor-promoting agents may, then,

activate a program of gene expression in those cells that have already been genetically altered by initiating agents.

The multistage and multifactorial nature of the biological process, ultimately leading to carcinogenesis, is recognized both in experimental and clinical oncology. The demonstration of two distinct stages (initiation and promotion) in animals indicates that there are apparently two stages in environmentally induced human cancer. Complex interactions between carcinogen-modifying factors (endogenous and exogenous) and tumor promoters probably play an important role in the induction and progression of neoplasia. Because promotion is a reversible process and requires continuous exposure for a period of time, in contrast to the rapid and irreversible phenomenon of initiation of carcinogenesis, the modulation and interruption of the promotion step seems to be the obvious and only feasible way of cancer prevention. Therefore, identification of endogenous and exogenous tumor promoters and the understanding of their mode of action at the molecular level will facilitite the design of methods for the control and prevention of cancer.

Proposed Course:

This project will continue in order to better define the mechanism(s) of tumor promotion. We plan to make monoclonal antibodies against the purified receptor for active phorbol and ingenol esters and to partially sequence the receptor protein, synthesize the corresponding polynucleotide sequences and clone the receptor gene(s). Further investigation of EL will be also given high priority. In addition, we will continue work on the isolation and characterization of all the growth-stimulatory and growth-inhibitory factors. The isolation, characterization and mode of action of putative endogenous growth-promoting and differentiation-modulating substances (endogenous tumor promoter[s]) should provide useful information for understanding the mechanisms of growth, differentiation and carcinogenesis. These studies have the potential to provide clues for designing strategies to reverse and suppress the process of neoplastic development.

Publications:

Shoyab, M.: Affinity modulation of epidermal growth factor membrane receptors by biologically active phorbol and ingenol esters. In Chandra, P. (Ed.): Biological Markers of Neoplastic Transformation. New York, Plenum Press, 1983, pp. 307-320.

Shoyab, M.: Enhancement by fluphenazine of DMBA-induced mammary tumorigenesis in rats. Cancer Lett. 18: 297-303, 1983.

Shoyab, M.: Specific high affinity membrane receptors for biologically active phorbol and ingenol esters. In Reid, E. (Ed.): Methodological Survey in Biochemistry and Analysis. New York, Plenum Press (In Press)

Shoyab, M., Todaro, G. J. and Tallman, J. F.: Certain neuroleptic and antiphyschotic tricyclic drugs competitively inhibit interaction between tumor-promoting phorbol esters and their specific receptors. Cancer Lett. 16: 171-175, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05150-04 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chemical Carcinogenesis and Cocarcinogenesis In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

U. R. Rapp Visiting Scientist, LVC, NCI

COOPERATING UNITS *(if any)*

Department of Pathology, University of Helsinki, Finland

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	0.3	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

An in vitro system was established for the quantitative chemical transformation of rat epithelial cells (RC-E). These cells can also be transformed by C3H MuLV in conjunction with 12-O-tetradecanoylphorbol-13-acetate (TPA). Transformed cell clones obtained from soft agar generally were virus nonproducers. The purpose of these experiments was to sequence-label TPA-promotable cellular tumor genes and, thus, make possible their isolation by molecular cloning. RC-E cells have also appeared to provide the first system for two-stage carcinogenesis in culture with ethylnitrosourea (ENU) as carcinogen and TPA as tumor-promoter. The observation of cocarcinogenesis of RC-E cells with MuLV and TPA promoted a search for factors homologous to TPA in normal sera. Such a transforming factor was found and purified from normal mouse serum.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

None

Objectives:

To develop improved in vitro assays for chemical transformation of epithelial cells; to improve the methodology of the assay systems to permit the processing of large numbers of carcinogen-treated cells and shorten the time required for transformation experiments; to isolate, using techniques of cocarcinogenesis, new cell-derived tumor genes after their linkage with or incorporation into type C viral genomes; and to clone the active tumor gene(s) from a chemically transformed cell.

Methods Employed:

A basic goal of chemical carcinogenesis research is the identification of cellular genes which bring about the transformed phenotype of cells after carcinogenesis exposure. Assays for quantitative chemical carcinogenesis and cocarcinogenesis with cultured mouse cells have been widely accepted as model processes that occur during the development of spontaneous tumors. While this methodology has been highly successful in delineating details of the metabolic activation of carcinogens and the gradual transformation of cells by such agents and tumor promoters, it has not yielded much information regarding the genes and genetic mechanisms involved.

RNA tumor viruses, on the other hand, while not being serious candidates as causative agents for most human tumors, have provided the first isolates of cellular genes which are apparently sufficient to induce malignant transformation. To combine the advantages of both systems, experiments have been initiated to identify the genes involved in chemical transformation and the regulation of their expression in vitro. Efforts to identify such genes have included: (1) screening of chemically transformed cells for the expression of known type C virus-transduced tumor genes; (2) isolation of additional transforming viruses from cocarcinogenesis experiments to widen the repertory of tumor genes for screening; (3) attempts to identify genes promotable by 12-O-tetradecanoylphorbol-13-acetate (TPA) by sequence-labeling with murine leukemia virus (MuLV) genomes; and (4) isolation of transforming gene(s) from extensively characterized, chemically transformed cells (i.e., MCA5) by transfection and molecular cloning. It was demonstrated recently that MCA5 cell DNA contains, or yields upon transfection, an active version of the oncogene Ki-ras. The evidence for its involvement in chemical transformation of C3H/10T1/2 cells, however, is weak, resting solely on its activity in the transfection assay which may be selectively sensitive to a subset of cellular oncogenes. We, therefore, plan to include in our studies flat revertants derived from MCA5 cells as part of a genetic analysis of oncogene activation in MCA-induced C3H/10T1/2 cell transformation. Moreover, we will be using untransformed C3H/10T1/2 Cl 8 cells, the parent of MCA5, as the recipient for DNA transfection.

Major Findings:

1. Use of epithelial rat cells for transformation in vitro with ethylnitrosourea (ENU) and TPA. Rat embryo epithelial (RC-E) cells were transformed in vitro using ethylnitrosourea (ENU) as a carcinogen either by itself or in conjunction with the tumor promoter TPA. The frequency of transformation in the absence of TPA was 5×10^{-4} at 10 μg/ml of ENU. Growth of ENU-treated cells in TPA-substituted medium increased the transformation frequency eight-fold. Morphologically transformed cells were isolated from individual colonies growing in soft agar and analyzed for the production and deposition of pericellular glycoproteins. The ENU-transformed cells were able to retain pericellular matrix structures, as were the nontransformed RC-E control cells. Cell surface labeling revealed differences in the glycoprotein patterns between the cells. The ENU-transformed cells produced fibronectin and procollagen type I as their major glycoproteins. Procollagen type I was produced and deposited in the cell layers in altered form, i.e., procollagen 1-trimer, as shown by polypeptide analysis. The results indicate that ENU can induce malignant transformation of RC-E cells in culture and modify their production and deposition of pericellular glycoproteins. Clonal lines of RC-E cells transformed by ENU and TPA will be compared with those transformed by MuLV and TPA (see below). This comparison will include the restriction enzyme sensitivity pattern of transforming DNA from both sets of transformed cells.

Significance to Biomedical Research and the Program of the Institute:

The overall goal of this work is to characterize cellular genes that are associated with the induction of cancer as it occurs spontaneously or after induction with carcinogens. In consideration of the fact that carcinomas are especially prevalent in humans, special emphasis has been placed on transformation of epithelial cells. A family of cell-derived transforming genes isolated as part of type C viruses might provide the tools necessary for both an understanding of mechanisms of transformation and the development of strategies to neutralize their action.

Proposed Course:

1. Molecular cloning of the gene(s) responsible for oncogenic transformation in chemically transformed mouse cells.

Evidence from transfection experiments with chromosomal DNA has shown (Shih et al., Proc. Natl. Acad. Sci. USA, 76: 5714, 1979) that chemical transformation of cells may be achieved by the activation of single tumor genes. Similarly, an oncogene-transducing retrovirus has been isolated from IdUrd-induced chemically transformed cells. These "activated" cellular oncogenes may be altered as a consequence of carcinogen treatment or may represent derepressed normal genes. In order to study this question, recombinant DNA technology is being used to isolate such a gene(s) from transformed cells. Once this transforming gene(s) is available it will be possible to isolate its normal counterpart by screening a gene library made from normal cellular DNAs. The strategy for these experiments is as follows:

a) Construction of a gene library containing a collection of recombinant phages representing the entire genome of the chemically transformed cells. The total cellular DNAs from MCA5 cells were isolated and partially digested with S2u3A. The 15-20 kilobase (kb) DNA fragments were isolated from a gel electrophoresis which separated the partially digested DNAs according to size. A novel vector, lambda 1059, was used to clone these DNA fragments at BamHI sites. Only the recombinant phages will grow on E. coli strains lysogenic for phage P2. A gene library consisting of 5 x 10^5 clones could be used to screen for the recombinants containing the transforming gene(s) which should be present with a larger than 99% probability.

b) Screening the recombinant library for the transforming gene(s). A mixture of recombinant DNAs isolated from the entire gene library is used to transfect untransformed C3H/10T1/2 Cl 8 cells and to isolate transformed colonies grown in soft agar. A positive result indicates that such a transforming gene(s) has been cloned in vectors along with other cellular genes. In order to isolate the recombinant containing this gene(s), we will follow a subselection procedure. The entire gene library is divided into ten sub-libraries, each containing one-tenth the number of the recombinants in the original library. The DNA mixture isolated from each of these sub-libraries is assayed for its biological activities. The sub-libraries, containing the transforming gene(s), are identified and should represent a 10-fold enrichment. Similarly the positive sub-libraries will again be divided into ten subdivisions and the mixture of DNAs from each subdivision will be assayed for the presence of the transforming gene(s). After 6-7 cycles of enrichment, single clones, each containing a specific transforming gene, may be isolated.

2. TPA enhancement of transformation of mouse MMCE and rat FRE cells by IdUrd-induced C3H MuLV.

The basis for the observed transformation in conjunction with TPA may either be the presence of directly transforming virus in the IdUrd-induced virus population or result from a two-stage carcinogenesis with MuLV as the mutagen/carcinogen. Epithelial rat cells transformed by IdUrd-induced C3H MuLV in conjunction with TPA are presently being analyzed for MuLV sequences in the cellular genome and also for RNA transcripts initiated from within the viral genome. Transformed cells which contain only one copy of complete or defective MuLV will be chosen for further experiments.

3. Use of cocarcinogenesis for the isolation of new acutely transforming mouse type C viruses.

The observation has been made that NFS/N mice, inoculated with MuLV as newborns and receiving butylnitrosourea (BNU) at 4 weeks of age developed virus-positive lung tumors at high incidence. Some of these lung tumors yielded viruses which in turn induced lung tumors and peritoneal tumors in the absence of BNU. BNU by itself induces virus-negative lung tumors as well as lymphomas. MuLV, in the absence of BNU, induced lymphomas with a long latency period. We plan to investigate whether lung tumor-derived, in addition to peritoneal tumor-derived, MuLV contains a directly transforming component which has acquired a tumor gene and also can induce tumors in other target tissues in MuLV infected mice. The isolation of additional transforming mouse type C viruses is expected.

4. Screening of chemically transformed C3H/10T1/2 fibroblast and MMCE and RC-E epithelial cells for the expression of previously characterized viral oncogenes.

Publications:

Rapp, U. R., Gunnell, M. and Marquardt, H.: Normal mouse serum contains peptides which induce fibroblasts to grow in soft agar. J. Cell. Biochem. (In Press)

Rapp, U. R. and Keski-Oja, J.: TPA enhances transformation of epithelial MMCE cells by C3H/MuLV in vitro. Cancer Res. 42: 2407-2411, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05171-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Transforming Growth Factors Produced by Malignant Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

H. Marquardt Expert, LVC, NCI

COOPERATING UNITS *(if any)*

California Institute of Technology, Pasadena, CA

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Low molecular weight human, mouse and rat transforming growth factors (TGFs) were isolated from tissue culture media conditioned by: (1) two human metastatic Ormelanoma tumor lines, (2) a Moloney murine sarcoma virus-transformed mouse 3T3 cell line; and (3) two Fischer rat embryo fibroblast cell lines, nonproductively transformed, one by Snyder-Theilen feline sarcoma virus and the other by Abelson murine leukemia virus. These TGFs compete for binding to the cellular epidermal growth factor (EGF) receptor. The purification of each TGF was achieved by gel permeation chromatography, followed by reverse phase high pressure liquid chromatography using sequentially acetonitrile and 1-propanol in the presence of aqueous trifluoroacetic acid. The amino-terminal sequences of rat, mouse and human TGFs showed extensive sequence homology among polypeptides from different species and cell types. The complete primary structure of rat TGF was determined. Rat TGF is a single chain polypeptide, with a calculated molecular weight of 5,600, which displays 33% sequence homology with murine EGF and 44% sequence homology with human urogastrone. These results emphasize that TGFs which are associated with the control of cell growth and differentiation are highly conserved through vertebrate evolution and are chemically distinct, but are evolutionarily related to EGFs found in normal cells and tissue fluids.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George J. Todaro	Medical Officer	LVC, NCI
Joseph E. De Larco	Research Chemist	LVC, NCI
Daniel R. Twardzik	Research Chemist	LVC, NCI
John R. Stephenson	Visiting Scientist	LVC, NCI

Objectives:

To purify and characterize transforming growth factors (TGFs) released by malignant cells in order to chemically and biologically compare the isolated polypeptides with known growth factors, such as epidermal growth factor (EGF), and to determine their role in malignant transformation.

Methods Employed:

Biochemical and biological laboratory techniques were developed or adapted to isolate and purify TGFs from serum-free supernatants of cultured cells. Various standard techniques were used to characterize and compare the purified growth factors.

Major Findings:

1. Determination of the amino-terminal sequences of TGFs. TGFs were purified from serum-free medium conditioned by retrovirus-transformed Fischer rat embryo fibroblasts, mouse 3T3 cells and two human melanoma cell lines. The purification of each TGF was monitored in a radioreceptor assay based on receptor cross-reactivity with mouse submaxillary gland epidermal growth factor (EGF) and was achieved by gel permeation chromatography of the acid-soluble, TGF-containing activity, followed by reverse phase high pressure liquid chromatography using sequentially acetonitrile and 1-propanol in the presence of aqueous trifluoroacetic acid. The amino-terminal sequences of rat, mouse and human TGFs were determined. Extensive (>90%) sequence homology was found among TGF polypeptides from different species and cell types.

2. The complete primary structure of rat TGF. The complete amino acid sequence of rat TGF was deduced from microsequence analysis data of reduced and S-carboxyamidomethylated TGF and Lys-c peptides, and from results of carboxypeptidase digestion of modified rat TGF. Rat TGF is a single chain polypeptide with a calculated molecular weight of 5,600 which displays 33% sequence homology with murine EGF and 44% sequence homology with human urogastrone.

Significance to Biomedical Research and the Program of the Institute:

A new and unique family of growth factors was isolated and purified to homogeneity from different species (human, mouse, rat) and different cell types (melanoma cells and virally transformed fibroblasts). The isolated growth factors are highly conserved through vertebrate evolution and are associated

with the control of cell growth and differentiation. The demonstrated sequence homology between TGFs and EGFs emphasizes the evolutionary relationship between growth factors derived from neoplastic and normal cells. Thus, TGFs can utilize the EGF membrane receptor expressed on normal and transformed cells to mediate certain biological responses or to support the cells' own growth. The determination of the complete primary structure of TGF has been a major advance toward understanding the development and maintenance of neoplasia.

Proposed Course:

Studies in progress suggest that TGFs isolated from human tumor cells in tissue culture and from the urine of cancer patients are chemically nearly indistinguishable. Monoclonal antibodies to TGF have been prepared and will be used to develop sensitive and specific radioimmunoassays to correlate TGF production and the development of neoplasia.

Publications:

De Larco, J. E., Preston, Y. H., Marquardt, H. and Todaro, G. J.: Characterization of purified SGF and its relationship to EGF. In Galeotti, T., Cittadini, A., Neri, G. and Pappa, S. (Eds.): Membranes in Tumor Growth, Developments in Cancer Research. New York, Elsevier Biomedical Press, 1982, pp. 431-436.

Marquardt, H., Hunkapiller, M. W., Hood, L. E., Twardzik, D. R., De Larco, J. E., Stephenson, J. R. and Todaro, G. J.: Transforming growth factors produced by retrovirus-transformed fibroblasts and human melanoma cells: Amino acid sequence homology with epidermal growth factor. Proc. Natl. Acad. Sci. USA. (In Press)

Pike, L. J., Marquardt, H., Todaro, G. J., Gallis, B., Casnellie, J. E., Bornstein, P. and Krebs, E. G.: Transforming growth factor and epidermal growth factor stimulate the phosphorylation of a synthetic, tyrosine-containing peptide in a similar manner. J. Biol. Chem. 275: 14628-14631, 1982.

Rapp, U. R., Gunnell, M. and Marquardt, H.: Normal mouse serum contains peptides which induce fibroblasts to grow in soft agar. J. Cell. Biochem. 21: 29-38, 1983.

Todaro, G. J., Marquardt, H., Twardzik, D. R., Johnson, P. A., Fryling, C. M. and De Larco, J. E.: Transforming growth factors produced by tumor cells. In Owens, A. (Ed.): Tumor Cell Heterogeneity: Origins and Implications. New York, Academic Press, 1982, Vol. 4, pp. 205-224.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05174-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Control of Cellular Differentiation by Extracellular Signals

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

A. Rizzino Expert, LVC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The long range goal of this project is to understand the molecular mechanisms that control cellular differentiation during mammalian embryogenesis. Multipotent embryonal carcinoma cells are ideally suited for this purpose. These cells not only differentiate into a wide variety of cell types that are derived from the three embryonic germ layers, they also mimic, both morphologically and biochemically, important stages of early mammalian development. However, to fully utilize this model system one must be able to direct the differentiation of multipotent embryonal carcinoma cells to only one cell type. This project describes culture conditions under which two multipotent embryonal carcinoma cell lines differentiate primarily, and possibly exclusively, into parietal extraembryonic endoderm - one of the first cell types to appear during mammalian development. To avoid the inherent problems of serum-containing media, defined media have been developed that support the growth and/or the differentiation of several different embryonal carcinoma cell lines. The defined medium contains fibronectin, insulin, transferrin, and high density lipoprotein in place of serum. In this defined medium, multipotent embryonal carcinoma cells rapidly differentiate, with over 90% undergoing commitment within one cell cycle. After 72 hours, a pure population of differentiated cells has formed. More importantly, at least 85% of the cells exhibit the properties of parietal extraembryonic endoderm. Upon further examination of this new cell culture model, it was discovered that fibronectin and laminin promote the differentiation of multipotent embryonal carcinoma cells. This provided the first evidence that these molecules, which are developmentally regulated, may directly influence early mammalian development. This possibility is supported by the finding that early mouse embryos cultured in the absence of serum require fibronectin or laminin in order to develop beyond the blastocyst stage. Studies are in progress to determine the mechanisms by which fibronectin and laminin influence differentiation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

None

Objectives:

It is now well-established that extracellular signals play a significant role in the control of cellular differentiation during early mammalian development. In order to identify these signals and to study their molecular modes of action, many investigators are examining the differentiation of multipotent mouse embryonal carcinoma (EC) cell lines. These cell lines not only mimic important stages of early mammalian development but also differentiate into a wide variety of cell types (e.g., both cardiac and skeletal muscle, nerve and glial cells, cartilage, adipocytes, etc.). However, it is very difficult to identify and study extracellular signals that control differentiation in such complex populations. Therefore, the initial aim of this project was to develop a cell culture model in which an entire population of multipotent EC cells differentiates into only one cell type. Having achieved this aim during the initial stages of this project, two major objectives remain the focus of the project: (1) identify the extracellular signals that block differentiation and those that promote differentiation and (2) determine the molecular mechanisms by which these factors influence the process of differentiation.

Methods Employed:

Due to the complexity and undefined nature of serum, it is very difficult to identify the factors that influence differentiation. With this problem in mind, a defined medium was developed several years ago for studying the growth and differentiation of the EC cell line F9 (Rizzino, A. and Crowley, C., Proc. Natl. Acad. Sci. USA, 77: 457-461, 1980). In this medium (referred to as EM-3), serum is replaced with insulin, transferrin and the attachment factor fibronectin. In EM-3, as in serum-containing media, little or no differentiation of F9 occurs unless an inducer, such as retinoic acid, is added. In the presence of retinoic acid, F9 EC cells undergo irreversible cellular differentiation and form cells that exhibit the properties of parietal endoderm (parietal endoderm begins to form at the end of the 4th day of gestation and is one of the first cell types to form during mammalian development). The only major difference between the growth of F9 EC cells in EM-3 and in serum-containing media is that F9 will clone in serum-containing media but cannot in EM-3. This difference is eliminated when EM-3 is supplemented with high density lipoprotein (HDL). The fact that F9 EC cells can only differentiate into a few cell types (it is a restricted EC cell line) raises several important questions: (1) Do multipotent EC cells, which can differentiate into a wide variety of cells derived from each of the three embryonic germ layers, proliferate in EM-3 or in EM-3 plus HDL? (2) Do multipotent EC cells require and utilize the same inducers of differentiation as restricted EC cells? Therefore, the first step was to examine the behavior of several different EC cell lines in the absence of serum. In most experiments, two multipotent EC cell lines were used: OC15S1 and 1003. However, in order to verify

the relevance of these results to early development, mouse embryos have been examined under similar culture conditions. In all EC cell experiments, a variety of markers, including ultrastructure and the production of plasminogen activator, has been used to monitor, and to aid in the identification of, the differentiated cells.

Major Findings:

1. The differentiation of two multipotent embryonal carcinoma cell lines into parietal endoderm-like cells. In striking contrast to F9 EC cells, two multipotent EC cell lines, OC-15S1 and 1003, do not proliferate in EM-3 supplemented with HDL, but rapidly and irreversibly differentiate. Under these conditions, greater than 90% of the EC cells differentiate within 12 hours (greater than 99% by 24 hours) and none of the EC cells remain undifferentiated after 48 hours. The differentiated cells that form exhibit a uniform morphology throughout the culture, and their morphology is characteristic of parietal endoderm. This is supported by an ultrastructural examination of the differentiated cells. As expected of parietal extraembryonic endoderm, the differentiated cells are almost completely devoid of microvilli and do not exhibit the numerous cytoplasmic vacuoles that are characteristic of visceral endoderm. In addition, at least 85% of the differentiated cells secrete plasminogen activator, and only the type of plasminogen activator (the tissue activator type) that is unique to parietal extraembryonic endoderm is secreted. Further support for this finding is the absence of alpha-fetoprotein (a marker during early development for visceral endoderm) and the absence of receptors for epidermal growth factor and platelet-derived growth factor (both are expressed by visceral extraembryonic endoderm and several other embryonic cell types). On the basis of these results, it is clear that at least 85%, and possibly 100%, of the differentiated cells exhibit the properties of parietal extraembryonic endoderm.

2. Fibronectin and laminin influence the cellular differentiation of multipotent embryonal carcinoma cells. In an attempt to understand why multipotent EC cells differentiate in EM-3 plus HDL, each of the components of the defined medium was examined for its ability to influence differentiation. This led to the discovery that fibronectin, a major component of extracellular matrices, can promote the differentiation of OC-15S1 and 1003 in serum-containing media. Under these conditions, 65-75% of the cells differentiated. However, fibronectin will only promote differentiation if it is bound to the surface of the culture dish. (Although serum contains relatively high concentrations of fibronectin, it does not bind to the culture dish. Consequently, culture dishes must be coated with fibronectin prior to the addition of serum, otherwise it has no effect on differentiation. As a result of this finding, it is now clear that, contrary to what was commonly believed, fibronectin in serum-containing media does not function as an attachment factor. This has been verified by culturing several different cell lines in fibronectin-depleted, serum-containing media.) The finding that fibronectin induces the differentiation of several multipotent EC cell lines raised the possibility that other components of extracellular matrices might also promote EC cell differentiation. Therefore, laminin, a component of basement membranes and an attachment factor for F9 EC cells (Rizzino et al., J. Supramol. Struct., 13: 243-253, 1980), was tested for its effect on the growth and differentiation of multipotent EC cells. When laminin was substituted for fibronectin in the defined medium, the same complete differentiation of the multipotent cells

occurred, and laminin was also able to promote the differentiation of the multipotent EC cells in serum-containing media. In contrast to fibronectin and laminin, another macromolecule that has been shown to affect attachment and spreading of other cells, polylysine, does not promote the differentiation of OC-15S1 or 1003. Moreover, polylysine does not support the attachment of either multipotent EC cell line when used in place of fibronectin in the defined medium. It is evident from these findings that the differentiation of OC-15S1 and 1003 is dependent on the type of substratum to which they attach.

3. Serum factors interfere with the differentiation of multipotent EC cells. The finding that approximately 70% of the multipotent EC cells differentiate on fibronectin- or laminin-coated dishes in serum-containing media contrasts with the complete differentiation observed in the defined medium. The major reason for this result is that one or more serum factors block differentiation. Attempts to better understand this result have led to the finding that as the cells proliferate they remove the inhibitory factors from the serum-containing media. These factors have been shown to be stable to extremes of pH and are non-dialysable. However, the nature of these molecules and their mechanisms of action remain to be identified.

4. Embryonal carcinoma cells and early mouse embryos respond to the same factors for attachment and growth. In order to verify that the results obtained with EC cells are relevant to early mammalian development, mouse embryos at the blastocyst stage were cultured under conditions similar to those used for EC cells. In these experiments, the results obtained with embryos in defined media were compared to those obtained in serum-containing media. (In serum-containing media, blastocysts attach to the surface of the culture dish after 40-48 hours and begin to differentiate at that point. Initially, the trophoblasts differentiate and form cells that secrete plasminogen activator [the urokinase type]. During the subsequent 24 to 48 hours, the inner cell mass begins to differentiate and the first cells to appear are primary endoderm, which then specializes into parietal and visceral extraembryonic endoderm.) When mouse blastocysts are cultured in defined medium containing insulin, transferrin and HDL but lacking an attachment factor, they attach very poorly to the surface of the tissue culture dish and fail to develop further. However, if fibronectin (or laminin) is also added, the blastocysts attach firmly to the culture dish and the trophoblasts differentiate (as evidenced by their production of plasminogen activator). Shortly thereafter, the inner cell mass, which has grown considerably, as it does in serum-containing media, begins to differentiate, forming extraembryonic endoderm. However, development of the inner cell mass does not appear to continue beyond this point in defined media. Apparently, additional factors are required at this stage during development and most have not been identified. However, as reported in a related project (The possible role of transforming growth factors during the early stages of mammalian development - Z01CP05248-02 LVC), platelet-derived growth factor stimulates the growth of differentiated cells derived from EC cells and from mouse blastocysts. While these results indicate that the development of mouse blastocysts does not proceed as far in defined media as it does in serum-containing media, it is evident that EC cells and mouse embryos respond to many of the same factors.

Significance to Biomedical Research and the Program of the Institute:

The major goal of this project is to identify and study extracellular signals (hormones, attachment factors, etc.) that control differentiation. Thus far, the above studies have proven to be important in several ways. First, the entire population of two different multipotent EC cell lines irreversibly differentiates into one major cell type (parietal endoderm) when the cells are cultured in EM-3 plus HDL. This is very significant since the conditions developed previously for the differentiation of multipotent EC cells did not result in homogeneous formation of any particular cell type and, in most cases, reproducibility was poor. Second, attempts to understand why the multipotent EC cells differentiate in the defined medium led to the finding that fibronectin and laminin (both of which are found in extracellular matrices) induce EC cell differentiation. This finding is important in light of the fact that both fibronectin and laminin are carefully regulated during early mammalian development and they appear prior to the formation of parietal and visceral extraembryonic endoderm. Thus, it seems likely that fibronectin and laminin play a major role in the formation of extraembryonic endoderm during development. This is consistent with the finding that fibronectin and laminin promote the attachment and differentiation of early mouse embryos. Third, the use of defined media has revealed important differences between restricted and multipotent EC cell lines. Fourth, the defined medium EM-3, which was developed for the growth of F9 EC cells, has proven invaluable in a related project; namely, the study of transforming growth factors produced by EC cells. The importance of the current project lies not only in furthering our understanding of differentiation during embryogenesis but ultimately should help to evaluate a new approach to cancer therapy. It has been argued [Pierce, In Sherman, M. and Solter, D. (Eds.): Teratomas and Differentiation. Academic Press, 1975, pp. 3-12] that the stem cells of certain tumors (e.g., squamous cell carcinomas, neuroblastomas and teratocarcinomas) have the capacity to differentiate in vivo to form harmless post-mitotic cell types. Under normal conditions, the frequency of differentiation is usually low, but it may be possible to retard or completely block the growth of certain tumors by inducing their stem cells to differentiate. However, before we can adequately evaluate such an approach, we must determine how, and to what extent, the extracellular environment influences cellular differentiation.

Proposed Course:

In order to focus on the mechanisms that control cellular differentiation during early mammalian development, the following studies are proposed. (1) Examine the mechanisms(s) by which fibronectin and laminin promote differentiation. For these studies, it is planned to isolate mutant embryonal carcinoma cells that no longer respond to fibronectin or laminin by differentiating. Initially, the binding of fibronectin and laminin to parent and mutant EC cells will be examined. In addition, since fibronectin and laminin appear to promote the differentiation of multipotent EC cells by affecting cell shape, the influence of these molecules on the cytoskeleton will be examined. (2) Develop a defined medium that supports the long-term growth of multipotent EC cells. This will not only lead to the identification of the macromolecular requirements for growth but will also help to better understand why the multipotent EC cells differentiate when cultured in the defined medium EM-3 plus HDL. Equally important, a defined medium that supports growth will be the most efficient means of identifying the

serum factors that interfere with the differentiation of multipotent EC cells. (These inhibitors are of considerable interest because they suppress differentiation and therefore suppress the loss of tumorigenicity that accompanies the differentiation of EC cells.) Once these inhibitory factors have been identified, the mechanisms by which they influence differentiation will be examined.

Publications:

Rizzino, A.: Fibronectin and laminin promote the differentiation of two multipotent embryonal carcinoma cell lines. In Silver, L. M., Martin, G. R. and Strickland, S. (Eds): Teratocarcinoma Stem Cells. New York, Cold Spring Harbor Press (In press)

Rizzino, A.: The growth and differentiation of embryonal carcinoma cells in defined media. In Sirbasku, D., Sato, G. and Pardee, A. (Eds.): Growth of Cells in Hormonally Defined Media. New York, Cold Spring Harbor Press, 1982, pp. 209-218.

Rizzino, A.: Model systems for studying the differentiation of embryonal carcinoma cells. Cell Biol. Int. Rep. (In Press)

Rizzino, A.: Two multipotent embryonal carcinoma cell lines irreversibly differentiate in defined media. Dev. Biol. 95: 126-136, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05175-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transforming Growth Factors in Urine of Patients with Disseminated Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

D. R. Twardzik Research Chemist, LVC, NCI

COOPERATING UNITS *(if any)*

Dept. Pediatrics, Children's Memorial Hospital, University of Oklahoma, Oklahoma City, OK; Dept. Urology, Univ. Chicago Medical School, Chicago, IL; Dept. Endocrinology, School of Medicine, Vanderbilt Univ., Nashville, TN

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The urine of patients with a variety of pediatric malignancies including small cell carcinoma, osteogenic sarcoma and rhabdomyosarcoma contains an acid- and heat-stable, high molecular weight complex which competes for binding to epidermal growth factor (EGF) and promotes the anchorage-independent growth of non-transformed cells in semi-solid media. High levels of this activity were not found in the urine of patients with acute or chronic lymphocytic leukemia or in the majority of healthy control children. The transforming activity associated with the high molecular weight, patient-derived activity is a result of the interaction of EGF related peptides in the complex and a transformation potentiating factor (TPF) which is functionally unrelated to EGF. In addition, the urine of some cancer patients also contains a low molecular weight transforming growth factor related to those released by tumor cells in culture. These tumor-associated and tumor-specific activities may, in addition to being useful clinical markers of neoplasia, also provide information on the role of host-and tumor-specific growth factors in the mechanism(s) of tumor progression in vivo.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

John R. Stephenson	Visiting Scientist	LVC, NCI
Fred H. Reynolds, Jr.	Expert	LVC, NCI
Stephen A. Sherwin	Clinical Associate	BRMP, NCI

Objectives:

High and low molecular weight growth factor activities which compete for epidermal growth factor (EGF) receptors and stimulate anchorage-independent cell growth in semi-solid media will be purified from the urine of patients with disseminated cancer. Their biochemical and biological properties will be compared to human EGF (urogastrone) and transforming growth factor (TGF) released by tumor cells in culture. In addition, both a pediatric clinical study and an animal model will be designed to examine the usefulness of urine-derived growth factors as markers of the transformed phenotype.

Methods Employed:

Urine was collected from previously untreated cancer patients and human tumor-bearing athymic mice, and peptides solubilized utilizing an acid-ethanol extraction procedure. The high molecular weight TGF (30,000-35,000 M_r) was initially resolved from urogastrone and other urinary peptides by gel filtration under acidic conditions. TGF activity was identified by both radiolabeled EGF competition assays on EGF receptor-rich human carcinoma cells and stimulation of normal rat kidney cells to form progressively growing colonies in soft agar. Further purification was achieved by reverse phase high performance liquid chromatography (HPLC) on C_{18} μ Bondapak columns utilizing different solvent elution systems. The purified peptide(s) was compared in both structure and function to human EGF and TGFs released from human tumor cells in vitro.

Major Findings:

1. Detection of high molecular weight transforming growth factor-like activities in urine of pediatric cancer patients. Urine specimens from patients with a variety of pediatric malignancies selected for disseminated disease and an equal number of control children were examined for the presence of transforming growth activities. Peptides were solubilized with acidified ethanol from 25 ml of previously frozen early morning void urine and chromatographed on Bio-Gel P-30 columns. Aliquots of alternate fractions were tested for EGF-competing and soft agar growth stimulating activities. The urine from a typical patient, in addition to containing EGF which elutes in the region of the 6,000 M_r insulin marker, also contains a peak of EGF-competing activity eluting slightly larger than the 29,000 M_r carbonic anhydrase marker. This 30,000-35,000 M_r EGF-competing activity coeluted with soft agar growth stimulating activity. All urine specimens examined, including urine from healthy children, also contained an 8,000 M_r soft agar growth-stimulating activity which is chromatographically separable from EGF.

A scattergram representing the ratio of the high molecular to low molecular weight soft agar growth stimulating activity (TGF activity) in urine from 18 cancer patients and an equal number of controls is presented in Chart I. Eleven out of 18 patients scored higher than 0.5, with most patients scoring in the range of 1.0 to 2.0. The majority of patients whose urine did not contain detectable levels of the high molecular weight TGF-like activity were diagnosed as having either chronic or acute lymphocytic leukemia. Highest levels of activity were found in lymphoma patients and a patient with undifferentiated small cell carcinoma. In contrast to the low levels of TGF-like activity found in urine from adult controls, urine derived from healthy children contained higher background levels of high molecular weight, soft agar growth stimulating activity.

2. Requirement of a transformation potentiating factor for urine-derived "transforming" activity. The low molecular weight, 8,000 M_r "transforming" activity found in all urine specimens examined was further purified by high performance liquid chromatography on C_{18} μ Bondapak columns. This activity can be resolved into two components following elution with acetonitrile, neither of which by themselves supports the growth of non-transformed cells in semi-solid media. The EGF-competing activity elutes at 40-42% acetonitrile as a doublet; both species cross-react equivalently in a homologous radioimmunoassay for human urogastrone. The second component of the low molecular weight transforming activity elutes at 38% acetonitrile and does not compete with EGF for membrane receptors. In the presence of nanogram amounts of EGF this factor is extremely potent in stimulating the growth of both normal rat kidney and normal human foreskin fibroblasts in semi-solid media. This peptide has been purified to homogeneity by HPLC and migrates on SDS-polyacrylamide gel electrophoresis as a single band with an apparent molecular weight of 8,600. In addition to EGF, the low molecular weight EGF-competing activity derived from the urine of a patient with a well-differentiated gastric adenocarcinoma also contains TGF. This peptide, unlike EGF, elutes from μ Bondapak columns in the range of 18-20% acetonitrile, is not immunologically related to EGF and, thus, is similar to TGFs produced by transformed cells in culture. Following HPLC purification, the urine-derived TGF also requires the presence of a second factor, transformation potentiating factor (TPF), in order to promote anchorage-independent cell growth.

3. Comparison of growth factors functionally related to EGF in the urine of normal and human tumor-bearing athymic mice. Acid-ethanol-extracted urine from 8-12-week-old BALB/c athymic mice contains a major peak of EGF-competing activity which elutes from Bio-Gel P-100 columns in the region of the 6,000 M_r insulin marker. This peptide is indistinguishable from mouse submaxillary gland EGF when tested in a homologous radioimmune assay for murine EGF. In addition to EGF, acid-ethanol-extracted mouse urine also contains a minor peak of EGF-competing activity eluting with an apparent molecular weight of 20,000 M_r. The mean concentration of this 20,000 M_r growth factor in normal nude mouse urine as determined by the radioreceptor assay is 0.7 ng of EGF equivalents per mg of acid-ethanol solubilized urine peptides. A significant increase in the levels of this factor is seen in urine collected from mice bearing tumors following the subcutaneous injection of a human rhabdomyosarcoma cell line designated A673. The amount of EGF-competing activity eluting in the 20,000 M_r region derived from the urine of tumor-bearing mice is approximately 8-10-fold higher relative to the comparable activity found in an equivalent amount of normal mouse urine. Highest

titers of the 20,000 M_r EGF-competing activity are found in urine derived from mice in which the original A673 tumor is trocar passaged. A similar, but less exaggerated, response relative to the human rhabdomyosarcoma cell line A673 was also seen in athymic mice bearing tumors following inoculation of the human melanoma cell line A375. The component which elutes in the 20,000 molecular weight region in both normal and tumor-bearing urines was further characterized using high performance liquid chromatography in order to determine if the factor which responds to tumor burden has elution characteristics on HPLC similar to the factor found in low levels in normal urine. The 20,000 M_r growth factor from control mouse urine is resolved into three EGF-competing activities on C_{18} μ Bondapak columns. The major activity elutes at an average acetonitrile concentration of 32%, whereas two minor peaks of EGF-competing activity eluting at approximately 30.5% and 35% acetonitrile are also seen. The 20,000 M_r component found in tumor-bearing mouse urine gives a similar EGF-competing profile when examined on HPLC. In addition to the EGF-competing activities eluting in the 32-35% range of acetonitrile, the 20,000 M_r component from tumor-bearing urine also contains an EGF-competing activity eluting in the region of 20% acetonitrile. This activity is not found in control urine and corresponds in elution position to the 20,000 M_r TGFs released by A673 cells into conditioned media. Dilutions of comparable amounts of EGF equivalents as determined by the radioreceptor assay of both control and tumor bearer-derived activities demonstrate immunological cross-reactivity when tested with antisera against mouse EGF. At least 5 more EGF receptor equivalents of the HPLC purified factor relative to EGF were required to displace 50% of ^{125}I-EGF bound to antibody. In contrast, transforming growth factors derived from the urine of tumor-bearing mice eluting at 20% acetonitrile do not demonstrate any cross-reactivity when tested at similar concentrations with this particular antisera.

Significance to Biomedical Research and the Program of the Institute:

In addition to the potential usefulness of urine-derived, tumor-specific and tumor-associated growth factor(s) as a clinical marker of tumor burden prior to and following therapy, these studies also provide information on the role of host- and tumor-specific growth factors on the mechanism(s) of tumor progression in vivo.

Proposed Course:

A more specific assay with sensitivity in the picomole range will be developed for detecting TGFs in the urine of cancer patients. Synthetic peptides corresponding to the known sequence of rat TGF will be used to produce both polyclonal and monoclonal antisera for use in development and application of said TGF assay.

Publications:

Marquardt, H., Hunkapiller, M. W., Hood, L. E., Twardzik, D. R., De Larco, J. E., Stephenson, J. R. and Todaro, G. J.: Transforming growth factors produced by retrovirus-transformed rodent fibroblasts and human melanoma cells: Amino acid sequence homology with epidermal growth factor. Proc. Natl. Acad. Sci. USA (In Press).

Sherwin, S. A., Twardzik, D. R., Bohn, W. H., Cockley, K. and Todaro, G. J.: High molecular weight transforming growth factor activity in the urine of patients with disseminated cancer. Cancer Res. 43: 403-407, 1983.

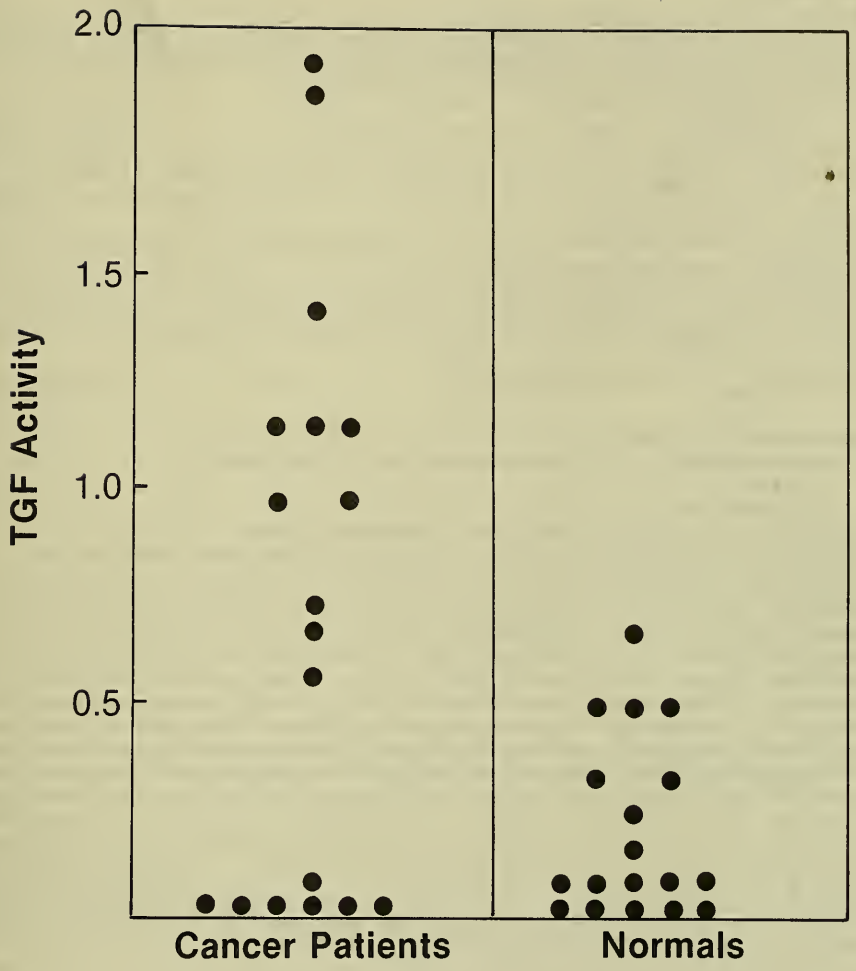
Todaro, G. J., Marquardt, H., Twardzik, D. R., Johnson, P. A., Fryling, C. M. and De Larco, J. E.: Transforming growth factors produced by tumor cells. In Owens, A. H., Jr. (Ed.): Tumor Cell Heterogeneity: Origins and Implications. New York, Academic Press, 1982, Vol. 2, pp. 205-224.

Twardzik, D. R., Sherwin, S. A., Ranchalis, J. E. and Todaro, G. J.: The urine of normal, pregnant and tumor bearing humans contains transforming growth factors. J. Natl. Cancer Inst. 69: 793-798, 1982.

Twardzik, D. R., Todaro, G. J., Reynolds, F. H., Jr. and Stephenson, J. R.: Similar transforming growth factors (TGFs) produced by cells transformed by different isolates of feline sarcoma virus. Virology 124: 201-207, 1983.

Zoelen, E. J., Twardzik, D. R., van Oostwaard, Th. M. J., van der Saag, P. T. and de Laat, S. W.: Transforming growth factors produced by a neuroblastoma cell line in a chemically defined serum-free medium. Proc. Natl. Acad. Sci. USA (In Press)

Figure 1



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05180-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Evolution and Sequence Organization of Mammalian Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

R. E. Benveniste Medical Officer, LVC, NCI

COOPERATING UNITS *(if any)*

University of California School of Medicine, Davis, CA; Yale University, New Haven, CT; Wayne State University, Detroit, MI; New England Regional Primate Center, South Borough, MA

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.1	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Nucleic acid hybridization studies using cloned retroviral DNA are being used to examine the sites of integration and sequence organization of viral sequences in mammalian cells. One of the goals of these studies is to characterize the endogenous RNA tumor viral genomes in normal and malignant human tissues. Sequences related to various subclones of the endogenous baboon type C virus have been cloned from a gorilla DNA library. These gorilla viral sequences will be used to probe various human tissues. In addition, the concept of retroviruses as transposable elements between species is being used as a marker for evolutionary rates of mammalian divergence. We have measured precisely the time of interspecies virus transfer and used this to measure the rates of accumulation of nucleotide substitutions in the mammalian lineages that have diverged after the viral transfer occurred. An endogenous virus is being cloned from carnivore cell DNA that is related to the colobus monkey type C virus, and will be used as a probe in carnivore evolutionary studies. A complete phylogenetic tree of carnivore evolution has been derived by molecular hybridization techniques - it differs in many respects from the classical relationships derived by anatomical considerations and morphometric measurements.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Kurt J. Stromberg	Medical Director	LVC, NCI
Stephen J. O'Brien	Geneticist	LVC, NCI
Daniel K. Haapala	Microbiologist	LVC, NCI

Objectives:

To study the evolution and organization of primate and feline retroviruses within the mammalian genome. To use the time of interspecies viral transfer as a defined marker for the rates of evolution of various species. Recombinant DNA techniques will be employed to develop probes appropriate for the detection of virus-related sequences in human cells and tissues.

Methods Employed:

The viruses used were the Old World monkey isolates from this laboratory: the baboon type C virus, the colobus-macaque-rhesus class of type C viruses, and the langur type D viruses. The feline viruses included RD-114 and feline leukemia virus (FeLV). Primary cell lines from various feline and ape species were developed and maintained. These lines were used in virus isolation, host range, and viral interference experiments as well as to study the control of viral transcription. Replication of retroviruses was detected by assaying the pellet obtained after high speed centrifugation of supernatant fluid from cells for reverse transcriptase activity. Radioimmunoassays for various type C viral proteins were also employed to characterize new isolates. The cloning of proviral DNA was performed using various plasmids as vectors, as well as phages constructed from lambda. Restriction enzyme maps were generated for various retroviruses. Cloned proviral DNA was used as a probe for cloning viral sequences integrated in the genomes of mammalian species. The expression of primate retroviral sequences in cells was investigated with RNA blotting procedures after selection of poly A-containing molecules by oligo dT cellulose chromatography.

Major Findings:

1. Characterization of retroviruses isolated from various macaque species. An examination of various rhesus monkey (Macaca mulatta) organs has shown a preference for type C viral antigen expression in the placenta. Separate cocultivations of isolated trophoblasts from ten rhesus monkey placentas with cell lines from heterologous mammalian species led to rapid isolation of type C rhesus retroviruses in four of ten cases. These four retrovirus isolates have been designated MMC-2 through MMC-5. Five of the remaining six sets of cocultivations grew simian foamy virus and were discontinued. Distinction of these viral isolates from the initial rhesus isolate (MMC-1) and the previous isolate from the stumptail monkey, Macaca arctoides (MAC-1), could be made by liquid DNA hybridization, although not by limited restriction endonuclease digestion.

Both MAC-1 and MMC-1 were obtained in single long term cocultivation experiments (over seven months). The present isolates, MMC-2 through MMC-5, were detected in two to five weeks. Consequently, primary trophoblast cells represent a useful differentiated cell type for isolation of infectious retrovirus from this primate species. In addition, in a collaborative study, several isolates from Macaca cyclopis are being compared by restriction endonuclease digestion to the previous isolates from Macaca arctoides and Macaca mulatta.

2. Detection of retroviral sequences in normal and malignant human tissues. A gorilla DNA library has been constructed in λ WES. λ B and plaques containing nucleic acid sequences that hybridize to subcloned restriction fragments of the baboon type C genome have been purified. These gorilla sequences are being mapped and will be used as probes for endogenous human viral sequences.

3. Cloning of an endogenous carnivore virus related to the colobus monkey type C virus. The only infectious carnivore viruses isolated to date include RD-114 and FeLV, both of which are viruses recently acquired by interspecies transmission. These viruses are found in multiple copies in the DNA of six closely related Felis species. The colobus monkey type C virus detects distantly related sequences in carnivore DNA. A DNA library has been made of leopard cat DNA (which lacks RD and FeLV viral sequences) and plaques are being purified that hybridize to cloned subgenomic colobus virus DNA fragments. These carnivore viral sequences will be examined for their distribution in carnivore species and as a marker in carnivore evolutionary studies.

4. The transfer of retroviruses from primates to felines as a marker for studies of evolutionary rates. Earlier work had shown a transfer of viruses from primates to felines sometime during the past several million years. The virus acquired by cats (and 6 of their descendant species) is called RD-114. We have now shown that this transfer occurred at the time of the gelada-baboon ancestor or about 4-6 million years ago. Using this transfer as a marker for evolutionary time, we have studied the rate of accumulation of mutations in those primate and feline species that have diverged since the time of virus transfer. The rate of mutation has been the same in both lineages, and the rate of base-pair substitutions in DNA is therefore independent of generation time in these two mammalian orders.

5. Carnivore evolution and phylogeny as derived by DNA hybridization. A phylogenetic tree of the carnivores has been derived from thermal stability measurements of nonrepetitive cellular DNA. The data include 30 species of carnivores, with 8 of them being used as index species. The computer programs developed by Drs. Fitch ("Neighborliness") and Dayhoff ("Mattop") were used to derive phylogenies. The data obtained differ in many respects from that obtained by classical methods such as anatomical comparisons and morphometric measurements. For example, the lesser panda and skunk seem to represent distinct families of carnivores since they are equidistant from a phylogenetic perspective from all other carnivores. In collaboration with Dr. S. O'Brien, the giant panda was shown to belong to the Ursidae family. The pandas, raccoons, and bears were also studied from a variety of evolutionary perspectives, utilizing DNA hybridization, isozyme mapping, and immunological distance, and compared to previously obtained data on karyology and comparative anatomy.

Significance to Biomedical Research and the Program of the Institute:

The discovery and evolutionary tracing of the numerous primate retroviruses represents an important program of study. Earlier investigations revealed that endogenous retroviral DNA sequences are present in primate cellular DNA and are inherited as stable Mendelian units. In certain cases, these viruses can be transmitted from one species to an evolutionarily distant one and subsequently incorporated into the germ line. The viral sequences are, therefore, subject to the same evolutionary processes as the cellular DNA sequences.

Recently, recombinant DNA technology coupled with restriction enzyme analysis has provided selective and specific probes which can detect subviral human gene sequences related to the various animal retroviruses. Thus, in higher primates, it appears that complete viral sequences exist which are only rarely, if ever, expressed as a virus particle. This is potentially very important and may provide a means to eventually isolate and characterize a bona fide human retrovirus and permit an assessment of its role in human neoplasia.

One of the key questions in evolutionary biology is whether mutations in DNA accumulate as a function of chronological time or as a function of generation time of a species. The only previous "clocks" available were the fossil record or the dates of continental drift, both of which are imprecise. The transfer of retroviruses between species offers a unique tool for fixing a point in evolutionary time that is completely independent of any assumptions of geologic time.

Proposed Course:

We will attempt to clone those human viral sequences related to endogenous gorilla type C viral DNA sequences in order to generate a restriction map of the endogenous human viruses. Endogenous carnivore viral sequences are also being cloned that are present in all families of carnivores and will be used to study their origin from primates and integration sites, and as a tool to study carnivore, especially, feline evolution. Since retrovirus DNA sequences evolve at a faster rate than nonrepetitive cellular DNA, these viral sequences could be used to study the phylogeny of closely related carnivore species.

Publications:

Stromberg, K. J. and Benveniste, R. E.: Efficient isolation of endogenous rhesus retrovirus from trophoblast. Virology (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05202-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure, Function, and Utilization of Eukaryotic Promoter Sequences

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

G. E. Mark Expert, LVC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structure, function and utilization of eukaryotic promoter sequences required for the specific initiation of RNA transcription are being studied. The endogenous retrovirus CPC-1 isolated from Colobus polykomos kidney cells has proven to be a unique probe for the modulators of eukaryotic gene expression. Specific goals are: (1) to elucidate the specific DNA signal sequences (promoters) which control efficient transcription; (2) to evaluate the evolutionary fate of retroviral sequences; and (3) to develop efficient eukaryotic cloning vectors. The CPC-1 long terminal repeat (LTR) has been sequenced and shown to contain two overlapping promoters. Comparison of these sequences with those of transcriptionally inactive parent proviruses shows that the promoter initiation domain covers at least 10 nucleotides (-32 to -23). The active promoter has been subcloned and used to express a truncated tk gene ligated into the viral cap site. Data suggest that amplification of endogenous proviral sequences has been the result of gene transposition. A DNA sequence in sheep related to the CPC-1 promoter has been found to be responsible for the insertional activation of bovine leukemia virus.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Tom I. Bonner Guest Researcher LVC, NCI

Objectives:

Eukaryotic cells are capable of controlling the expression of at least a part of their genome by regulation of RNA transcription. Such specialized genes as those coding for ovalbumin, hemoglobin, and immunoglobulins have been shown to be under such control, as have the sequences for endogenous avian retroviruses. The mechanism(s) which account for the switching on and off of genes during development are poorly understood. Since prokaryotic gene regulation involves the efficiency with which RNA polymerase recognizes and interacts with specific DNA signal sequences (promoters), we may speculate that this interaction is also important in the eukaryote. The complexity of the eukaryotic genome has necessitated the development of additional levels of transcriptional modulation involving the methylation state and the local chromatin structure of individual cistrons. The endogenous (CPC-1) virus isolated from Colobus polykomos following BUDR treatment and long-term cocultivation with a human carcinoma cell line, A549, provides a probe to dissect the modulators of eukaryotic gene expression. Enigmatically, CPC-1 exists in colobus monkey tissue as a repressed gene, in spite of the fact that 50 to 70 copies are present. C_rT analysis has shown that none of these genes are expressed as RNA transcripts. By defining the events which allowed the expression, and subsequent replication, of the virus genome we hope to gain a better understanding of those factors which switch genes on and off. Molecularly cloned DNA sequences from CPC-1, 5CH3 (an endogenous chimpanzee retrovirus sequence), M432 (an endogenous murine retrovirus), leuk-2 and Carc 3 (murine retroviruses with oncogenic potential) have been used to (1) evaluate the sequences required for RNA polymerase II-directed transcription, (2) examine the evolutionary fate of retroviral sequences, and (3) develop retrovirus-guided eukaryotic cloning vectors.

Methods Employed:

Functional promoter sequences have been cloned from both the endogenous murine virus, M432, and colobus virus, CPC-1, employing unintegrated DNA intermediates. The long terminal repeats (LTRs) were sequenced (Maxim and Gilbert method) to determine the primary sequence of presumptive promoter regions. Functionality of the CPC-1 promoter was examined in vitro employing the transcription extract described by Manley. Activity in vivo has been evaluated by DNA-mediated transfection of thymidine kinase-negative (tk^-) cells with recombinant DNA constructs, each containing the CPC-1 promoter sequences and the herpes simplex tk gene from which transcription initiation and termination sequences had been removed. Subsequent transformation to the tk^+ phenotype and the more sensitive transient expression assay were used to quantitate promoter activity. To develop eukaryotic cloning and expression vectors, retroviral-containing plasmids have been constructed utilizing oligodeoxyribonucleotide linkers, DNA resections by the enzymes BAL-31 and nuclease S_1, insertions of SV40 augmentation sequences

and thymidine kinase DNA. The efficiency of several constructs was predetermined from Maxim-Gilbert DNA sequencing of the DNAs to be joined.

Major Findings:

1. The DNA sequences responsible for promotion and possible control of RNA polymerase II-directed transcription. The mechanism of transcriptional regulation in eukaryotes involves the molecular architecture of the template DNA, dictated in part by the nucleotide sequence of the transcriptional promoter. The LTR-derived from the replication-competent CPC-1 retrovirus has been partially sequenced to determine the nature of its promoter region. Two overlapping promoter regions were discovered, each consisting of a canonical TATA box preceded by an identical transcriptional modulation sequence (CCAATCATA). Analysis of stable in vivo transcripts revealed that their initiation was directed by the farther downstream promoter. The duplicitous nature of the CPC-1 promoter region suggested that its transcriptional efficiency might be quite high. This was substantiated by C_0t analysis of productively infected A549 cells which revealed the presence of an extraordinarily high number of viral transcripts (5-10,000 copies/cell) in these cells. The activity and specificity of in vivo transcription were demonstrated in vitro employing restriction enzyme-truncated, cloned CPC-1 and a cell-free extract. To ascertain the cellular origin of the CPC-1 viral promoter sequences, two endogenous proviral LTRs were cloned from colobus kidney cells. Nucleotide sequence and in vitro transcription comparisons of the three DNAs revealed: (1) the CPC-1 promoter originated from colobus proviral sequences; (2) of the 160 nucleotides sequenced, the endogenous promoters differed from CPC-1 and each other by four base changes; and (3) although both binding (CCAAT) and initiation (TATA) sites were unaltered in the endogenous promoters, these DNAs were transcriptionally inactive. The in vitro transcription assay employed in this study measures only the presence or absence of the proper initiation sequences. The above results extend the domain of these sequences to include nucleotides -32 to -23 upstream from the cap site (the RNA startpoint) and demonstrate the utility of point mutations to define essential regions. To assess the functioning of the CCAAT region of the endogenous LTRs, and to substantiate the in vitro transcription results, constructs have been made containing the thymidine kinase gene at the cap site. Thus, the activity of the viral promoter is proportional to the expression of the tk gene, and may be measured by transfection of tk cells with the recombinant DNA. The CPC-1 promoter-tk construct is active and is capable of stable biochemical transformation of tk^- cells. The sensitivity of this assay is being increased by assaying for the transient expression, in the transfected cells, of tk enzyme or specific mRNA. One of the endogenous promoters has been subcloned so that it can replace the CPC-1 promoter in this assay. Should this construct prove to be inactive, as expected, point mutations will be introduced into the endogenous promoter and up mutations can be selected by their tk^+ genotype. The alterations which are required for promoter functionality will subsequently be determined at the nucleotide sequence level.

2. Structure and evolution of endogenous retroviruses. The endogenous parent of CPC-1 exists essentially as one of a multigene family of 50-70 members (a similarity shared by most endogenous retroviruses). The origin and evolution of these sequences are in doubt. The difficulty of CPC-1 isolation suggested that

its repression was the result of multiple factors. This was supported by the following observations: (1) both cloned endogenous proviral promoters are transcriptionally inactive; and (2) recent Southern blot analyses of the endogenous proviruses revealed all of them to be hypermethylated (internally, as well as over their promoter regions). These findings are inconsistent with the high proviral copy number being the result of superinfections (an RNA intermediate is required); rather, a model of gene conversion would be favored involving the provirus as a transposable element.

Various regions of the CPC-1 genome have been sequenced to determine the location and reading frames of its genes. The nucleotide sequence of p15E was provided to Dr. Stephen Oroszlan as its amino acid sequence. Comparison of the genes at the extremities of a retrovirus of fowl (REV), and colobus and baboon families of retroviruses show that this avian virus evolved from a primate virus containing a 5' terminus related to the colobus family and a 3' terminus related to the baboon family. It would appear that viable recombination between non-identical retroviruses is possible in nature.

In addition to the sequences of p15E and part of the gp70 derived from CPC-1, the entire sequence of a murine mink cell focus-forming virus (MCF) env gene recombinant has been used to analyze the corresponding domain of an endogenous human retroviral genome (in collaboration with Dr. Maurice Cohen, Litton Bionetics, Inc., Frederick Cancer Research Facility). Preliminary results suggest the human retroviral env gene is translatable into a protein which contains a hydrophobic leader sequence and hydrophobically flanked, post-translational cleavage sites analogous to those of murine and primate origin. The patterns of glycosylation sites are different for the three genes sequenced, indicating that each interacts with separate cellular receptors. Structural requirements dictate conservation of specific glycoprotein domains; thus, broadly reactive antibodies raised in rabbits against synthetic peptides representing these domains may be capable of detecting the expression of this and other human retroviruses.

Recent experiments point to the reproductive defectiveness of the bovine leukemia virus (BLV) as being a consequence of the lack of transcriptional modulation sequences in its LTR (Dr. James Casey, Louisiana State University). Analysis of a productively infected sheep cell line revealed a BLV provirus integrated next to a CCAAT sequence related to that found in the U3 region of the CPC-1 LTR. Several questions need to be answered. Since these U3 sequences are not conserved between known retroviruses, how did CPC-1 obtain these sequences? Are they part of an operon in eukaryotes? If so, are their expressions regulated, or do they simply act as enhancer sequences? A collaboration has been initiated with Dr. Casey to investigate the nature of these sequences.

3. Retrovirus LTRs hold significant promise as useful eukaryotic cloning vectors. To date, the DNA sequence to be expressed has been placed either upstream or downstream from the LTR so as to use its transcription termination or initiation signals, respectively. The CPC-1 promoter has been found to be transcriptionally highly efficient. It also contains a unique SacI restriction site at the transcription cap site. Herpes simplex virus (HSV) tk DNA, lacking its own promoter and terminator sequences, has been ligated, in both orientations, into the SacI site of a subcloned CPC-1 partial LTR, making use of SacI linkers. Only the clone with tk in the correct orientation is capable of

biochemical transformation of tk^- cells. Thus, the viral promoter and termination signals are functional. This vector is presently being modified to exclude the plasmid poison sequences and include the SV40 origin of DNA replication and enhancer sequences. The result should be a vector which will replicate autonomously in both E. coli and an appropriate mammalian cell, while being capable of high expression of the inserted sequence in the latter cells.

According to the promoter insertion model of viral carcinogenesis, an integrated retrovirus may provide an active promoter upstream of a cellular oncogene. The increased expression of this cellular gene results in cell transformation. It is possible that the presence of enhancer sequences in the LTRs of oncogenic retroviruses stimulates downstream expression. The frequency of this event is small, being proportional to the frequency of viral integration. To increase this frequency, a recombinant is being developed which lacks functional gag proteins (so that vRNA is not removed from the cytoplasm by virus particle formation) and the termination sequence between the gag and polymerase genes (the expression of reverse transcriptase should increase significantly). In addition, the SV40 augmentation sequences are being inserted near the right LTR. It is hoped that the increased availability of viral RNA, coupled with substantial intracellular amounts of reverse transcriptase, will result in continuous integration of viral genomes.

Significance to Biomedical Research and the Program of the Institute:

The molecular mechanisms which underlie the regulation of transcription in eukaryotic cells are largely unknown. Understanding these mechanisms will lead to a broader comprehension of the factors which are able to modulate genes during cellular differentiation and neoplastic transformation. The importance of promoter insertion to enhance the expression of a restricted cellular gene has been established for avian leukosis and will undoubtably be extended to other oncogenic events. We are in a position to decipher some of the regulatory factors, while simultaneously applying the knowledge to obtain the expression of medically relevant genes.

Proposed Course:

CPC-1 promoter sequences provide a unique tool to define the mechanisms which regulate gene expression. These sequences will be used to (1) more fully describe functional regions via determining the consequences of point initiations, and (2) to stably introduce new genetic information into eukaryotic cells.

Publications:

Bonner, T. I., Birkenmeier, E. H., Gonda, M. A., Mark, G. E., Searfoss, G. H. and Todaro, G. J.: A family of retroviral sequences found in chimpanzee but not human DNA. J. Virol. 43: 914-924, 1982.

Callahan, R., Hood, M., Birkenmeier, E. and Mark, G.: The molecular cloning of the novel M. cervicolor popaeus endogenous retrovirus M432 and the sequence of its LTR. J. Virol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05223-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of Mitogenesis in Tumor Promotion

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

N. H. Colburn Expert, LVC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.2	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

One hypothesis for tumor promotion proposes that promoters function to stimulate proliferation of initiated cell populations which produces either (1) clonal selection of progressing cells or (2) increased probability of gene rearrangements or fixation of DNA damage. Recent work in this laboratory suggests that the promoting activity of phorbol esters in JB6 mouse epidermal cells is not due to a release from quiescence type of mitogenic stimulation by the tumor promoter. Evidence for this is: (1) some mitogen-resistant (M-) variants show promotable (P+) phenotype; and (2) di-2-ethylhexyl phthalate (DEHP) promotes anchorage-independence but does not function as a mitogen in JB6 cells. These 12-O-tetradecanoylphorbol-13-acetate (TPA) mitogen-resistant variants are currently being used to discover biochemical events which determine the mitogenic response. Two such events which appear to mediate the mitogenic response to TPA are epidermal growth factor (EGF) (or other growth factor) binding to EGF receptors and stimulated hexose transport. Supporting a requirement for EGF receptors in TPA mitogenesis is our recent observation that reconstitution of M- EGF receptorless cells with membranes containing EGF receptors restored not only binding and mitogenic response to EGF, but also mitogenic response to TPA. In support of a role for stimulated hexose transport is our observation that a mitogen-sensitive (M+) revertant of an M- cell line regained the stimulated hexose uptake response to TPA missing in the M- parental line.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Marion Copley Staff Fellow LEP, NCI

Objectives:

To determine whether promoter-dependent mitogenic stimulation is required for promoting activity in JB6 mouse epidermal cells. To determine whether promotion of anchorage-independent growth of JB6 cells can occur under conditions in which promoter-induced mitogenesis does not, and to determine whether cell lines selected from promotable JB6 lines for 12-O-tetradecanoylphorbol-13-acetate (TPA) mitogen resistance are coselected for nonpromotability. To determine biochemical or physiologic events that are required for mitogenic stimulation by phorbol esters.

Methods Employed:

Selection for mitogen resistance using colchicine plus TPA at plateau density. Assay of anchorage-independent growth and mitogenesis response by increased cell number and labeling index at plateau density after mitogen introduction. Determination of ^{125}I-labeled epidermal growth factor (EGF) binding and of ^{3}H-2-deoxyglucose uptake.

Major Findings:

1. Promotion of transformation without promoter-induced mitogenesis. Previous studies showed that JB6 variants selected for resistance to mitogenic stimulation by TPA (M-) were not consistently coselected for resistance to promotion of anchorage-independence. Recently we have found that the mouse liver tumor promoter di-2-ethylhexyl phthalate (DEHP), which promotes anchorage-independence in JB6 cells, does not produce a mitogenic response in JB6 cells. Hence, it appears that mitogenesis is not a requirement for promotion of anchorage-independence in JB6 cells by either TPA or DEHP.

2. Hexose transport may be involved in mediating the mitogenic response to TPA. The TPA mitogenesis-resistant (M-) variants of JB6 show high basal hexose uptake and no stimulation of uptake in response to TPA, whereas their sensitive counterparts (M+) show low basal uptake and stimulation by TPA. The JB6 clonal lines which are resistant to promotion of transformation by TPA and other promoters are not distinguishable from their sensitive counterparts on the basis of hexose transport. Thus, promoter-stimulated hexose transport may be a required event in mitogenesis, but not in promotion by TPA. Recent results have shown that (1) the TPA mitogenic response is limited by glucose concentration, and (2) an M+ revertant from an M- line also acquired the TPA stimulation of hexose uptake.

3. EGF receptors may be required for mitogenic response to TPA. TPA mitogen resistant (M-) JB6 cells obtained by selection consistently showed little or no EGF binding. This suggested the hypothesis that EGF receptors may be required

for TPA mitogenesis. Insertion of membrane fractions containing EGF receptors into TPA mitogen-resistant EGF receptorless cells as described by Bishayee et al., (Proc. Natl. Acad. Sci. USA 79: 1893, 1982) restored EGF binding, mitogenic response to EGF and mitogenic response to TPA, thus confirming the hypothesis. The magnitude of restored response was 25-50% of the parental M+ cell response.

Significance to Biomedical Research and the Program of the Institute:

Whether tumor promoters act primarily or entirely as mitogens to bring about tumor promotion has been one of the major unanswered questions in carcinogenesis for some time. Recent reports (Kennedy, A. and Little, J., Cancer Res. 40: 1915, 1980; Peraino et al., Cancer Res. 40: 3268, 1980) have suggested that promotion can occur without mitogenesis. Now this conclusion has been strengthened considerably by using an independent approach to demonstrate that TPA mitogenresistant clones are promotable. It is now possible to deal separately with the question of which responses to TPA are on the mitogenesis pathway and which are on the promotion pathway.

Proposed Course:

Continued use of the TPA mitogen-resistant variants will be made to elucidate required events in mitogenesis, especially the possible relationship between EGF receptors and hexose uptake response. New mitogen resistant variants will be sought by nonselective cloning from the original JB6 cells so that generalizations can be further assessed. We hope to phase down this project in favor of other projects sometime soon since we have obtained most of the answers sought in this inquiry.

Publications:

Colburn, N. H., Gindhart, T. D., Hegamyer, G. A., Blumberg, P. M., Delclos, B., Magun, B. E. and Lockyer, J.: The role of phorbol diester and EGF receptors in determining sensitivity to TPA. Cancer Res. 42: 3093-3097, 1982.

Copley, M., Gindhart, T. D. and Colburn, N. H.: Hexose uptake as an indicator of JB6 mouse epidermal cell resistance to mitogenic activity of TPA. J. Cell. Physiol. 114: 173-178, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05225-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Membrane Biochemical Determinants of Tumor Promotion

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

L. Srinivas Visiting Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

Developmental and Metabolic Neurology Branch, NINCDS, NIH, Bethesda, MD;
University of Texas System Cancer Center, Smithville, TX

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of cell surface gangliosides in promotion of transformation in "promotable" JB6 mouse epidermal cells is being studied. We have previously reported that there is a substantial decrease in cellular membrane trisialoganglioside (GT) synthesis in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) in the TPA-sensitive, promotable cells, but not in the resistant counterparts. To understand the mechanism of the promotion-relevant decrease in GT synthesis, an oxidative degradation pathway of sialoglycoconjugate was investigated. To study the effect of specific oxidation of cell surface sialic acid, sodium meta periodate ($NaIO_4$) was used. When oxidized by $NaIO_4$, JB6 cells were transformed. This transformation was inhibited by retinoic acid and other sialoglycoconjugates. Since TPA produces elevated reactive oxygen moieties it is hypothesized that cell surface glycolipids may be targets. Another mouse skin promoter, benzoyl peroxide (a generator of reactive oxygen), was entrapped in liposomes and found to induce promotion of transformation in JB6 cells. This promotion of transformation was not inhibited by retinoic acid but was inhibited by the ganglioside GT. Continuing efforts are aimed at isolation of JB6 cells that are resistant to the GT response to TPA. A requirement for the GT response in promotion would suggest that these variants should be promotion-resistant. A new cell line has been developed from parental JB6 cells which is resistant to low glutamine concentrations and is also resistant to the GT response to TPA. In addition, this cell line appears to have been coselected for promotion resistance, thus arguing further for a causal relationship of the GT synthesis response to TPA to promotion of neoplastic transformation. In a collaborative study the synthesis of asialo GM1 in stimulated macrophages was correlated to the order of stimulation and binding of fluoresceinated antibody to asialo GM1.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on the Project:

Nancy H. Colburn	Expert	LVC, NCI
Howard Holden	Microbiologist	BRMP, NCI

Objectives:

To elucidate the involvement of gangliosides in transformation of JB6 mouse epidermal cells and to study specifically the mechanism of reduced trisialoganglioside (GT) synthesis. Specifically to (1) investigate oxidative degradation via reactive oxygen as a possible mechanism of the decrease in G_T synthesis in response to 12-O-tetradecanoylphorbol-13-acetate (TPA); (2) compare the reactive oxygen generator benzoyl peroxide to TPA with respect to promotion of transformation, G_T synthesis response and sensitivity to G_T, and inhibition of promotion by retinoic acid; (3) select a cell line that is resistant to the G_T response to TPA and see if it is coselected for promotion resistance; (4) determine whether decreased G_T synthesis may be required to maintain the tumor cell phenotype; and (5) chemically correlate synthesis of asialo GM_1 in activated macrophages.

Methods Employed:

Sodium meta periodate ($NaIO_4$) at 2 mM was used to oxidize surface sialic acids. Gangliosides were added as liposomes to inhibit the promotion of transformation. Benzoyl peroxide was entrapped in liposomes of cholesterol and phosphatidyl choline and used as a promoter. Low glutamine-resistant cells were selected by a gradual decrease to 1/1000th of normal glutamine content. Phenotype was characterized by resistance to promotion of anchorage-independent growth by TPA and ganglioside synthesis profile. Response to TPA was determined by following terminal four-hour incorporation of ^{14}C-glucosamine into gangliosides. Ganglioside profiles of several transformed cell lines were studied by 2-^{14}C-glucosamine labeling and isolation of the ganglioside by chloroform:methanol, separation by thin layer chromatography (TLC) and quantitation by liquid scintillation counting and scanning.

Major Findings:

1. Oxidation of cell surface sialic acid leads to promotion of transformation. TPA produces diminished superoxide dismutase (SOD) activity in JB6 mouse epidermal cells, an effect that is expected to produce elevation of reactive oxygen. This decrease in SOD is inhibited by the addition of G_T. To specifically study whether cell surface sialic acid-containing molecules were targets for oxidation, sodium meta periodate ($NaIO_4$), a specific oxidant, was used. This oxidation of cell surface sialic acids leds to promotion of transformation in the TPA-promotable (P+) but not in non-promotable (P-) cells. The transformation induced by $NaIO_4$ was inhibited by retinoic acid and sialoglycoconjugates, including G_T, but was not inhibited by preoxidized G_T. In vitro, cell-free oxidation of G_T by $NaIO_4$ yielded further confirmation that G_T was oxidized and now appeared to be

behaving like another ganglioside, G_{D1b}, in its behavior on TLC. It is hypothesized that if G_T was converted by oxidation the cell would read it as though G_T was functionally lost and behave like a transformed cell.

2. Benzoyl peroxide, a reactive oxygen generator, works as a promoter in the JB6 mouse epidermal system. To test the hypothesis that reactive oxygen generation can mediate promotion of transformation in JB6 cells, benzoyl peroxide, an in vivo mouse skin promoter, was chosen. To allow sufficient exposure time and to gain access to the cell membrane the unstable benzoyl peroxide was entrapped in liposomes of cholesterol and phosphatidyl choline and added to the cells two to three times at 2-day intervals. Benzoyl peroxide was active as a promoter in a dose-dependent manner from 10^{-9} to 10^{-3} M. This was not inhibited by retinoic acid (as was TPA promotion) but was inhibited by G_T. This indicated that (1) reactive oxygen probably mediates TPA promotion via a G_T-sensitive step; and (2) the promotion pathways for TPA and benzoyl peroxide differ by at least one step, namely a retinoic acid sensitive step.

3. JB6 cells selected for resistance to the GT response to TPA are also resistant to TPA promotion of transformation. Glutamine is an essential nutrient for cells to maintain oxidative and glycolytic pathways, as well as glycoconjugate synthesis. Normally cells may be sensitive to the oxidative action of TPA, but if deprived of glutamine they may slow down oxidation and, hence, become TPA-resistant. Another hypothesis is that if G_T is essential for normal cells, cells deprived of glutamine may synthesize glycoconjugates at a lowered rate and compensate by lowering G_T catabolic activity, even in the presence of inducers, allowing this sialoglycoconjugate to have an extended half-life. JB6 cells were selected with a stepwise lowering of glutamine to 1/1000 of the normal medium concentration until a cell line showing a constant 1-day doubling time in low glutamine was obtained. These cells, when tested for their capability to respond to promotion of transformation, were resistant to TPA. Furthermore, like other P- cells, they do not undergo lowered G_T synthesis in response to TPA promotion. This resistance was not due to a slower growth rate of selected cells. These cells express the same phenotype even if they are switched to normal glutamine media, thereby showing that they have a stable phenotype. This constitutes independent support for a causal relationship between reduced G_T synthesis and promotion of transformation in JB6 cells.

4. Lowered GT synthesis appears to be necessary for expression of the tumorigenic phenotype. Several mouse epidermal cell lines transformed by TPA were studied. It appeared that all the transformed (tumorigenic, anchorage independent) cell lines showed a lowered G_T synthesis compared to their non-transformed counterparts. The accumulating ganglioside was G_{D1a}. When TPA-resistant (P-) cells were transfected with cloned Moloney mos DNA, they acquired a transformed phenotype (positive in focus assay and soft agar assay). In these transformed cells, G_T synthesis was lowered compared to the non-transformed controls. The accumulating ganglioside was GM_1. This shows that both chemical transformation and viral DNA transformation lead to low G_T synthesis, possibly by different pathways because the accumulating gangliosides are different. Decreased G_T synthesis was also observed when other tumor promoters, such as epidermal growth factor (EGF) and mezerein, were used as promoters in JB6 cells. The accumulating ganglioside was G_{D1a}, just as for TPA.

5. Expression of asialo GM1 is chemically correlated to the anti-asialo GM1 fluorescent antibody binding to stimulated macrophages. When stimulated by peptone and other agents, macrophages express graded, elevated asialo GM_1, thus demonstrating that to be a marker for activated macrophages. Dr. Howard Holden demonstrated enhanced asialo GM_1 antibody binding to stimulated cells as compared to non-stimulated cells. The asialo GM_1 from stimulated cells was isolated and separated after 24 hrs. of 1-^{14}C-galactose labeling and was found to be correlated to the level of antibody binding. A chemical study confirms that there is elevated synthesis of asialo GM_1 in stimulated macrophages.

Significance to Biomedical Research and the Program of the Institute:

The ganglioside G_T appears to inhibit promotion of transformation. If it prevented tumor promotion in experiments in vivo it could be used effectively in cancer prophylaxis. The possibility arises that the P+ gene product could be a TPA-inducible neuraminidase with specificity for G_T.

Proposed Course:

(1) Attempts will be made to isolate and characterize a membrane-bound neuraminidase which is responsible for catalyzing degradation of G_T and to determine its inducibility or activation by tumor promoters. (2) The low glutamine-resistant LJ cells will be further investigated to understand their basis for resistance to TPA-induced promotion of transformation. (3) G_T will be tested in vivo for an ability to inhibit tumor promotion. In collaboration with Dr. T. J. Slaga and Dr. S. Fischer, skin painting experiments with TPA as promotor and G_T as a possible inhibitor have been undertaken. (4) The consequences of reduced G_T synthesis leading to neoplastic transformation will be investigated.

Publications:

Colburn, N. H., Wendel, E., Hegamyer, G. A., Talmadge, C. B., Dion, L. D., Srinivas, L. and Gindhart, T. D.: The use of promoter-resistant variants to elucidate the role of specific membrane and gene-level changes in process of tumor promotion. In Borzsonyi, M. and Yamasaki, H. (Eds.): The Role of Cocarcinogens and Promoters in Human and Experimental Carcinogenesis. Lyon, France, IARC Scientific Publications (In Press)

Srinivas, L. and Colburn, N. H.: Membrane glycolipid changes associated with premalignant progression, malignancy and metastasis. In Kaiser, H. E. (Ed): Progressive Stages of Malignant Neoplastic Growth. England, Pergamon Press (In Press)

Srinivas, L., Gindhart, T. D. and Colburn, N. H.: Tumor promotor resistant cells lack trisialoganglioside response. Proc. Natl. Acad. Sci. USA 79: 4988-4991, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05226-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Surface Receptors and Signal Transduction in Tumor Promotion

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

N. H. Colburn Expert, LVC, NCI

COOPERATING UNITS *(if any)*

Division of Radiation Oncology, University of Arizona, Tucson, AZ; Atomic Energy of Canada, Ltd., Chalk River, Ontario, Canada

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.4	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Phorbol diester binding sites appear stable biologically, show no defect in number, affinity for H-3-labeled phorbol-12,13-dibutyrate (PDBu) or down modulation in preneoplastic cells selected for resistance to 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mitogenesis or promotion of transformation. Nor does malignant transformation detectably perturb these binding sites in mouse or human cells. Loss of epidermal growth factor (EGF) receptors correlates with loss of mitogenic response to TPA in monolayer culture, but has no effect on H-3-PDBu binding or TPA promotion of transformation. The roles of reactive oxygen production and divalent cation transport are being probed as possible mediators of signal transduction following receptor binding. Benzoyl peroxide, when applied to cells in liposomes during a period of 2 to 4 days, induces anchorage-independent transformation. This is inhibited by trisialoganglioside (GT), but not by retinoic acid, thus suggesting at least one difference between the promotion pathways for TPA and benzoyl peroxide. DNA strand breaks may be required for promotion of transformation of JB6 cells by benzoyl peroxide but are not required for promotion by TPA.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Thomas Gindhart	Expert	LEP, NCI
Leela Srinivas	Visiting Fellow	LVC, NCI

Objectives:

To determine the role of phorbol diester receptors in promotion of transformation of JB6 mouse epidermal cell lines by 12-O-tetradecanoylphorbol-13-acetate (TPA). A major question to be addressed is: What is the physiologic system most immediately perturbed by phorbol diesters? The strategy of this research is to characterize the biochemical effector mechanism of the phorbol diester binding site. Identification of the events which occur most immediately following occupancy of the binding site is the initial objective, and clear separation of promotion-relevant distal events from those unrelated to tumor promotion is the final objective. Specific studies are aimed at determining sensitivity of phorbol diester-induced promotion to inhibitors of (1) reactive oxygen (H_2O_2 [hydrogen peroxide] and benzoyl peroxide have been shown to be promoters in JB6 cells in our laboratory) and (2) calcium metabolism.

Methods Employed:

Assay of anchorage-independent growth in response to various inducers and inhibitors that produce or modulate events postulated to be mediators of signal transduction. Assays of superoxide and superoxide dismutase, intracellular calcium concentrations by atomic absorption, and calcium flux.

Major Findings:

1. Phorbol diester binding sites are biologically stable. Selection for TPA-resistant variants has not, in any instance, coselected for a receptorless state for phorbol esters; nor is malignant transformation associated with any alteration in these receptors.

2. EGF receptors are not required for promotion of transformation by TPA but may be required for mediating mitogenic response to TPA. See Project Number Z01CP05223-03 LVC.

3. Benzoyl peroxide, a promoter of mouse skin carcinogenesis, also promotes JB6 cell transformation. Benzoyl peroxide at concentrations of 10^{-9} to 10^{-5} M, when applied in liposomes 2 to 3 times during a period of 2 to 4 days, is active in promoting anchorage-independence. This is inhibited by trisialoganglioside (G_T), but not by retinoic acid, thus suggesting at least one difference between the promotion pathways for TPA and benzoyl peroxide. DNA strand breaks are produced in JB6 cells by benzoyl peroxide, but not by TPA (Bowden et al., in press), suggesting that DNA strand breaks are not required for promotion of transformation by TPA but could be required for promotion by benzoyl peroxide.

4. Superoxide, but not hydrogen peroxide, may be involved in mediating promotion of transformation by TPA. Highly purified catalase showed no activity for inhibition of promotion of transformation by TPA, thus indicating that H_2O_2 is not a mediator of promotion by TPA in JB6 cells. TPA lowers superoxide dismutase activity in JB6 cells as it does in mouse skin, suggesting the possibility that elevation of superoxide could be involved in promotion by TPA. Mild oxidation with $NaIO_4$ also had promotion activity (See Project Number Z01CP05141-04 LVC). We conclude that reactive oxygen and oxidation of certain cellular targets may be involved in mediating promotion, but the reactive oxygen species must be other than H_2O_2.

Significance to Biomedical Research and the Program of the Institute:

The stability of the phorbol ester receptor suggests that it is part of a basic physiologic mechanism whose functioning is essential for cell survival. Attempts to control preneoplastic progression or tumor growth through pharmacologic blockade of this binding site would be expected to encounter severe, undesirable side effects since every mammalian cell type tested to date, except erythrocytes, possesses these binding sites and responds strongly to phorbol diesters at low concentrations. Reactive oxygen generation and calcium influxes may be involved in tumor promotion. Manipulation of these systems appears to offer more promising new approaches to cancer control than blockade of the phorbol diester binding site. Blockade of EGF binding sites may modulate early stages of preneoplastic progression evidenced by mitogenic responses to phorbol esters. Various EGF receptor blocking agents are currently under consideration for relevance as determined in this system.

Proposed Course:

Continuation of these studies towards identification of promotion-relevant signaling events following phorbol diester binding in order to pinpoint the factor(s) which allows a cell to respond to TPA with anchorage-independent growth to include the following: (1) Identification of the target molecules attacked by reactive oxygen. Membrane lipids such as gangliosides may be important targets. (2) Determination of whether certain antioxidants act as antipromoters. (3) Determination of whether selection of JB6 cells for resistance to TPA-induced elevation of oxygen radicals coselects for promotion resistance. (4) Determination of whether promotion of transformation is dependent on extracellular and intracellular calcium metabolism. (5) Determination of whether calcium resistance is one of a set of necessary components for determining sensitivity to promotion of anchorage-independence. (6) Determination of whether there is differential regulation of calcium-dependent protein kinase in promotion-sensitive and -resistant cells.

Publications:

Colburn, N., Gindhart, T., Dalal, B. and Hegamyer, G.: The role of phorbol ester binding and down modulation in responses to promoters by mouse and human cells. In Rice, J., Langenbach, R. and Nesnow, S. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Press, 1982, pp. 189-200.

Colburn, N. H., Gindhart, T. D., Hegamyer, G. A., Blumberg, P. M., Delclos, B., Magun, B. E. and Lockyer, J.: The role of phorbol diester and EGF receptors in determining sensitivity to TPA. Cancer Res. 42: 3093-3097, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05228-03 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genes for Promotability

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

N. H. Colburn Expert, LVC, NCI

COOPERATING UNITS *(if any)*

Laboratories of Molecular Biology and Biological Carcinogenesis, Litton Bionetics, Inc. Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.6	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This research aims to identify the changes in the genetic material or its regulation which must occur for mammalian cells to undergo neoplastic progression in response to tumor promoters. Results suggest that promotability behaves as a dominant trait. Transfection of whole cell DNA from promotion-sensitive (P+) cells into promotion-resistant (P-) cells results in transfer of promotion sensitivity. Transfection of DNA from nonpromotable cells yields a response to TPA that is no higher than the untransfected background. Transfected promotability is stable. The restriction enzyme sensitivity (resistance to BglII) and size class (around 10 Kb) of the P+ gene has been characterized. Epidermal tumor cell DNA can be expressed in P+ but not in P- cells suggesting a permissive genotype for the former. Current efforts are directed to cloning the P+ gene, with an ultimate objective of isolating and elucidating the function of the P+ gene product. The basis for resistance of P- cells will be investigated as will the relationship of P+ genes to transforming genes. It is expected that P+ genes specify an event involved in induction of malignancy, which may not be involved in maintenance of the malignant phenotype.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Michael Lerman	Visiting Scientist	LEP, NCI
Thomas Gindhart	Expert	LEP, NCI

Objectives:

To determine the number and nature of genetically distinct steps required for tumor-promoting phorbol diesters and other promoters to induce neoplastic transformation of JB6 mouse epidermal cell lines. To characterize the genetic loci in terms of their structure, regulation and function(s). The ultimate objective is to identify regulatory mechanisms at the DNA level through cloning and sequencing of the relevant genes.

Methods Employed:

DNA transfection using calcium phosphate DNA precipitates to transfer DNA from promotable cells into nonpromotable cells. Soft agar assay with or without the promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) for determination of biological activity. Restriction enzyme analysis to characterize DNA sequences showing activity for promotion sensitivity. Fractionation of DNA fragments to determine gene size. Ligation to appropriate vectors for cloning in E. coli.

Major Findings:

1. Promotability appears to be a dominant trait. A JB6 promotion-insensitive (P-) clonal line was fused with a JB6 promotion-sensitive clonal line after first allowing the two cell lines to phagocytose differentially labeled fluorescent beads. The products were sorted on the FACS IV cell sorter and the P+/- fusion product saved and assayed for promotion of anchorage-independence. Of three sorted P+/- fusion products all three were promoted to anchorage-independence in response to TPA at levels similar to the P+ parental line, thus indicating apparent dominance of the P+ trait.

2. Promotability can be transferred by transfection of genomic DNA from P+ into P- cells. When DNA isolated from one of two P+ lines, JB6 Cl 41 or JB6 Cl 22, was precipitated with calcium phosphate and used to treat either of the P- cell lines JB6 Cl 30 or JB6 Cl 25, the transfected cells yielded 200-400 agar colonies per 10^6 cells in response to TPA. Control P- Cl 30 cells transfected with Cl 30 DNA yielded only 40 colonies per 10^6 cells, a value indistinguishable from that obtained without DNA transfection. Several other unrelated DNAs, when transfected, showed little or no activity for transferring promotability. NIH/3T3 cells do not show promotability after transfection of P+ DNAs, indicating that (1) promotability is determined by genetic components present in both P+ donor and P- recipient cells; or (2) that promotability is determined by genetic components in the P+ DNA, but NIH/3T3 cells synthesize a suppressor of expression of the P+ donor sequences.

3. Transfected promotability is stable. In order to obtain enrichment of cells that received DNA without promoting transformation of the cells, co-transfection with the dominant E. coli marker Eco gpt followed by growth in selective medium was carried out. P+ lines established from Eco gpt-positive colonies were stable for at least 8 passages. The P- lines obtained after co-transfection with a P- DNA were also stable. Secondary transfection of P- Cl 30 cells with DNA from P+ cells obtained by primary transfection yielded P+ cells at a frequency similar to the frequency obtained for primary transfection.

4. The restriction enzyme sensitivity of the P+ gene(s) has been determined. P+ DNA appears to be sensitive to EcoRI, BamHI, HindIII, XhoI, SalI and PVUII, and resistant to BglI and BglII digestion when assayed by transfection and TPA induction of anchorage-independence. Secondary transfectants show the same restriction enzyme sensitivity.

5. Tumor cell DNA is expressed in P+ but not in P- cell recipients. DNAs from several chemically transformed (tumorigenic, anchorage-independent) mouse epidermal cell lines showed no transforming activity on transfection into NIH/3T3 cells or JB6 P- cells. One line did, however, produce anchorage-independent transformation on transfection into either of two P+ cell lines. This suggests that P+ cells contribute a gene product (or lack of it) needed to provide a permissive state for expression of tumor cell phenotype.

6. Tumor promoters switch off collagen synthesis by regulation of available collagen mRNA. We have found that exposure of JB6 cells to TPA produces nearly complete absence of translatable and hybridizable mRNA for type I collagen. This is the first evidence that tumor promoters regulate transcription of specific genes in a system in which promotion of transformation is occurring.

Significance to Biomedical Research and the Program of the Institute:

Identification of genetic information required for inducibility of preneoplastic progression should lead to improved mechanisms of tumor prevention and control.

Proposed Course:

(1) Clone the P+ DNA sequences. (2) Determine whether transforming sequences and promotability sequences are the same or different on the basis of restriction enzyme sensitivity then by hybridization with cloned probes. (3) Determine whether P+ cells obtained from P- cells by transfection of P+ DNA acquire the biochemical responses to tumor promoters which have been suggested by other experiments as being required events in the promotion process. (4) Determine whether P- cells owe their promotion resistance to (a) lack of P+ genes, (b) rearrangement of P+ genes, or (c) presence of P+ genes that are not transcribed. (5) Isolate the P+ gene product(s) and determine its function. (6) Determine the relationship of P+ genes to the various oncogenes recently described.

Publications:

Colburn, N. H., Talmadge, C. B. and Gindhart, T. D.: Transfer of phorbol ester promotability by transfection of DNA from promotable into nonpromotable cells. In Cohn, W. E. (Ed.): Progress in Nucleic Acid Research and Molecular Biology. New York, Academic Press, 1983, Vol. 29, pp. 107-110.

Colburn, N. H., Talmadge, C. B. and Gindhart, T. D.: Transfer of sensitivity to tumor promoters by transfection of DNA from sensitive into insensitive mouse JB6 epidermal cells. Mol. Cell. Biol. (In Press)

Dion, L. D., Bear, J., Bateman, J., De Luca, L. and Colburn, N. H.: Inhibition by tumor promoting phorbol esters of procollagen synthesis in promotable JB6 mouse epidermal cells. J. Natl. Cancer Inst. 69: 1147-1154, 1982.

Sobel, M. E., Dion, L. D., Vuust, J. and Colburn, N. H.: Tumor promoting phorbol esters inhibit procollagen synthesis at a pretranslational level in JB6 mouse epidermal cells. Mol. Cell. Biol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05246-02 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation and Characterization of Growth Regulatory Factor from Human Tumor Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

K. K. Iwata Staff Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

University of Rochester, Rochester, NY; Electron Microscopy, Program Resources, Inc., Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	1.1	2.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Serum-free conditioned media from several human tumor cell lines (e.g., epidermoid carcinomas, melanomas, bronchogenic carcinomas, and rhabdomyosarcomas) and from NIH/3T3 cells transfected with DNA from a human lung carcinoma were observed to produce a class of factors which inhibits growth of human melanoma and carcinoma cells in soft agar and in monolayer cultures. These inhibitors of tumor cell growth have been designated tumor inhibiting factors (TIFs). Normal human fibroblasts and epithelial cells, however, are stimulated to proliferate by these same factors. This project is directed towards the purification and characterization of this new class of factors that inhibits tumor cell growth. We have determined some of the biochemical and biophysical properties of TIF. We have also identified a rich source of TIF in certain normal human tissues. This should facilitate its purification and characterization. This source should yield sufficient TIF for sequencing, antibody production, and in vivo experiments.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

George J. Todaro Medical Officer LVC, NCI

Objectives:

To purify and characterize a new class of growth regulatory factors which inhibits tumor cell growth but is not cytotoxic to normal human cells. Sources of tumor inhibiting factors (TIFs) will be sought which will yield sufficient quantities of TIFs for sequencing and antibody production. To study the molecular mechanism(s) of TIF action which determines its inhibitory activity on tumor cells, but not normal cells. To conduct in vivo experiments to develop conditions for future potential clinical applications of TIF in cancer therapy.

Methods Employed:

Biochemical and biological techniques previously developed in this laboratory were used to obtain factors from sera, tissue extracts and serum-free supernatants from cultured cells. New techniques were developed to assay tumor cell growth inhibitors. TIF was purified using gel permeation chromatography followed by several chromatographic steps using reverse phase, high performance liquid chromatography (HPLC) and several different gradient solvent systems.

Major Findings:

1. Tumor cell inhibiting factors in conditioned medium. Supernatant fluids from human tumor cell lines (e.g., rhabdomyosarcomas, melanomas, and carcinomas) were observed to contain factors which, when purified, inhibit the growth of tumor cells in soft agar and monolayer cultures. These TIFs, however, stimulated the growth of normal human fibroblasts and epithelial cells. These factors from human tumor cells inhibited the growth of normal mink lung epithelial cells and slightly stimulated rat and mouse cells. The response of different species to these factors appears to vary.

Using a rapid and sensitive assay, which was developed in the course of this project, the major tumor inhibiting activities were found to fractionate by gel permeation chromatography into three molecular weight classes (5,000, 10,000, and 20,000). Reverse phase HPLC and an organic solvent gradient further resolved the 10,000 molecular weight TIF into three distinct activities which eluted at different solvent concentrations. It has not been resolved whether any of the three 10,000 molecular weight TIFs are related to each other or to the 5,000 or 20,000 molecular weight TIFs.

The 10,000 and 20,000 molecular weight forms of TIF have been the most characterized. Both are trypsin-sensitive and heat-stable at 56°C for 30 minutes. The 10,000 molecular weight TIF is heat-stable at 100°C, but the 20,000 molecular weight form is not. Another distinguishing feature between the 10,000 and the

20,000 molecular weight TIFs is the ability to inhibit the growth of mink epithelial cells. Normal mink epithelial cells are very sensitive to inhibition by the 10,000, but not by the 20,000, molecular weight TIF. A549 human lung carcinoma cells are inhibited by both the 10,000 and the 20,000 molecular weight TIFs. We observed at least two different forms of TIFs with different biological and biophysical properties.

In addition to developing a purification scheme for the TIFs using gel permeation chromatography and reverse phase HPLC, we have developed procedures for stabilizing the biological activity of the TIFs. The TIF activities become very labile at the end of several purification steps.

2. Tumor inhibiting factors affect gross cell surface and internal cytoskeletal changes in tumor cells. Scanning electron microscopy of human lung carcinomas, melanomas, and normal human fibroblasts treated with TIF shows considerable cell surface changes only in the transformed cells. Lung carcinoma cells show dramatic changes in cell-cell contact and in the distribution of microvilli. Normal human fibroblasts treated with TIF were indistinguishable from the untreated control.

Fluorescent microscopic studies using antisera against actin show lung carcinoma cells treated with TIF to have microfilaments which are much thicker and directionally oriented than the untreated control. Normal human fibroblasts treated with TIF were indistinguishable from the untreated control.

3. Tumor cell inhibitory factors in tissues. Recently, we have found that extracts from certain normal human tissues provide a rich source of TIF. The yield of TIF from several grams of tissue is greater than that obtained from hundreds of liters of conditioned media. The use of tissue extracts to obtain TIF has saved a considerable amount of time and effort previously devoted to tissue culture, production, harvesting, and processing conditioned media, in addition to the cost. All of the biological and physical properties of the tissue-extracted, 10,000 molecular weight TIF appear to be identical to those of the TIF derived from the conditioned media. This would suggest that the tissue-extracted TIF is related or identical to TIF released into conditioned media. The potential quantities of TIF which can be processed from tissue extracts makes feasible the sequencing of TIF, the production of antibody against TIF, and in vivo experiments. The information yielded from the thorough characterization of the tissue-extracted TIF through the use of sequence information and antibody against TIF would allow further characterization of TIFs from conditioned media.

4. Antagonistic relationship between tumor growth factor (TGF) and tumor inhibiting factor (TIF). TGF and TIF can be isolated from media conditioned by a rhabdomyosarcoma cell line (A673). Soft agar growth of lung carcinoma cells is enhanced by this TGF and inhibited by the TIF. Soft agar growth of lung carcinoma cells can be modulated by the ratio of TGF/TIF so that there is an apparently antagonistic relationship between the TGF and the TIF isolated from the same source. This would lead one to speculate on the mechanism of growth regulation by the rhabdomyosarcoma which produced both of these factors. Tumors may produce both growth stimulatory and inhibitory factors to regulate their rate of proliferation.

b. Immune suppressor activity. Crude extracts of certain human tissues contain a factor which suppresses phytohemagglutinin (PHA) stimulation of human lymphocytes. Whether or not this activity is related to the immune suppressor activity reported in the literature has yet to be determined.

Significance to Biomedical Research and the Program of the Institute:

Factors which inhibit tumor cell growth but not that of normal cells provide new tools to study the control of tumor cell proliferation. The characterization of these factors and their biological activities would provide a further understanding into the mechanisms which control tumor cell proliferation. Antisera against TIF could be used diagnostically to identify or isolate TIFs characteristic for a given tumor. In vivo studies using TIF could lead to clinical applications in conjunction with other cancer therapies.

Proposed Course:

The various classes of TIFs will be further purified and characterized. Sufficient quantities will be purified for sequence analysis, the production of monoclonal antibodies, and further studies into the mode(s) of action of these factors on tumor cells. Eventually, recombinant DNA technology will be used to produce quantities of TIFs for in vivo and in vitro studies. Successful in vivo experiments could lead to eventual clinical applications.

Publications:

Massague, J., Czech, M. P., Iwata, K., De Larco, J. E. and Todaro, G. J.: Affinity labeling of a transforming growth factor receptor that does not interact with epidermal growth factor. Proc. Natl. Acad. Sci. USA 79: 6822-6826, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05248-02 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Possible Role of Transforming Growth Factors During Early Mammalian Development

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

A. Rizzino Expert, LVC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Transforming growth factors are recognized as an important class of factors because they confer a malignant phenotype on non-neoplastic cells. At present the functions of these factors are poorly understood. This project examines the roles of transforming growth factors during early mammalian development. Using early mouse embryos and embryonal carcinoma cells, it is shown that early embryonic cells release factors with transforming growth factor activity. However, studies with embryonal carcinoma cells demonstrate that these cells release an entirely different category of transforming growth factors. Unlike the transforming growth factors purified and characterized previously, the factors released by embryonal carcinoma cells do not bind to receptors for epidermal growth factor nor do they act synergistically with epidermal growth factor. Attempts thus far to identify the factors released by embryonal carcinoma cells suggest that several different factors are released, including one that exhibits the properties of platelet-derived growth factor. It is not yet known whether this particular factor is responsible for any of the transforming growth factor activity released by embryonal carcinoma cells, but highly purified platelet-derived growth factor itself does stimulate the growth of endoderm derived from embryonal carcinoma cells and early mouse embryos.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

None

Objectives:

During the past five years, a wide variety of cells have been found to release a new class of factors. Currently, these factors are referred to as transforming growth factors (TGFs) because of their ability to induce anchorage-independent growth of non-transformed cells. One of the major questions concerning TGFs relates to their function(s). In the case of malignant cells, it has been proposed that the ectopic production of TGFs contributes to the malignant phenotype that they express. However, TGFs may also have normal physiological functions and it has been suggested that they play an important role during embryogenesis. In light of the importance of TGFs, this project examines the possible role of TGFs during the early stages of mammalian development. In particular, three points are addressed: (1) Do the TGFs described thus far influence the growth and/or differentiation of early embryonic cells? (2) Do early embryonic cells produce TGFs? (3) If early embryonic cells produce factors with TGF-like activity, what is the nature of these factors and are they similar to the TGFs described in other systems?

Methods Employed:

Two model systems were selected for these studies: mouse embryonal carcinoma (EC) cell lines and cultured mouse embryos. EC cells are particularly well-suited to this project because (1) they are biochemically and morphologically very similar to the cells of the inner cell mass (the cells of the inner cell mass are totipotent and give rise to the embryo proper); and (2) the differentiation of EC cells in vitro can be directed to specific cell types, including those that form during early development. Equally important is the fact that EC cells, in contrast to early embryos, can be easily grown in large quantities, which is necessary in order to isolate and characterize any TGF-like factors produced by the cells. Therefore, EC cells have been employed in most experiments and early mouse embryos have been used primarily to verify that the conclusions reached with EC cells are relevant to early mammalian development. In order to examine the influence of the TGFs isolated and characterized thus far (alpha TGF and beta TGF), EC cells and their differentiated cells have been cultured in the defined medium developed for EC cells (Rizzino, A. and Crowley, C., Proc. Natl. Acad. Sci. USA. 77: 457-461, 1980). This is necessary since fetal calf serum, which is used to culture EC cells, is known to contain molecules with properties similar to alpha TGF and beta TGF. Although the concentration of these factors in serum is likely to be low, it is equally likely that there is sufficient activity in serum to at least partially mask the response to added TGFs. For the same reason, attempts to isolate and purify TGF-like factors from media conditioned by EC cells must be undertaken in defined media. For these studies, two EC cell lines, F9 and PC-13, were specifically selected. F9 cells were selected because they can be induced by retinoic acid (RA) to

differentiate into parietal and visceral extraembryonic endoderm. (In vivo, parietal and visceral endoderm begin to form at the end of the 4th day of gestation and, thus, are two of the first cell types to appear during early mammalian development.) PC-13 cells were selected because their differentiated cells, which also form after RA treatment, exhibit a large increase in the ability to bind epidermal growth factor (EGF) and to respond to EGF as a mitogen. Thus, it seemed likely that the behavior of these differentiated cells could be influenced by TGFs, in particular by those that bind to EGF receptors.

Another important reason for selecting F9 and PC-13 EC cells is that both cell lines condition their culture media, and this raised the possibility that EC cells produce factors with TGF activity. This possibility was initially examined by determining whether media conditioned by EC cells could promote the growth of non-transformed cells (normal rat kidney cells - NRK) in soft agar. Subsequent studies tested for the presence of alpha TGF and beta TGF. A radioreceptor assay that utilizes the competition between alpha TGF and EGF for binding to membrane receptors was employed to test for alpha TGF. For beta TGF, a bioassay was employed. This assay takes advantage of the fact that EGF dramatically potentiates the ability of beta TGF to promote the growth of NRK cells in soft agar.

Major Findings:

1. Response of embryonal carcinoma cells and their differentiated cells to TGFs. The first question addressed was: Do alpha TGF and beta TGF interfere with the ability of RA to induce differentiation? This was examined by treating F9 and PC-13 EC cells with RA in the presence and absence of alpha TGF and/or beta TGF. No effect on the differentiation of either cell line was observed. Next, the question of whether these TGFs affect the growth of the EC cells and/or their differentiated cells was examined. Neither TGF influenced the growth of F9 or PC-13 EC cells, whereas the growth of the differentiated cells derived from both EC cell lines was stimulated by alpha TGF. In contrast to alpha TGF, beta TGF does not stimulate the growth of the differentiated cells nor does it appear to affect the cells in any other fashion. The finding that the differentiated cells respond to alpha TGF was examined further. As expected, the growth of the differentiated cells was stimulated by EGF, which in turn suggested that differentiation is accompanied by a significant increase in the ability to bind EGF. This was found to be the case: The differentiation of PC-13 and F9 EC cells resulted in increases in EGF binding of 24.5- and 3.8-fold, respectively, relative to the undifferentiated parental EC cells.

2. Production of TGF-like factors by embryonal carcinoma cells. The failure of F9 and PC-13 EC cells to respond to TGFs, plus their ability to condition their culture media, raised the possibility that EC cells produce molecules with TGF-like activity. This possibility was examined by preparing conditioned media from F9 and PC-13 EC cells and testing the ability of the conditioned media to induce NRK cells to grow in soft agar. Both EC cell lines were found to release factors that promote the growth of NRK cells in soft agar. This suggested that EC cells do indeed release TGFs into their culture media. However, upon further examination, the factors released by EC cells have been found to differ from the TGFs that have been characterized thus far--alpha TGF and beta TGF. No alpha TGF or

EGF could be detected in the EC cell-conditioned media by radioreceptor assay. Given this finding, it was possible to directly assay for beta TGF in the conditioned media. No beta TGF was detected by the bioassay employed. This finding has been verified by preparing extracts of EC cells according to the method used to detect beta TGF in other cells. This is an important finding since all cells tested until now have been found to produce beta TGF (Roberts et al., Proc. Natl. Acad. Sci. USA. 78: 5339-5343, 1981). In light of these results, it is clear that the factors released by EC cells represent an entirely different class of factors, which merits further investigation. For this purpose, a simpler assay will be needed in order to isolate and characterize these factors. One alternative would be to design an assay in which these factors are examined for activity in the absence of serum. Toward this end, a defined medium has been developed for the growth of NRK cells in monolayer. Studies are now in progress to develop conditions in which NRK cells can be grown in soft agar in the absence of serum.

3. Embryonal carcinoma cells produce platelet-derived growth factor (PDGF) or a related factor and their differentiated cells respond to it. In order to characterize the factors released by EC cells, media conditioned by these cells were tested for the presence of known factors. Using a radioreceptor assay for PDGF, EC cells have been found to release molecules that compete with the binding of PDGF to membrane receptors on 3T3 cells. In light of these results, EC cells and their differentiated cells were tested for a response to PDGF itself. No response to PDGF was observed with EC cells, but PDGF was found to stimulate the growth of the differentiated cells. The parental EC cells were also found to bind very little PDGF, whereas their differentiated cells bound significant levels. As a consequence of differentiation, the binding of PDGF increases over 50-fold and the average differentiated cell expresses approximately 10,000 receptors for PDGF. As expected from these results, the differentiated cells were found to respond by increased growth in media conditioned by EC cells. At present, it is uncertain whether the TGF-like factors released by EC cells could be due, at least in part, to the PDGF activity released by the cells. Further investigation is required to resolve this point since it appears that EC cells release a number of different factors.

4. Early mouse embryos release factors with TGF-like activity. In order to determine whether the production of TGF-like factors by EC cells is a reflection of their embryonic characteristics or of their malignant properties, early mouse embryos were examined for the release of TGF-like factors. Since EC cells are most closely related to the cells of the inner cell mass at the 4th day of gestation, mouse blastocysts were selected for this study. However, at this stage of development each embryo is composed of only 64 cells (12-14 inner cell mass cells and approximately 50 trophoblast cells). Therefore, an assay suitable for detecting the release of TGF-like factors from a small number of cells had to be developed. Rather than prepare conditioned media from the blastocysts, which is impractical, a different approach was taken. Mouse blastocysts were cultured in tissue culture dishes long enough for them to attach to the surface of the culture dish. They were then overlaid with two layers of agar and a final layer of agar containing NRK cells. In this way, the embryos and the NRK cells are physically separated from one another and easily distinguished from one another. Under these conditions, any TGF-like factors released by the embryos diffuse through the agar and promote the growth of NRK cells in soft agar. Using this

assay, mouse embryos cultured to approximately the 5th day of gestation were found to release molecules that promote the growth of non-transformed cells in soft agar. Moreover, both trophoblasts (obtained by treating mouse embryos with BUDR) and inner cell masses (isolated by immunosurgery) were found to release factors with TGF-like activity. In light of these findings, the effect of PDGF was examined. As in the case of the differentiated cells derived from EC cells, the growth of extraembryonic endoderm derived from mouse blastocysts was stimulated by highly purified PDGF. The production of TGF-like factors in both the EC cell system and the embryo model system strongly suggests that these factors reflect embryonic characteristics rather than malignant properties. This finding, and the effect of PDGF in both systems, confirms the expectation that EC cells represent an appropriate model system for examining the role of TGF-like factors during the early stages of mammalian development.

Significance to Biomedical Research and the Program of the Institute:

Transforming growth factors are recognized as an important class of new factors. Although they were initially discovered in malignant cells, it has recently become clear that normal cells contain similar molecules. This project provides strong evidence that early embryonic cells produce and release factors that exhibit the characteristic property of a TGF--the ability to promote anchorage-independent growth of non-transformed cells. More importantly, this project argues that early embryonic cells do not produce either of the TGFs isolated and purified from other systems, but produce an entirely different category of TGFs. It is evident from this finding that TGFs represent a complex class of factors and it is expected that they will be found to influence the behavior of cells in many different ways. As predicted, this project also demonstrates that EC cells provide an appropriate model system for examining the influence of TGFs during early embryonic development. In this regard, the discovery that early mouse embryos release factors with TGF-like activity was a direct result of the findings with EC cells. This takes on special significance, since it would be most impractical to isolate and purify TGFs from very early embryos. Clearly, work on the embryonic forms of TGFs must rely, at least for the time being, on using the EC cell model system. This work will not only examine a new category of TGF-like factors but also determine why embryonic cells require the presence of factors that confer a malignant phenotype on non-neoplastic cells. Ultimately, this will improve our understanding of the relationship between embryonic and malignant cells.

Proposed Course:

In order to better understand the role of TGFs during early mammalian development, the following studies are proposed: (1) Purify and further characterize the TGF-like factors produced by EC cells. (2) Determine if the PDGF-like factor produced by EC cells is responsible for the TGF-like activity of these cells. In this regard, it will be determined whether the production of the PDGF-like factor decreases after the differentiation of EC cells. (My most recent studies indicate that differentiation results in reduced production of TGF-like activity). (3) Determine whether multipotent and restricted EC cells release the same endogenous growth factors. (4) Determine whether the factors derived from EC cells influence their own differentiation. (It is known that chondrocytes and

myoblasts produce factors that promote chondrogenesis and myogenesis, respectively). (5) Determine if the factors released by EC cells influence the differentiation of early mouse embryos in vitro.

Publications

Rizzino, A.: The growth and differentiation of embryonal carcinoma cells in defined media. In Sirbasku, D., Sato, G. and Pardee, A. (Eds.): Growth of Cells in Hormonally Defined Media. New York, Cold Spring Harbor Press, 1982, pp. 209-218.

Rizzino, A.: Model systems for studying the differentiation of embryonal carcinoma cells. Cell Biol. Int. Rep. (In Press)

Rizzino, A., Orme, L. S. and De Larco, J. E.: Embryonal carcinoma cell growth and differentiation: Production of and response to molecules with transforming growth factor activity. Exp. Cell Res. 143: 143-152, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05249-02 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Involvement of Human Oncogenes in Chromosomal Translocations

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

N. Heisterkamp Visiting Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

Erasmus University, Rotterdam, The Netherlands

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The complete human c-abl oncogene has been isolated from a cosmid library. By analysis of a series of mouse-human somatic cell hybrids using a c-abl-specific probe, human c-abl could be assigned to chromosome 9. Subsequently, it was demonstrated that in chronic myelogenous leukemia (CML) c-abl is translocated from chromosome 9 to 22, the Philadelphia chromosome (Ph1). This finding argues for a possible involvement of c-abl in CML and demonstrates, for the first time, that this translocation is reciprocal. Using a similar approach, the translocation of c-sis from chromosome 22 to 9 could be demonstrated in the Philadephia chromosome. Sequences encoding the tyrosine phosphorylation acceptor region of the human c-abl oncogene have been identified and their nucleic acid sequences determined. An extensive sequence homology between this region of c-abl and the acceptor regions of the v-src, v-yes and v-fes family of viral oncogenes was shown to exist. These findings argue that these different oncogenes with tyrosine protein kinase activity were probably derived from a common progenitor and may represent members of a diverse family of cellular protein kinases.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

John Groffen	Visiting Fellow	LVC, NCI
John R. Stephenson	Visiting Scientist	LVC, NCI
Fred H. Reynolds, Jr.	Expert	LVC, NCI

Objectives:

The major objective of this project is a determination of the possible involvement of cellular homologs of viral oncogenes, in particular, c-abl, in human tumorigenesis.

Methods Employed:

Southern blotting and hybridization to identify defined genes in human cellular DNA. Molecular cloning of human cellular genes in phage, plasmid or cosmid vector systems. Application of expression plasmids to obtain translation products, which will be used for immunization of rabbits. In addition, nucleic acid sequencing and techniques to introduce cloned DNA into cultured mammalian cells were employed.

Major Findings:

1. Detection of v-abl homologous sequences in human lung carcinoma DNA. A contiguous region of a cellular DNA sequence, 64 kilobases (kb) in length and representing overlapping cellular inserts from three independent cosmid clones, has been isolated from a representative library of human lung carcinoma DNA partially digested with MboI. Within this region of the cellular genome, v-abl homologous sequences are dispersed over a total region of around 32 kb. These sequences represent the entire v-abl cellular homolog, are colinear with the viral v-abl transforming gene, and contain a minimum of seven intervening sequences. By Southern analysis of a series of mouse-human somatic cell hybrids using a c-abl-specific probe, human c-abl could be assigned to chromosome 9.

2. Association of specific chromosomal translocations with CML. Several relatively specific, chromosomal translocations are known to be associated with particular human cancers. One of these, the Philadelphia translocation, t(9;22)(q34;q11), is observed in over 90% of chronic myelocytic luekemias (CML). Translocation of the q11 to qter segment of chromosome 22 to chromosome 9 results in a deleted form of chromosome 22, referred to as the Philadelphia (Ph1) chromosome. Using c-abl-specific hybridization probes, it could be shown that in the Philadelphia translocation, c-abl is translocated from chromosome 9 to 22q (the Ph1 chromosome in CML). Because of the small size of the segment of chromosome 9 which translocates to chromosome 22 and the localization of immunoglobulin λ light chain sequences on chromosome 22, c-abl appears to map in close proximity to λ sequences in the Ph1 chromosome.

3. Claiming of c-sis and its association with CML. Another acute transforming retrovirus, the simian sarcoma virus, is a genetic recombinant between a non-transforming retrovirus and cellular sequences of woolly monkey origin. Part of human c-sis was molecularly cloned; because human c-sis is translocated from chromosome 22 to chromosome 9 in CML, its regional localization could be determined (22, q11 to qter).

4. Commonality among the tyrosine phosphorylation acceptor region of c-abl and v-src, v-yes and v-fes. Others have demonstrated that v-src, v-yes and v-fes exhibit considerable sequence homology, particularly in the region of their phosphorylation acceptor sites. In the human, c-abl oncogene sequences encoding the tyrosine phosphorylation acceptor region have been identified and their nucleic acid sequences determined. Comparison of this sequence with the acquired sequences of other retroviruses established extensive homology between this region of c-abl and the acceptor regions of the v-src, v-yes, and v-fes family of viral oncogenes, as well as more distant relatedness to the catalytic chain of the mammalian cAMP-dependent protein kinase. These findings argue that, of the homologs of retroviral oncogenes with tyrosine protein kinase activity examined to date, all were probably derived from a common progenitor and may represent members of a diverse family of cellular protein kinases.

Significance to Biomedical Research and the Program of the Institute:

A determination of the role of cellular homologs of viral oncogenes, in particular c-abl, in naturally occurring human tumors should improve our understanding of the molecular basis of malignant transformation and lead to the development of new approaches to cancer detection and control.

Proposed Course:

More studies will be initiated, or are in progress, to elucidate the role of the human c-abl oncogene in CML. In collaboration with Dr. F. Reynolds and Dr. J. R. Stephenson experiments are in progress to obtain antipeptide antisera and/or monoclonal antibodies specific for human c-abl. The amino acid sequences of these peptides were derived from the nucleotide sequence of human c-abl. In addition, fusion proteins of human c-abl and bacterial proteins will be made by molecular cloning in expression vectors and used in an alternative approach to obtain antisera specific for human c-abl. The availability of these antisera (or monoclonal antibodies) will be very helpful in elucidating the role of the c-abl gene products in humans and in understanding the role of c-abl in CML.

Publications:

de Klein, A., van Kessel, A. G., Grosveld, G., Bartram, C. R., Hagemeijer, A., Bootsma, D., Spurr, N. K., Heisterkamp, N., Groffen, J. and Stephenson, J. R.: A cellular oncogene (c-abl) is translocated to the Philadelphia chromosome in chronic myelocytic leukemia. Nature 300: 765-767, 1982.

Groffen, J., Heisterkamp, N., Reynolds, F. H., Jr. and Stephenson, J. R.: Human c-abl phosphotyrosine acceptor site. Nature (In Press)

Groffen, J., Heisterkamp, N., Stephenson, J. R., Geurts van Kessel, A., de Klein, A., Grosveld, G. and Bootsma, D.: c-sis is translocated from chromosome 22 to chromosome 9 in chronic myelocytic leukemia. J. Exp. Med. (In Press)

Heisterkamp, N., Groffen, J. and Stephenson, J. R.: The human v-abl cellular homologue. J. Mol. Appl. Genet. 2: 57-68, 1983.

Heisterkamp, N., Groffen, J., Stephenson, J. R., Spurr, N. K., Goodfellow, P. N., Solomon, E., Carritt, B. and Bodmer, W. F.: Chromosomal localization of human cellular homologues of two viral oncogenes. Nature 299: 747-749, 1982.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05250-02 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Identification of Oncogenes and their Possible Involvement in Human Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

J. Groffen Visiting Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

Imperial Cancer Research Fund, London, England; University of California, Irvine, CA

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Snyder-Theilen feline sarcoma virus (ST-FeSV)- or Abelson murine leukemia virus (A-MuLV)-transformed mink cells exhibit a relatively high reversion rate compared to the nontransformed phenotype. The phenotypically normal cells have not rearranged the integrated retrovirus. However, it could be demonstrated that the retroviral sequences, in comparison with those in transformed cells, are highly methylated. These findings demonstrate that viral oncogene-mediated transformation can be regulated by methylation. Acute promyelocytic leukemia is frequently associated with a reciprocal 15-17 chromosomal translocation. The c-fes oncogene, which has been mapped to chromosome 15, was shown to be translocated to chromosome 17 in this disease. Using a probe specific for v-fms, a human cosmid library was screened for v-fms homologous sequences. A contiguous region of cellular DNA of approximately 64 kilobases (kb) in length was isolated. Within this region of the DNA, v-fms homologous sequences are dispersed over a total region of around 32 kb and represent the entire human cellular homolog of v-fms. By analysis of human-mouse somatic cell hybrids, human c-fms could be localized to chromosome 5 band q34. This chromosomal localization is of interest in view of reports of a specific deletion involving approximately two thirds of the q arm of chromosome 5 in acute myelogenous leukemia.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Nora Heisterkamp	Visiting Fellow	LVC, NCI
John R. Stephenson	Visiting Scientist	LVC, NCI

Objectives:

To accomplish the molecular cloning of the human c-fms and c-fes sequences in order to study their potential role in human tumorigenesis.

Methods Employed:

Southern blotting and hybridization were used to identify defined genes in human cellular DNA, as well as molecular cloning of human cellular genes in phage, plasmid or cosmid vector systems. Cloned genes were introduced into cultured mammalian cells.

Major findings:

1. Regulation of transformation by methylation. Mink cells morphologically transformed by either Snyder-Theilen feline sarcoma virus (ST-FeSV) or Abelson murine leukemia virus (Abelson-MuLV) exhibit relatively high rates of reversion to the nontransformed phenotype. The proviral DNAs are conserved within the revertant lines and have not undergone changes in integration sites due to translocations or other genomic rearrangements. Loss of the transformed phenotype was shown to be associated with increased cytosine methylation of proviral DNA sequences, while levels of methylation resume control values upon spontaneous retransformation of revertant clones. Following molecular cloning and transfection to Rat-2 cells, ST-FeSV proviral DNAs from revertant and transformed cells induced similar numbers of transformed foci. These findings demonstrate regulation of viral oncogene-mediated transformation by cytosine methylation.

2. Localization of a translocation in acute promyelocytic leukemia. Somatic cell hybrids have been constructed between a mouse cell line and blood leukocytes from a patient with acute promyelocytic leukemia (APL) showing the $15q^{+};17q^{-}$ chromosome translocation frequently associated with this disease. One hybrid contains the $15q^{+}$ translocation chromosome and very little other human material. It was demonstrated that the c-fes oncogene which has been mapped to chromosome 15, is not present in this hybrid and is, therefore, probably translocated to the 17q chromosome. Analysis of the genetic markers present in this hybrid has enabled a more precise localization of the translocation breakpoints on chromosomes 15 and 17.

3. Detection of the human cellular homolog of v-fms. The integrated form of McDonough FeSV proviral DNA, including cellular flanking sequences, was molecularly cloned from nonproductively transformed Fischer rat cells. Acquired cellular-derived (v-fms) sequences within the cloned proviral DNA were mapped

from between 2.6 and 5.5 kilobases (kb) from the 5' long terminal repeat (LTR). Using a series of molecular probes representing subgenomic regions of the viral v-fms gene, a cosmid library of human lung carcinoma DNA was screened for v-fms homologous sequences. Three cosmid clones containing overlapping v-fms homologous cellular DNA inserts, representing a contiguous region of a cellular DNA sequence of approximately 64 kb in length, were isolated. Within this region of human genomic DNA, v-fms homologous sequences are dispersed over a total region of around 32 kb. These sequences represent the entire human cellular homolog of v-fms, are colinear with the viral v-fms transforming gene, and contain a minimum of four intervening sequences.

4. Assignment of c-fms to chromosome 5. The human cellular homolog of transforming sequences represented within the McDonough strain of feline sarcoma virus (c-fms) was assigned to chromosome 5 by analysis of a series of mouse-human somatic cell hybrids containing variable complements of human chromosomes. Regional localization of c-fms to band q34 on chromosome 5 was accomplished by analysis of Chinese hamster-human cell hybrids containing terminal and interstitial, deleted forms of chromosome 5. The localization of c-fms to chromosome 5 (q34) is of interest in view of reports of a specific, apparently interstitial, deletion involving approximately two-thirds of the q arm of chromosome 5 in acute myelogenous leukemia cells.

Significance to Biomedical Research and the Program of the Institute:

A determination of the role of cellular homologs of viral oncogenes in human tumorigenesis should improve our understanding of the molecular basis of malignant transformation and lead to the development of new approaches to cancer detection and control.

Proposed Course:

Future studies will be directed towards more extensive investigations to elucidate the possible relationship between acute myelocytic leukemia (AML), APL and c-fms, c-fes, respectively. This will involve the isolation of specific probes from the cloned human c-fms and c-fes genes which subsequently will be used to screen total cellular DNA of AML and APL patients for the presence of DNA rearrangements.

Publications:

Groffen, J., Heisterkamp, N., Blennerhassett, G. and Stephenson, J. R.: Regulation of viral and cellular oncogene expression by cytosine methylation. Virology 126: 213-227, 1983.

Groffen, J., Heisterkamp, N., Shibuya, M., Hanafusa, H. and Stephenson, J. R.: Transforming genes of avian (v-fps) and mammalian (v-fes) retroviruses correspond to a common cellular locus. Virology 125: 480-486, 1983.

Heisterkamp, N., Groffen, J. and Stephenson, J. R.: Isolation of v-fms and its human cellular homologue. Virology 126: 248-250, 1983.

Heisterkamp, N., Groffen, J., Stephenson, J. R., Spurr, N. K., Goodfellow, P. N., Solomon, E., Carritt, B. and Bodmer, W. F.: Chromosomal localization of human cellular homologues of two viral oncogenes. Nature 299: 747-749, 1982.

Sheer, D., Hiorns, L. R., Stanley, K. F., Goodfellow, P. N., Swallow, D. M., Povey, S., Heisterkamp, N., Groffen, J., Stephenson, J. R. and Solomon, E.: Genetic analysis of the 15;17 chromosome translocation associated with acute promyelocytic leukemia. Proc. Natl. Acad. Sci. USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05330-01 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Urinary Growth Factors in Human Neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

K. J. Stromberg Medical Director, LVC, NCI

COOPERATING UNITS *(if any)*

Division of Endocrinology, Vanderbilt University, Nashville, TN; Laboratory of Pathology, Division of Cancer Biology and Diagnosis, NCI, Bethesda, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues Fluids ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

High molecular weight growth factor (HMW GF) was efficiently absorbed by trimethylsilyl-controlled pore glass beads (TMS-CpG beads) from the bulk urine of a patient bearing a glioblastoma multiforme. After a second cycle of TMS-CpG absorption, HMW GF had a molecular weight of approximately 28,000 using Bio-Gel P-100 chromatography. Following an estimated 95% surgical resection of tumor, no appreciable growth factor activity of this sort was detectable in a comparable 48-hour urine collection. This brain tumor-associated HMW GF activity contained only about 20% of the radioreceptor activity of the standard small molecular weight human epidermal growth factor (SMW hEGF) while maintaining full immunoreactivity to the SMW hEGF. Consequently, the HMW GF associated with brain tumor burden in this patient appears similar to the high molecular weight form of human EGF (HMW hEGF) previously reported in low concentration in normal human urine. Thus, the HMW GF may be of host cell, rather than uniquely of tumor cell, origin.

The process of horizontal recruitment in transplantation tumor biology is defined as the induction of malignancy in adjacent, presumably normal, host cells of tumor-bearing animals. We have reported a human chondroblastic tumor in a nu/nu mouse expressing a mixed histologic composition. The bipartate tumor was injected into nu/nu rats. A growth factor of high molecular weight was demonstrated in the urine of the tumor-bearing rats which may be of rat or human origin.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Daniel R. Twardzik Research Chemist LVC, NCI

Objectives:

To study the expression of a family of urinary growth factors in human cancer patients in respect to their histologic type and their extent of tumor burden, to develop novel growth factor isolation procedures employing hydrophobic absorption, and to use nu/nu rats and mice bearing human tumors as model systems.

Methods Employed:

Malignant astrocytoma, grade III or grade IV (glioblastoma multiforme), the most aggressive cancer of the central nervous system, was selected for emphasis because (1) the issue of occult metastasis is avoided (extra-CNS spread of this malignancy is exceedingly rare; (2) repeated tumor debulking occurs (to provide a natural human model for correlation of growth factor activity with tumor burden over an extended period of time); (3) it addresses the question of whether or not the high molecular weight growth factor (HMW GF) traverses the blood-brain barrier to reach the kidneys for clearance; and (4) if the HMW GF was a tumor cell product, and not simply a host factor which responded to tumor burden, a differential concentration might be found between urine and cerebral spinal fluid.

A simple procedure has been developed for rapid processing of large volumes of either human or rat urine based on growth factor absorption on to trimethylsilyl-controlled pore glass beads (TMS-CpG beads) and selective elution with appropriate concentrations of acetonitrile. The presence of growth factor(s) was assessed by stimulation of growth of normal rat kidney cells (NRK) in soft agar, and competition with ^{125}I-labeled epidermal growth factor (EGF) for binding to membrane receptors. Athymic mice and rats were employed to passage human carcinomas of various histologic types and housed in metabolic cages to facilitate efficient urine collection.

Major Findings:

1. Development of a method for separating growth factor from bulk urine. High molecular weight growth factor (HMW GF) was efficiently absorbed by TMS-CpG beads from the bulk urine of a patient bearing a glioblastoma multiforme. Following a second cycle of TMS-CpG absorption, HMW GF had a molecular weight of approximately 28,000 using Bio-Gel P-100 chromatography. Following an estimated 95% surgical resection of tumor, no growth factor activity of this sort was detectable in a comparable 48-hour urine collection. This brain tumor-associated HMW GF activity contained only about 20% of the radioreceptor activity of the standard small molecular weight human epidermal growth factor (SMW hEGF) while maintaining full immunoreactivity to the SMW hEGF. Consequently, the HMW GF associated with brain

tumor burden in this patient appears similar to the high molecular weight form of human EGF (HMW hEGF) previously reported in low concentration in normal human urine. Thus, the HMW GF may be of host cell, rather than uniquely of tumor cell, origin.

2. Three human tumors were established in rats and used as a model for correlation of urine concentration of growth factor and tumor mass. Five human tumors from nu/nu mice, representing three histologic types from lung and stomach neoplasias, have been established in nu/nu rats. Ample urine from rats bearing these human tumors was collected in order to identify which tumor results in the most abundant urinary growth factor production. Additionally, urine samples were collected over a prolonged time period during which periodic surgical debulking occurred. This will permit examination of growth factor production in response to tumor burden.

3. TGF may mediate the initiation of horizontal recruitment of tumor cells. A human chondroblastic osteosarcoma transplanted into the nu/nu mouse continues to express biochemical and histological markers similar to the original tumor. Morphologically, the majority of the cells were of the chondroblastoid type with a minor component of fibrosarcoma. By isozyme analysis and karyology, the tumor is human with a minor contamination of mouse. However, all attempts to grow the human component of this mixed tumor in vitro have resulted in murine cell lines. A murine cell line (TC 715), established from this bipartate tumor, causes fibrosarcomas in nu/nu mice and forms colonies in soft agar. Urine isolated from nu/nu rats injected with chondrosarcoma tumor from the mouse contains a growth factor of a high molecular weight (30-35,000 daltons). This factor competes in radioreceptor assays with epidermal growth factor and also promotes the growth of phenotypically normal rat kidney cells in soft agar. Consequently, it is a transforming growth factor (TGF) and may be of human origin because no high molecular weight TGF activity has been detected in the murine cell line, TC 715.

Significance to Biomedical Research and the Program of the Institute:

Urinary transforming growth factors may prove to be useful markers of pre-clinical cancer development in man. Although considerable resources are currently being devoted to the basic molecular biology of TGFs, relatively little emphasis has been placed on developing an approach to their evaluation and possible application in pre-clinical human cancer detection. Identification of the origin (host or tumor) and correlation with clinical condition (tumor bulk) of the HMW GF is an essential initial step in this process.

The occurrence of horizontal recruitment has been known for some time but the relatively recent increase in the use of immunodeficient animals as recipients of human xenografts has brought the phenomenon to the attention of investigators working with grafted tissue. We have recently reported the formation of a bipartate tumor containing human chondroblastic cells and a mouse fibrosarcoma. A transforming growth factor of high molecular weight was present in the urine of nu/nu rats bearing this tumor and was absent from the media of the recruited murine cell line (TC 175), suggesting that TGFs of human or rat origin could mediate the initial steps of horizontal recruitment.

Proposed Course:

Quantitative procedures for large-scale HMW GF isolation and characterization using pooled preoperative urines from patients with malignant astrocytomas are currently being developed. This will assist in identifying the HMW GF in tumored urine as equivalent to HMW hEGF and establishing it to be of host cell, rather than uniquely of tumor cell, origin. The long-term objective is to follow individual bulk urine collection and concentration on TMS-CpG beads with high pressure liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis using silver staining of gels to quantify the relative presence of each member of the family of urinary growth factors (HMW hEGF/HMW GF, SMW hEGF, alpha TGF and beta TGF). Thus, a profile of urinary growth factor expression would be available to relate to the clinical state during which the urine sample was collected.

Publications

Tralka, T. S., Yee, C., Rabson, A. B., Wivel, N. A., Stromberg, K., Rabson, A. S. and Costa, J.: Murine type-C retroviruses and intracysternal A-particles in human tumors serially passaged in nude mice. J. Natl. Cancer Inst. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05331-01 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and Biological Activity of Oncogene-Encoded Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

F. H. Reynolds, Jr. Expert, NCI

COOPERATING UNITS *(if any)*

Laboratory of Molecular Virology and Carcinogenesis, Litton Bionetics, Inc., Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.3	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structure and biological activity of three oncogene-encoded proteins were studied. Proteins encoded by two oncogenes, v-fes and v-abl, are tyrosine specific protein kinases which phosphorylate both themselves and other substrates. The phosphorylation sites on P120gag-abl were identified on three tryptic peptides and found to be 1, 6, and 7 residues distal to their tryptic cleavage sites. The sequence of one of these peptides was deduced from the corresponding DNA sequence of the human c-abl gene and the peptide was chemically synthesized. Immunoprecipitated P120gag-abl efficiently phosphorylated this peptide. Tryptic peptides prepared from v-fes gene-encoded polyproteins were found to have a similar phosphorylation site at a position seven residues distal to the cleavage site. A phosphoglycoprotein of 150,000 daltons (P150) was identified as a substrate for the v-abl- and v-fes-encoded protein kinases; it was found to be a serine-specific protein kinase. P150 was shown to be distinct from each of several previously demonstrated substrates for tyrosine-specific protein kinases, including vinculin, several glycolytic enzymes and P39. Its phosphorylation may be involved in the regulation of cellular events resulting in expression of the transformed phenotype. Expression of this protein was restricted to epithelial cells and carcinomas, suggesting that it may be a useful tumor marker. Cells transformed by both the v-abl and v-fes genes were found to secrete a tumor growth factor that is structurally related to epidermal growth factor. This factor, in concert with a relatively ubiquitous potentiating factor, stimulated the growth of cells in soft agar. A third oncogene studied, v-raf, was found to encode two proteins of 75,000 and 90,000 daltons that are not tyrosine-specific protein kinases. v-raf-transformed cells do not exhibit elevated levels of phosphotyrosine. The v-raf gene was found to differ from each of a number of other oncogenes examined and, thus, is thought to represent a unique new oncogene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

J. R. Stephenson	Visiting Scientist	LVC, NCI
J. Groffen	Visiting Fellow	LVC, NCI
N. Heisterkamp	Visiting Fellow	LVC, NCI
U. R. Rapp	Visiting Scientist	LVC, NCI
D. R. Twardzik	Research Chemist	LVC, NCI
G. J. Todaro	Medical Officer	LVC, NCI

Objectives:

The major objectives of this project are to identify the proteins encoded by both viral and cellular oncogenes and to study their mechanism(s) of transformation. Monoclonal antibodies recognizing determinants on these proteins should be useful for detection and therapy of human cancers.

Methods Employed:

A variety of cell lines transformed by v-fes, v-abl, v-fms and v-raf, as well as human tumor lines, are maintained for study. Techniques of modern biochemistry employed to study the oncogene-encoded proteins include immunological assays and precipitations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, high performance liquid chromatography, two-dimensional electrophoretic and chromatographic peptide and phosphoamino acid analysis as well as other methods of protein chemistry. Recently, solid phase peptide synthesis capability has been added to the laboratory. Monoclonal antibodies were generated using both mouse and rat hybridomas. Autoradiographic, ELISA and fluorescent methods of antigen detection are used.

Major Findings:

Major findings resulting from this project occurred in three areas: (1) proteins encoded by the v-fes and v-abl protein kinase genes; (2) proteins encoded by v-raf, a unique new oncogene; and (3) tumor growth factors produced by oncogene-transformed cells.

1. Proteins encoded by the v-fes and v-abl protein kinase genes. The sites of tyrosine phosphorylation of the v-fes- and v-abl-encoded proteins were investigated in detail. The Gardner and Snyder-Theilen strains of feline sarcoma virus (FeSV) encode 110,000 molecular weight (M_r) polyproteins as their primary translational products. Cells transformed by a variant of Snyder-Theilen FeSV express an 85,000 M_r polyprotein. Single tyrosine phosphorylation sites identified within each of these viral gene products were shown to represent major in vitro acceptors phosphorylated by the polyprotein kinases. By two-dimensional tryptic peptide analysis, these acceptor sites are highly related, although they exhibit minor differences in map position, while by analytical high performance liquid chromatography, all three elute at approximately the same acetonitrile concentration (21-23%). The tyrosine acceptor sites in these FeSV-encoded polyproteins

have been localized by sequential Edman degradation at a position seven amino acid residues distal to their trypsin cleavage site. The major tyrosine acceptors, two serine phosphorylation acceptors common to the p12 structural components of all three polyproteins, a single major phosphothreonine site, and several minor, less well-resolved acceptor sites are shown to be phosphorylated in vivo. In addition to the major tyrosine acceptor site initially phosphorylated in vitro, phosphorylation of secondary tyrosine acceptors was shown to occur to proportionately greater extents when in vitro phosphotransferase reactions were carried out for longer times or at higher temperatures. In addition, tryptic peptides containing two major, in vivo, $P120^{gag-abl}$ tyrosine phosphorylation acceptor sites were identified, phosphorylated in vitro, and purified to homogeneity. The tyrosine site in peptide (a) is localized at a position six residues distal to its trypsin cleavage site, whereas the tyrosine acceptor site in peptide (b) is at residue seven. A third peptide, (c), contains an amino-terminal phosphotyrosine residue; phosphorylation of this latter peptide only occurs to a significant extent in vivo. A restriction fragment containing DNA encoding the tyrosine-specific protein kinase phosphorylation site on peptide (b) in both v-abl and its human cellular homolog, c-abl, was identified and subjected to nucleic acid sequencing. The predicted peptide was chemically synthesized and tested as a substrate for $P120^{gag-abl}$ with positive results. In addition, all of the phosphorylation was found to be on tyrosine residues. This peptide and others specified by the human c-abl gene were used to prepare antisera. Similarly, monoclonal antibodies were prepared against the v-fes-encoded proteins. A series of hybridomas have been isolated which produce monoclonal antibodies directed against polyprotein gene products of the Gardner, Snyder-Theilen and McDonough strains of FeSV. Within these are representatives of several immunoglobulin classes, including IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgM. Antibody produced by one hybridoma recognizes immunologic determinants localized within a feline leukemia virus (FeLV) gag gene structural component (p15) common to polyproteins encoded by all three FeSV isolates, whereas antibody produced by additional hybridomas is specific for p30 and v-fes-encoded determinants. Both Gardner (GA) $P110^{gag-fes}$ and Snyder-Theilen (ST) $P85^{gag-fes}$ immunoprecipitated by antibody directed against p15 exhibit tyrosine-specific protein kinase activity, but lack such activity when precipitated by antibody specific for their acquired sequence (v-fes) components.

A cellular phosphoglycoprotein of 150,000 molecular weight (P150) has been identified and found to be a substrate for v-abl- and v-fes-encoded protein kinases. Phosphotyrosine was found on P150 isolated from [^{32}Pi]orthophosphate-labeled cells of several species transformed by these viruses, but not on P150 isolated from nontransformed control cells. P150 exhibits an associated serine-specific protein kinase activity which recognizes $P120^{gag-abl}$ and GA $P110^{gag-fes}$, as well as P150 itself, as substrates and uses [γ^{32}P] ATP as a phosphate donor. Interspecies antigenic determinants on P150 permitted its isolation from cells of all mammalian species tested, including mouse, rat, cat, dog, mink and a number of human tumor lines. Similar [^{35}S]methionine- and [^{32}Pi]orthophosphate-labeled tryptic petides were characteristic of P150s isolated from cells of several mammalian species, including human, indicating conservation of this protein throughout recent evolution. Upon immunoprecipitation in buffer containing a low concentration of nonionic detergent, mink P150 bound to and phosphorylated GA $P110^{gag-fes}$; although binding was also found in cells of other species transformed by v-fes, it was considerably weaker. These findings are in agreement with

our previous report of P150 incorporation into pseudotype virions obtained from v-fes transformed mink cells. Glycosylation of P150 was demonstrated by incorporation of [^{3}H]mannose, and the glycosylation site was localized to a single tryptic peptide. P150 was shown to be distinct from each of several previously demonstrated substrates for tyrosine-specific protein kinases, including vinculin, several glycolytic enzymes and P39. Phosphorylation of P150 on tyrosine residues may be involved in the regulation of cellular events resulting in expression of the transformed phenotype.

2. Proteins encoded by v-raf, a unique new oncogene. Methylcholanthrene-transformed C3H/10T1/2 mouse cells were treated with iododeoxyuridine and the virus induced was inoculated into mice. A new, acute transforming, type C retrovirus, designated 3611-MSV, and its gene, v-raf, was isolated. It transforms embryo fibroblasts and epithelial cells in culture and induces fibrosarcomas in vivo. Analysis of viral protein expression has led to the demonstration of 90,000 and 75,000 molecular weight polyproteins, both containing amino-terminal murine leukemia virus (MuLV) gag gene proteins, p15 and p12. The v-raf-encoded polyproteins lack detectable protein kinase activity, and 3611-MSV-transformed cells do not exhibit elevated levels of phosphotyrosine. This gene was molecularly cloned and a comparison of v-raf with previously isolated retrovirus oncogenes, either by direct hybridization or by comparison of restriction fragments of their cellular homologs, shows it to be unique. Transfection of NIH/3T3 and RAT-2 cells with cloned v-raf DNA leads to efficient transformation and expression of P75 and P90, containing NH_2-terminal gag gene-encoded components linked to the acquired sequence (v-raf) translational product.

3. Tumor growth factors produced by oncogene transformed cells. Fischer rat embryo cells transformed by v-abl, v-fes and v-fms release transforming growth factors (TGFs) into cell culture medium. These peptides stimulate phosphorylation of epidermal growth factor (EGF) membrane receptors and further promote anchorage-independent cell growth. Cells transformed by v-abl and v-fes produce high titers of TGF (60-200 nanogram equivalents [ng eq] EGF/liter) while cells transformed by v-fms produce TGF at only low levels (<10 ng eq EGF/liter). Upon purification, TGF progressively loses transforming activity. A second potentiating factor, when added to purified TGF preparations, restores transforming function. This factor has been partially purified and shown to be distinct from previously described growth factors.

Significance to Biomedical Research and the Program of the Institute:

The discovery of oncogene-encoded proteins and the tyrosine-specific protein kinase enzymatic reactivity of some of them has opened exciting new possibilities for the detection, management and therapy of human cancer. These proteins should be expressed in human cancers and, therefore, be detectable by standard, immunological assays using fluorescence, radioisotopes or the ELISA technique. Monoclonal antibodies to these proteins could prove useful for immunotherapy of human cancers.

Proposed Course:

This project represents a balanced approach to study the mechanism(s) of oncogene expression-mediated transformation in cell culture and to detect oncogene

expression in human cancers. The mechanism(s) of transformation by these oncogenes will be studied using tissue culture cells in an effort to identify additional substrates of the v-abl- and v-fes-encoded protein kinases and to explore the role of p150 in the transformed cell. A major effort to detect oncogene-encoded proteins in human cancers will involve synthetic peptides containing regions of the predicted c-abl protein used to generate hyperimmune rabbit sera and monoclonal antibodies. Several of these currently exist and others are at various stages of development. These reagents will be used in an attempt to detect expression of the human c-abl gene in cancerous cells by immunoprecipitation and Western blotting techniques. The recent finding of c-abl gene translocation in human chronic myelogenous leukemia (CML) has provided important insight into the likely target cell for c-abl expression. Sera from human subjects will be used in an attempt to detect antibodies to the Abelson protein in CML patients, and the possibility of c-fes and c-fms expression in human tumors will similarly be investigated.

Publications:

Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr. and Stephenson, J. R.: Structure and biological activity of v-raf, a new oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA (In Press)

Rapp, U. R., Reynolds, F. H., Jr. and Stephenson, J. R.: Isolation of a new mammalian type C transforming virus. In Pearson, M. L. and Sternberg, N. L. (Eds.): Proceedings of Gene Transfer and Cancer Workshop. New York, Raven Press (In Press)

Rapp, U. R., Reynolds, F. H., Jr. and Stephenson, J. R.: New mammalian transforming retrovirus: Demonstration of a polyprotein gene product. J. Virol. 45: 914-924, 1983.

Reynolds, F. H., Jr., Oroszlan, S., Blomberg, J. and Stephenson, J. R.: Tyrosine phosphorylation sites common to transforming proteins encoded by Gardner and Snyder-Theilen FeSV. Virology 122: 134-146, 1982.

Reynolds, F. H., Jr., Oroszlan, S. and Stephenson, J. R.: Abelson murine leukemia virus P120: Identification and characterization of tyrosine phosphorylation sites. J. Virol. 44: 1097-1101, 1982.

Twardzik, D. R., Todaro, G. J., Reynolds, F. H., Jr. and Stephenson, J. R.: Similar transforming growth factors (TGFs) produced by cells transformed by different isolates of feline sarcoma virus. Virology 124: 201-207, 1983.

Veronese, F., Kelloff, G. J., Reynolds, F. H., Jr., Hill, R. W. and Stephenson, J. R.: Monoclonal antibodies to feline sarcoma virus gag and fes gene translational products. J. Cell. Biochem. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05332-01 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Purification and Characterization of SGF-Related, EGF-Competing Growth Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

D. A. Pigott Visiting Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ectopic production of peptide growth factors by malignant cells, which are also capable of utilizing them (autocrine stimulation), confers a selective growth advantage to tumor tissues. Epidermal growth factor (EGF)-competing peptides are an example of these ectopic growth factors and sarcoma growth factor (SGF), produced by Moloney sarcoma virus-transformed murine cells, is the best-characterized example of these peptides. SGF accounts, however, for only a small portion (about 10%) of the total EGF-competing activity in serum-free conditioned medium. The majority of the EGF-competing activity is separated as a larger molecular weight species on the initial Bio-Gel P-60 column eluted with 1 M acetic acid. Ion exchange chromatography of these larger species on CM-cellulose at pH 5 reveals three resolvable activities and the highest specific activity fraction from this column is itself heterogeneous when chromatographed by reverse phase high pressure liquid chromatography (HPLC) on a Bondapak C18 column eluted by either acetonitrile or propanol. This indicates that these peptides differ structurally from SGF. Following purification to homogeneity, collaboration with other groups to define the primary and secondary structures of these molecules will permit enumeration of the structures of this whole family of SGF-like molecules. Comparison of these structures will help in defining the conserved regions necessary for both EGF receptor binding and mitogenesis. Equally important, comparison of the structures of the SGF family of peptides will indicate whether there is a single "SGF gene" (with peptides related to different initiation or termination codons), or whether there is a family of such genes in the murine cells which are activated following Moloney sarcoma virus transformation. To facilitate large-scale production of these EGF-competing growth factors, a variant of the parental cell line is being isolated which is capable of anchorage-independent growth in serum-free medium. Such a variant is currently being characterized for the continued production of these, and perhaps new, ectopic growth factors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Joseph E. De Larco Research Chemist LVC, NCI

Objectives:

To purify and characterize sarcoma growth factor (SGF)-related, epidermal growth factor (EGF)-competing peptides secreted by Moloney sarcoma virus transformed murine (3T3) cells (3B11-IC) to serum-free medium. To define, in collaboration with other groups, the primary and secondary structures of these molecules to permit enumeration of the structures of this whole family of "SGF-related" molecules. Comparison of the structures will help in defining the conserved regions necessary for both EGF receptor binding and mitogenesis. Equally important, comparison of the structures of this "SGF family" of peptides will indicate whether there is a single "SGF gene" (with peptides related to different initiation or termination codons), or whether there is a family of such genes in the murine cells which are activated following Moloney sarcoma virus transformation. To facilitate large scale production of these EGF-competing growth factors, a variant of the parental cell line is being developed which is capable of anchorage-independent growth in serum-free medium.

Methods Employed:

Tissue culture methods were used for the production and assay of EGF-competing growth factors from 3B11-IC cells. Confluent monolayers of cells were washed and exposed to serum-free medium for 48 hours. The conditioned medium was clarified by centrifugation and concentrated 25-fold using a hollow fiber concentrator. Acidification and desalting was achieved by dialysis against 1% acetic acid and the retained fraction further concentrated by lyophilization. The amount of EGF-competing activity in these samples and fractions generated by chromatography was determined using a radioreceptor assay and comparing the competition produced with a standard curve generated by the competition of a known preparation of unlabeled EGF with ^{125}I-labeled EGF. Purification of EGF-competing peptides involved sequential chromatography by sizing, ion exchange and hydrophobic methods. Isolation of serum-free, anchorage-independent cell variants involved a two-stage selection process. Serum-free anchorage-dependent variants were generated utilizing a high density culture employing μ-carriers in stationary cultures. Generation of serum-free variants selected for anchorage-independence utilized a high density culture in siliconized stirrer flasks and the repeated removal of cell debris.

Major Findings:

1. Detection of several distinct EGF-competing peptides. The initial step in purification of EGF-competing peptides utilized Bio-Gel P-60 gel filtration chromatography. The major activity eluted in a broad peak following the void volume and was well resolved from the later-eluting SGF activity. Upon chromatography on CM-52 at pH 5 the broad P-60 peak resolved into three fractions

containing EGF-competing activity. The latest-eluting activity from the CM-52 column contained the largest amount of activity at the highest specific activity. Further chromatography of this latest-eluting activity by reverse-phase high pressure liquid chromatography (HPLC) on a μ Bondapak C_{18} column eluted by acetonitrile or propanol revealed several peaks of EGF-competing activity eluting closely together at 23-25% acetonitrile or 14-16% propanol. Thus, 3B11-IC cells secrete several distinct EGF-competing peptides which vary structurally as indicated by size, pI and hydrophobicity differences.

2. Development of anchorage-dependent, serum-independent cell variants. Selection of cell variants capable of anchorage-dependent, serum-free growth has been successful and these cells have continued to grow in Dulbecco's minimum essential medium (DMEM) alone for the past year and continue to secrete EGF-competing activities. Recently, a variant(s) has arisen which appears to fulfill both the serum-free and anchorage-independent requirements. This variant is currently being expanded and its continued secretion of EGF-competing activities defined.

Significance to Biomedical Research and the Program of the Institute:

This work seeks to define the factors responsible for the uncontrolled proliferation of malignant cells. Furthermore, factors acting together with B-transforming growth factor, EGF or EGF-competing peptides contribute to the transformed morphology expressed by malignant cells and are capable of conferring the transformed phenotype on untransformed indicator cells. The data collected from the murine system appears to be directly applicable to certain human malignancies in vivo. Development of therapeutic agents for the treatment of these malignancies could involve suppression of the transformed phenotype and uncontrolled growth by interruption of the secretion or action of these ectopic growth factors. Alternatively, immunological therapeutics could be designed to destroy the cells producing these ectopic growth factors. These goals are consistent with the goals and mission of the section, the laboratory, the division and the NCI.

Proposed Course:

The project will continue in two phases. The first will consist of the purification to homogeneity of each of the EGF-competing activities and, in collaboration with other groups, the definition of the complete primary and secondary structures of these peptides. The second course will be to develop, characterize and utilize the serum- and anchorage-independent cells for the large-scale production of EGF-competing peptides. Further, it is anticipated that these cells will be found to produce new, as yet unidentified, growth factors which contribute to their anchorage- and serum-independent phenotypes.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05333-01 LVC

PERIOD COVERED

January 10, 1983 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Evolutionary Relationships of the Felidae: A Mitochondrial DNA Approach

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

M. George, Jr. Staff Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

Veterinary Resources Branch, DRS, NIH, Bethesda, MD; San Diego Zoo, San Diego, CA

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The cat family has served as an excellent model system for the study of viral-induced cancers. The Felidae has been divided into as few as 3 to as many as 20 different genera. To make any meaningful assumptions about the transmission of genes (viral or non-viral) across species lines within this family, a better understanding of its phylogeny is necessary. The aim of this study is to make in-depth comparative studies at the chromosomal, protein and DNA levels among members of the cat family. Mitochondrial DNA (mtDNA) has been chosen as an evolutionary probe. The molecule evolves rapidly, is maternally inherited and provides a high degree of resolution among closely related species. mtDNA has been isolated from the domestic cat, lion, cheetah and clouded leopard. The mtDNAs have been digested with 17 different restriction enzymes and cleavage maps have been constructed. The intra- and interspecific variations of these 4 index cat species have been measured. The construction of the cleavage maps have also aided in the preparation of mtDNA clones. The mtDNA clones will be used as molecular probes of mtDNA from each Felidae species (35 of 41) using "Southern" analysis of high molecular weight DNA from cultured cell lines. The comparative restriction maps of the mtDNA from the various species will be used to construct a molecular phylogeny of the Felidae. This phylogeny will be compared to phylogenetic topologies of the Felidae derived by other molecular and morphometric measurements.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Stephen J. O'Brien Geneticist LVC, NCI

Objectives:

1. The construction of mitochondrial DNA (mtDNA) cleavage maps from various members (domestic cat, cheetah, lion and clouded leopard) of the Felidae, with emphasis on the phylogenetic relationships found in this family. The determination of the extent of genetic variation within and among the different cat species.

2. Preparation and cloning of mtDNA restriction fragments (domestic cat mtDNA) into plasmid pBR322. The mtDNA clones will be used as molecular probes of the mtDNA found in the high molecular weight DNA derived from 35 Felidae species.

Methods Employed:

Mitochondrial DNA (mtDNA) was isolated and purified from tissue organs by differential centrifugation. This procedure also involves the use of CsCl density gradients. The purified mtDNA was subjected to digestion with 17 different restriction enzymes. The mtDNA fragments were end-labeled with ^{32}P using DNA polymerase I (large fragment) and separated by electrophoresis on vertical agarose slab gels. After autoradiography of the vacuum-dryed gels, the base-pair length of the fragments and genome sizes were determined. Construction of the cleavage maps involved multiple enzyme digestions. Other methods included the isolation and preparation of high molecular weight DNA from felid cultured cell lines; preparation and purification of mtDNA fragments for molecular cloning; nick-translation of mtDNA clones; and "Southern" blot analysis.

Major Findings:

1. Construction of mtDNA cleavage maps and analysis of the mtDNA fragment data for four feline species. mtDNA has been isolated from 4 index cat species: the domestic cat, cheetah, lion and clouded leopard. An average of 25 restriction enzyme sites has been mapped for each of the mtDNAs. The maps reveal that there are conserved restriction sites which are shared by other vertebrate mtDNAs. One conserved fragment (in the rRNA genes), which has been found in all vertebrate mtDNAs examined to date, is also present in cat mtDNAs. These lines of evidence suggest that the mtDNA gene order is probably the same in cats as in other species. Sizing of the fragments indicated that cat mtDNA may be larger than that of human mtDNA. When the 4 different mtDNAs are examined by electrophoresis, side by side, after restriction enzyme digestion, a measure of the genetic variation involved was obtained. By observing the number of shared fragments, we found interspecific variation to be very high. The degree of variation seen was much higher than that reported from DNA:DNA hybridization studies. An analysis of the intraspecific variation within 3 domestic cats examined showed only a

small amount of variation. We have also identified restriction fragments which will be the most useful for future cloning experiments.

Significance to Biomedical Research and the Program of the Institute:

This work complements the in-depth biochemical and genetic studies of the cat family initiated by Dr. S. J. O'Brien (Project # Z01CP04896-11 LVC). The phylogeny of this family, which is used as a model system for studies in viral carcinogenesis, is not fully understood. A phylogenetic analysis of this family, using mtDNA as fragment probes, will help resolve some of the uncertainty involving the classification of the Felidae. This, in turn, will allow the formulation of valid assumptions concerning the flow of genes (viral or non-viral) across species lines within this family. Additionally, we will learn more about the intra- and interspecific differences found in this family.

Proposed Course:

More individuals from the index species will be examined. Additional restriction sites are to be mapped in the 4 index species. This will provide a more accurate estimate of the genetic diversity found among them. It will also allow more reliable phylogenetic analysis and estimates of divergence times. Efforts will continue to construct mtDNA clones and screen the 35 additional feline species. This is necessary in order to get a true picture of the phylogenetic relationships found in this family. The levels of genetic variability within the Felidae must also be determined.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05334-01 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Correlates of Reproductive-Endocrine Traits in the Domestic Cat

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

D. E. Wildt Senior Staff Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.4	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The domestic cat serves as a valuable animal model because of its elaborate background in leukemia-sarcoma and the natural history of cancer. To ensure availability of animals with specific genetic backgrounds, considerable efforts are being made to define the reproductive-endocrine relationships, thereby maximizing reproductive potential within our breeding colonies. A series of comprehensive investigations have been conducted to (1) establish ovarian-endocrine relationships in the female cat during estrus, pregnancy, parturition and the postpartum interval; (2) study the influence of exogenous gonadotropins on ovarian function; and (3) determine testes-endocrine correlates in male cats. Overall, such information is being utilized to improve reproductive efficiency and breeding management within the feline colonies. Furthermore, techniques and findings from these studies have been applied and extended to reproductive, genetic and evolutionary analyses of both domestic and nondomestic Felidae, some of which are endangered.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

S. J. O'Brien	Geneticist	LVC, NCI
J. G. Howard	Biological Lab. Tech.	LVC, NCI
K. L. Goodrowe	Guest Researcher	VRB, DRS
P. M. Schmidt	Biological Lab. Tech.	VRB, DRS
E. J. Baas	Biologist	VRB, DRS

Objectives:

(1) To integrate reproductive-endocrine events associated with pregnancy and the postpartum interval of the female cat. (2) To develop hormonal treatments for artificially inducing ovulation and sexual behavior in various felids. (3) To establish endocrine norms and ejaculate characteristics in male felids.

Methods Employed:

Gonadal function was monitored in females by frequent ovarian examination using laparoscopy and in males by spermatozoal collection and evaluation following electroejaculation. Endocrine traits were assessed in both sexes using highly specific radioimmunoassays for pituitary, adrenal and gonadal hormones.

Major Findings:

1. Reproductive-endocrine relationships during pregnancy, parturition, lactation and the postpartum estrus. Ovarian-endocrine relationships in the domestic cat from estrus to mid-pregnancy were similar to events observed in females experiencing a non-mated, prolonged luteal phase (Biol. Reprod. 25: 15-28, 1981). However, serum progesterone levels were elevated in pregnant cats for extended durations at least equivalent to and sometimes longer than the pregnancy interval itself. It was unclear whether this additionally produced progesterone was of placental or ovarian origin, the latter possibly supplemented in some cats through the formation of accessory corpora lutea. It was apparent that the suckling stimulus had an inhibitory influence on tonic gonadal steroid and luteinizing hormone secretion during lactation which was alleviated by the weaning event. Postweaning elevations in estradiol and luteinizing hormone were then evident with resumption of sexual behavior occurring within 4 weeks. However, normal ovarian-hormonal-behavioral relationships appeared temporarily compromised with some females demonstrating a delayed or abbreviated postpartum estrus, ovulation failure and reduced post-ovulatory elevations in progesterone.

2. Ovulation induction. The domestic cat normally ovulates reflexly with multiple copulatory stimuli eliciting one or more surges of luteinizing hormone and then ovulation. These normal endogenous fluctuations in hormones can be either circumvented or artificially stimulated by the administration of exogenous gonadotropic hormones. Comparative testing of various drug preparations demonstrated that follicle stimulating hormone from a porcine extract (FSH-P)

was effective in stimulating vesicular follicle formation on ovaries sufficient to induce complementary sexual receptivity. Furthermore, the potential hyperstimulative (superovulatory) effect of this drug was remote even though FSH-P's potency was sufficient to overcome elevated progesterone titers and induce ovulation in females during mid-pregnancy. Comparative testing of FSH-P was conducted in various nondomestic felids. Ovulation was successfully induced in a high proportion of cheetahs, a moderate proportion of leopards and in a low proportion of tigers. The data suggested the potential of species specificity in response to a given hormonal regimen.

3. Ejaculate-endocrine norms. Standard ejaculate traits were established for the male domestic cat including information on spermatozoal concentration, motility and morphology. For comparative purposes, seminal traits were analyzed in a similarly treated nondomestic species, the cheetah. Striking differences were noted between the 2 Felidae, with the cheetah producing an ejaculate of inferior quality compared to the laboratory cat. Allozyme and 2-dimensional gel electrophoretic analyses demonstrated a marked lack of genetic variation in the cheetah, a finding which may be related to the poor reproductive potential in this species.

Analyses of circulating concentrations of testosterone, cortisol and progesterone permitted study of testes-adrenal relationships and the potential stress effects of routine laboratory procedures including anesthesia and electroejaculation. The latter procedures induced significant elevations in cortisol and progesterone with a simultaneous depressive influence on serum testosterone concentrations. A similar temporal response in altered adrenal hormone secretion was observed in other Felidae, including the cheetah and puma. However, the magnitude of the stress response in terms of absolute cortisol concentrations was species specific, with some felid groups apparently exhibiting greater stress susceptibility. A similar finding was evident in terms of androgen production, with Felidae species varying considerably in basal testosterone secretion.

Significance to Biomedical Research and the Program of the Institute:

Because the domestic cat plays a valuable role in the study of cancer, the continued availability of study animals with suitable genetic backgrounds is of utmost importance. Availability of any animal model can be ensured by the comprehensive understanding of reproductive processes. The investigation of reproductive-endocrine relationships establishes a baseline foundation which will eventually permit definitive control of reproductive function, including the perpetuation of species by artificial breeding. Achieving the latter goal will not only have application to propagating animal models for human disease but will be of significance in preserving endangered species.

Proposed Course:

Continued efforts will emphasize (1) hormonal control of testes function in the male cat; (2) artificial insemination of selected females experiencing natural or hormonally induced estrous activity; and (3) genetic relationships to the phenomenon of induced versus spontaneous ovulation.

Publications

Chakraborty, P. K., Wildt, D. E. and Seager, S. W. J.: Induction of estrus and ovulation in the cat and dog. Vet. Clin. N. Am. 12: 85-91, 1982.

Chan, S. Y. W., Chakraborty, P. K., Baas, E. J. and Wildt, D. E.: Ovarian-endocrine-behavioral function in the domestic cat treated with exogenous gonadotropins during mid-gestation. J. Reprod. Fertil. 65: 395-399, 1982.

O'Brien, S. J., Wildt, D. E., Goldman, D., Merril, C. R. and Bush, M.: The cheetah is depauperate in genetic variation. Science (In Press)

Phillips, L. G., Simmons, L. G., Bush, M., Howard, J. G. and Wildt, D. E.: Gonadotropin regimen for inducing ovarian activity in captive wild felids. J. Am. Vet. Med. Assoc. 181: 1246-1250, 1982.

Schmidt, P. M., Chakraborty, P. K. and Wildt, D. E.: Ovarian activity, circulating hormones and sexual behavior in the cat. II. Relationships during pregnancy, parturition, lactation and the postpartum estrus. Biol. Reprod. 28: 657-671, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05335-01 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Reproductive-Endocrine Relationships in the Inbred Miniature Pig

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

D. E. Wildt Senior Staff Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

Clemson University, Clemson, SC

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In 1972, the National Institutes of Health in collaboration with the Immunology Branch of the National Cancer Institute initiated a program to develop a highly inbred animal model suitable for tissue transplantation. The miniature pig was used and from its inception the experimental herd traditionally experienced conception rates and litter sizes considerably less than values reported for outbred, standard-sized pigs. To determine the causes of poor fertility and reduced reproductive performance, ovarian-endocrine relationships have been characterized in the miniature pig by studying reproductive hormonal profiles and ovarian morphology. The data have suggested that the reduced litter size in this particular strain is due to a deficient ovulation rate, potentially related to genetically altered endocrine function. Furthermore, ovulation number can be improved by gonadotropic therapy used in concert with synthetic steroids for synchronizing the reproductive cycle. Viable appearing embryos are detected following copulation; however, conception rates are reduced, probably due to a high incidence of cystic endometrial hyperplasia. As a consequence, this strain of miniature pig has proven to be a model for studying the adverse effects of increased homozygosity on altered reproductive/endocrine function and intra-uterine environment.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

L. D. Stuart	Head, Ungulate Unit	VRB, DRS
D. H. Sachs	Chief	IB, NCI

Objectives:

To establish ovarian-endocrine relationships and the causes of poor reproductive performance in an inbred strain of miniature pig.

Methods Employed:

In initial studies, adult female pigs were monitored daily for sexual behavior, and blood samples were obtained frequently throughout the estrous cycle and analyzed by radioimmunoassay for estradiol, progesterone and luteinizing hormone. Laparoscopy was performed frequently throughout the cycle and ovarian morphology described and photographed. A subsequent study was conducted to test the efficacy of a synthetic progesterone (allyl trenbolone) for estrous synchronization and the effectiveness of exogenous gonadotropins (pregnant mares' serum gonadotropin and human chorionic gonadotropin) for increasing ovulation rate. In this experiment, females were mated, subjected to surgery, the reproductive organs examined and early-stage embryos recovered by media flushing of the uterine horns.

Major Findings:

Determination and regulation of ovarian performance in the inbred miniature pig. Behavioral cyclicity patterns in the inbred miniature pig were similar to previous reports for outbred, standard-sized animals. However, the numbers of ovulations per female were markedly less than values published earlier for standard-sized females. Although temporal relationships in endocrine patterns were normal, serum titers of luteinizing hormone were elevated and progesterone depressed. The data were strongly indicative that the lack of genetic variation in this particular animal model had adversely influenced ovarian function probably through an indirect effect on the pituitary.

Ovarian performance, including ovulation rate, could be improved following estrous synchronization with allyl trenbolone and post-treatment therapy with pregnant mares' serum gonadotropin. Following copulation, resulting embryos appeared morphologically normal and viable based on successful in vitro culture for up to 72 hours after collection. However, these more recent studies also showed that uterine tissue integrity appeared adversely affected in the miniature pig. A high percentage (90%+) of females were diagnosed to be afflicted with cystic endometrial hyperplasia, some to the degree that maintenance of a pregnancy to term would be remote. The unique condition of the uteri was not determined to be related to any drug therapy.

Significance to Biomedical Research and the Program of the Institute:

The inbred miniature pig serves as an invaluable animal model in studies of histocompatibility and tissue transplantation. The present studies have demonstrated a biological link between the monomorphic genotype of this particular strain and reproductive potential, probably as a result of altered pituitary function. As a consequence, ovulatory activity is depressed and uterine integrity is disrupted; the etiological mechanism of the latter finding is still unknown. In addition to providing improved understanding of genetic-reproductive relationships, these studies provide impetus to further investigate methods for propagating the miniature pig to ensure adequate numbers of this model for future research use.

Proposed Course:

Further studies are planned to (1) evaluate the effect of cystic endometrial hyperplasia on embryonic viability in utero; (2) assess litter size in females treated with exogenous gonadotropins for increasing ovulation numbers; and (3) determine the potential of surgical transfer of miniature pig embryos to standard-sized recipients.

Publications:

Howard, P. K., Chakraborty, P. K., Camp, J. C., Stuart, L. D. and Wildt, D. E.: Correlates of ovarian morphology, estrous behavior and cyclicity in an inbred strain of miniature swine. Anat. Rec. 203: 55-65, 1982.

Howard, P. K., Chakraborty, P. K., Camp, J. C., Stuart, L. D. and Wildt, D. E.: Pituitary-ovarian relationships during the estrous cycle and the influence of parity in an inbred strain of miniature swine. J. Anim. Sci. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05336-01 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Developmental Studies in Manipulation and Freezing of Mouse and Cat Embryos

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

D. E. Wildt Senior Staff Fellow, LVC, NCI

COOPERATING UNITS *(if any)*

Veterinary Resources Branch, DRS, NIH, Bethesda, MD; Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The use of exogenous gonadotropic hormones has permitted the artificial induction of sexual activity and ovulation in the laboratory mouse and cat. Embryonic material from the former species is being applied to developmental studies permitting in vitro culture, manipulation, chronic cryopreservation of various inbred strains and the eventual recovery of such material for rederiving valuable models for future investigative research. Mouse embryos, freeze preserved in liquid nitrogen, thawed after 2 years storage and transferred into recipient females, have resulted in healthy, normal offspring. Technological advances in the mouse system are being applied and extended to the laboratory cat, a species previously shown to be an excellent model for the study of leukemia and sarcoma. Feline embryos at the morula stage of development have been collected and successfully cultured to the advanced blastocyst state in vitro. Methods for micromanipulation of the embryo will eventually be developed to allow microinjection of molecularly cloned genes which participate in transformation and inborn errors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

P. M. Schmidt	Biological Lab. Tech.	VRB, DRS
K. L. Goodrowe	Guest Researcher	VRB, DRS
C. T. Hansen	Geneticist	VRB, DRS
S. J. O'Brien	Geneticist	LVC, NCI

Objectives:

(1) To optimize methods to effectively collect, freeze preserve and transfer thawed embryonic material from inbred strains and outbred stocks of mice.
(2) To adapt and extend technologies of the mouse system to the laboratory cat, eventually allowing routine culture and transfer of normal and genetically altered feline embryos.

Methods Employed:

The following techniques were employed: (1) hormonal induction of ovulation and sexual behavior using gonadotropic therapy; (2) surgical procedures for embryo collection and transfer; (3) in vitro culture; and (4) dimethyl sulfoxide dilution and slow freezing techniques in an automated, programmable freezer unit.

Major Findings:

1. Cryopreservation of murine embryos varies with the mouse strain. A prerequisite to the cryobanking of embryonic material is the development of (1) successful ovulation induction regimens for any given mouse strain or stock and (2) laboratory skills for collection, handling, culturing and transferring embryos. Numerous drug therapies were tested with the mouse being most responsive to pregnant mares' serum gonadotropin for stimulating ovarian follicle development and human chorionic gonadotropin for inducing ovulation. Optimal results were obtained as follows: 4- to 8-cell oviductal embryos were frozen in a 1 M solution of dimethyl sulfoxide in phosphate buffered saline at a rate of $-0.5^{o}C$/minute to $-80^{o}C$ and then plunged into liquid nitrogen. Approximately 600 embryos were collected/week from 5 different mouse strains. Post-thaw survival rates of frozen embryos generally averaged 25%; however, the degree of recovery was highly dependent on the mouse strain employed. A total of 10 viable pregnancies have resulted from transfer of thawed embryos into the uterine horns of pseudopregnant recipient females. Although the described techniques were effective in cryopreserving mouse embryonic material, it was evident that considerable mouse strain/stock differences exist with respect to embryo recovery rates following both ovulation induction and freeze preservation. As such, this work continues to illustrate the complexity of freeze storing gametic or embryonic material and emphasizes that standard laboratory procedures useful in one mouse strain may require slight modification for successful use in related strains.

2. Development of methodology for retrieval of viable feline embryos. The mouse has served as a useful model in initiating developmental biology investigations in the domestic cat. Ovarian activity and sexual behavior including copulatory activity was successfully induced in approximately 75% of anestrous cats treated with follicle stimulating hormone. Embryos were collected surgically from both the oviductal and uterine regions of the reproductive tract and have developed in vitro from the morula to blastocyst stage when cultured in Hams-F10 media supplemented with fetal calf serum. These preliminary efforts have demonstrated the feasibility of hormonally controlled breeding and retrieval of viable embryonic material from the domestic cat.

Significance to Biomedical Research and the Program of the Institute:

The collection, manipulation, and transfer of embryonic material is finding increasing application in a variety of disciplines, particularly in biological research. The preservation of embryonic cells by freezing offers convenience, biological insurance against genetic drift and economical advantages. Embryo preservation provides a means by which a strain or species can be stored indefinitely with the potential to establish breeding populations at a later time.

Although these techniques in developmental biology offer considerable benefit, such methods, as well as more recent innovations in micromanipulation, have been restricted primarily to use in mouse and bovine systems. Our proposed adaptation of such technology to the domestic cat serves several major purposes. First, baseline information on embryonic morphology and culture requirements for felids in general is nonexistent. The ability to routinely transfer embryonic material from one domestic cat to another or to a close relative in the genus Felis would provide valuable mechanistic data on uterine compatibility within and among species and have considerable importance in the propagation of ecologically endangered Felidae. Of further significance, the availability of a feline embryo system would eventually permit the adaptation of micromanipulatory techniques currently in use in the mouse. Of particular interest would be the microinjection of molecularly cloned genes, which participate in transformation and inborn errors, into appropriately staged cat embryos which might then be transferred into recipient females. The experimental tracking of the fate of the gene implants would be the beginning of possible gene therapy for genetic diseases and cancer in an animal model system.

Proposed Course:

Continued efforts are to be made to improve techniques in both the mouse and cat systems. In the former, emphasis will be placed on analyzing the influence of genetic differences among strains on embryo recovery efficiency following ovulation induction and freeze storage. Most efforts in the domestic cat will initially strive to further establish species norms and successfully achieve sustained pregnancies following intra-animal embryo transfers. Plans also are being made to initiate a micromanipulation program adapting technologies to be developed in the mouse to our feline system.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05337-01 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of MCF-MuLVs Generated In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*

(Name, title, laboratory, and institute affiliation)

G. E. Mark Expert, LVC, NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mink cell focus-forming murine leukemia virus (MCF-MuLV) is a recombinant between ecotropic and endogenous xenotropic-related MuLV sequences. Some MCF viruses accelerate leukemia development upon intrathymic inoculation into young mice of their strain of origin. To determine the exact nature of the env sequences in the recombinant MCF CI-3 virus acquired in vitro, and to more precisely define the extent of recombinational exchange, as well as the deletion in another MCF virus (CI-4), the envelope gene sequences were determined and compared with those of ecotropic, xenotropic, and dualtropic MuLVs. Comparison of these sequences revealed several important features. (1) The extent of recombinational exchange in CI-3 is from 145 nucleotides 3' of the splice acceptor site for the envelope mRNA to nucleotide 1722, between the end of gp70 and the beginning of Prp15E. (2) Comparison of the deduced amino acid sequence of the CI-3 MCF gp70 with that of ecotropic gp70s reveals extensive conservation of the primary sequence in the carboxy half of the molecule, as well as in a centrally located polyproline domain. Two regions of substantive differences are found on either side of a region of partial homology, suggesting that either or both of these two variable regions may encode the receptor specificity and, consequently, the host range of these viruses. A nonidentity of xenotropic and MCF gp70s was observed, suggesting that xenotropic MuLVs are not the nonecotropic parent of the env gene of MCF-MuLVs. (3) The deletion present in the replication-defective CI-4 virus is bordered by an 11-nucleotide direct repeat in CI-3 viral DNA. (4) Comparison of the substituted sequence in CI-3 with that of Moloney (Mo)-MCF suggests a very close relationship, if not identity, between the endogenous dualtropic proviruses from which they were derived. Two major mechanisms for the formation of MCF-MuLVs may be envisioned; one involves recombination between unintegrated DNA intermediates representing an exogenous viral genome and an endogenous dualtropic proviral sequence. The sequence data is consistent with the env gene recombination event proceeding through the formation of H structures.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Ulf R. Rapp Visiting Scientist LVC, NCI

Objectives:

We have reported previously the isolation and molecular cloning of mink-cell focus-forming murine leukemia virus (MCF-MuLV) from cultures of iododeoxyuridine (IdUrd)-induced C3H/10T1/2 cells. One of the molecular clones, CI-3, induced anchorage-independent growth of epithelial mink lung cells in vitro. This latter activity required the presence of nontransforming helper MuLV and epidermal growth factor (EGF). Unintegrated DNA intermediates present in cultures of epithelial mink lung cells transformed in vitro were also the source of the molecular clone CI-4, a variant form of CI-3 virus, which contained a deletion in the p15E region of the envelope gene, as does spleen focus-forming virus (SFFV), another pathogenic MCF-MuLV.

The altered env gene products of MCF-MuLVs have been implicated as determinants of pathogenicity for both thymoma-inducing and erythroleukemia-inducing isolates. The viral envelope gene codes for two membrane proteins, gp70 and p15E, which are proteolytically cleaved from a glycosylated precursor (Prgp85). While both ecotropic MuLV envelope gene products have been obtained and partially sequenced, little information is available for MCF viral envelope proteins. To determine the exact nature of the env sequences in CI-3 acquired in vitro and to more precisely define the extent of recombinational exchange in CI-3, as well as the deletion in CI-4, we have determined the envelope gene sequence from CI-3 and CI-4 and compared it with that of other ecotropic, xenotropic and dualtropic MuLVs.

Methods Employed:

The isolation, molecular cloning and subcloning in pBR322 of the MCF-MuLV genomes pCI-2, pCI-3 and pCI-4 from epithelial mink lung cells transformed in vitro have been previously described by Dr. Rapp. DNAs (~10 µg) to be sequenced were digested with the appropriate restriction endonuclease and labeled using either T4 polynucleotide kinase or terminal deoxynucleotide transferase as described by Maxim and Gilbert. DNA fragments containing uniquely labeled ends were isolated from Seaplaque (Seakem) agarose gels. Products were separated in 8% or 20% polyacrylamide - 8 M urea sequencing gels, 0.4 mm thick, and visualized by autoradiography. DNA sequences were compiled and analyzed for protein sequence by the program of Queen and Korn.

Major Findings:

1. Structural characterization of the CI-3 DNA molecular clone. Comparison of these sequences with those of ecotropic AKR, Moloney (Mo) and Friend MuLVs, as well as with Mo-MCF, revealed several important features. (1) The extent of recombinational exchange in CI-3 is from 145 nucleotides 3' of the splice

acceptor site for the envelope mRNA to nucleotide 1722, between the end of gp70 and the beginning of Prp15E. No evidence for additional recombinational alteration was obtained. (2) The env gene substitution in CI-3 was virtually identical in sequence (18 changes out of 1158 nucleotides) to that present in Mo-MCF. The major difference was the location of the 3' MCF/ecotropic junction. (3) Comparison of the deduced amino acid sequence of the CI-3 MCF gp70 with that of ecotropic gp70s reveals extensive conservation of primary sequence (ecotropic-like carboxy half of the molecule) as well as a centrally located polyproline domain which may confer a flexible -helical conformation to this region. Two regions of substantive differences are found on either side of the region of partial homology. The CI-3 MCF gp70 sequence contains an additional glycosylation site and an insertion relative to the ecotropic gp70 sequence. Relative to the MCF sequences in CI-3, the ecotropic gp70 contains a polyproline-rich insertion upstream of the partial homology region and an additional glycosylation site downstream. The similarities seen throughout the remainder of the envelope gene suggest that either or both of these two variable regions may encode the receptor-specificity and consequently the host range of these viruses. The predicted amino acid sequence of a xenotropic MuLV was 87% homologous to that of CI-3, with the major differences residing in the twenty-five to thirty amino acids on either side of the region of partial homology. These changes may interfere with the penetration of mouse cells by xenotropic MuLVs. The nonidentity of xenotropic and MCF gp70s also suggests that xenotropic MuLVs are not the non-ecotropic parent of the env gene of MCF MuLVs. (4) The deletion present in the replication-defective CI-4 virus is bordered by an 11-nucleotide direct repeat in CI-3 viral DNA and may, therefore, be the result of either slippage during reverse transcription or homologous recombination within or between CI-3-like DNA proviruses. (5) The entire gp70 in CI-3 was derived from an endogenous dual-tropic provirus and the exchange during recombination was initiated and terminated within conserved sequences showing considerable (90% at the 5' border) or at least notable (80% at the 3' border) homology between endogenous and exogenous envelope genes. The sequence of the Prp15E portion of the env gene was probably derived from the ecotropic parent and not from the endogenous dualtropic provirus. Comparison of the substituted sequence in CI-3 with that of Mo-MCF suggests a very close relationship, if not identity, between the endogenous dualtropic proviruses from which they were derived. Thus, it appears that one specific provirus out of a large number of endogenous nonecotropic MuLVs is used for the formation of a recombinant MCF-MuLV, independent of whether the recombination occurs in lymphoid tissue in vivo or in fibroblast cells in vitro, or even in different mouse strains (C3H/He versus Balb/Mo). The close relatedness of MCF gp70 sequences in different MCF-MuLV is consistent with the finding that several independent isolates of MCF-MuLV appear to utilize the same cell surface receptor for infection. (6) Two major mechanisms for the formation of MCF-MuLVs may be envisioned. The data obtained here suggest that the recombination event proceeded through the formation of H structures during reverse transcription of heterozygous genomic RNAs. This displacement-assimilation mechanism is consistent with the envelope sequences of the pCI-2 and pCI-3 DNAs and their comparison to the Mo-MCF envelope sequences. Utilizing a procedure which we have shown generates MCF recombinants in vitro, we are now in a position to test some of the predictions of this and other recombination models in vitro.

Significance to Biomedical Research and the Program of the Institute:

Several mechanisms may create the apparent immortalization of differentiated cells. One such scheme may involve the cooperative interaction of exocrine and autocrine components with specific cellular receptors. The expression of endogenous retroviral sequences (the transmembrane envelope gene product) may mimic one of these components and result in the clonal expansion of the productive cells. This may represent the pre-metastatic phase of some malignancies.

Proposed Course:

The initial observation of in vitro transformation by the CI-3 MCF in conjunction with EGF will be re-examined now that the MCF has been characterized. Two areas will be investigated: (1) the role of βEGF cellular receptors and (2) the requirement for specific regions of the env gene.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05338-01 LVC

PERIOD COVERED

October 1, 1982 to September 30, 1983

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization, Mode of Action, and Evolution of the Oncogene raf

PRINCIPAL INVESTIGATOR *(List other professional personnel on subsequent pages.)*
(Name, title, laboratory, and institute affiliation)

G. E. Mark Expert, LVC, NCI

COOPERATING UNITS *(if any)*

Laboratory of Molecular Virology and Carcinogenesis, Basic Research Program, Litton Bionetics, Inc., Frederick, MD 21701

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new replication-defective, acute transforming retrovirus (3611-MSV) was recently isolated from mouse and molecularly cloned. Two gag-containing polyproteins (P75 and P90) are found in nonproducer transformed cells. The nucleotide sequence of 1.5 kilobases encompassing the transforming gene (v-raf) of 3611-MSV has been determined. v-raf sequences were found to have been inserted into the p30 region of an ecotropic murine leukemia virus (MuLV), with the concomitant deletion of the 2.4 kilobases extending to the middle of the polymerase gene. A 5-nucleotide direct repeat exists at each end of the v-raf sequences. A single nucleotide deletion, 10 bases upstream from the acquired sequences, places the oncogene in an open reading frame terminated by an amber triplet around 180 nucleotides from the 3' onc/MuLV junction. From the deduced amino acid sequence, a hybrid gag-raf polyprotein would have a molecular weight of around 75 kilodaltons. Consistent with the gag-x structure, we find that only the P75 polyprotein is modified by the fatty acid myristate, whereas only the P90 polyprotein is glycosylated. Comparison of the deduced v-raf amino acid sequence with other oncogenes revealed domains homologous to v-src and/or v-mos. c-raf-related sequences have been localized to human chromosome 3, a chromosome whose alterations are associated with small cell lung carcinoma and other familiar carcinomas. Human cells derived from small cell carcinomas express v-raf-specific RNA, as do other established human cell lines. Further characterization of v-raf specific proteins and mRNA will be presented.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel (other than the Principal Investigator) Engaged on this Project:

Ulf R. Rapp Visiting Scientist LVC, NCI

Objectives:

Activated cellular oncogenes are involved in tumorigenesis; perhaps proximally, perhaps distally. The recognition, isolation, and characterization of these potentially deleterious, cellular sequences will ultimately lead to an understanding of the biochemical functions which drive a cell to cancerous growth. Most oncogenes have been highly conserved through evolution since the appearance of metazoan organisms, hence the belief that their role in normal cellular development and differentiation is vital. Of the various ways of identifying transforming genes, transduction of such sequences by retroviruses is the most reliable. Recently, several new transforming, replication-defective retroviruses have been biologically isolated by Dr. Ulf R. Rapp. Initial characterization of one of these isolates (3611-MSV) revealed it to contain a partially deleted structural gene (gag) fused to a nonviral sequence. This alteration resulted in a virus which induced fibrosarcomas 4 weeks after the infection of newborn mice. The objectives of this project are three-fold: (1) to isolate, molecularly clone and nucleic acid sequence the transduced oncogene, as well as define the physical boundaries of the gene which limits its transforming activity; (2) to discern its mode of action from studies of its intracellular localization, its required protein domains, and its evolutionary ancestors; and (3) to identify its roles in normal cellular functions and human malignancy.

Methods Employed:

The integrated 3611-MSV provirus was detected in a transformed cellular clone utilizing a nick-translated env gene probe and standard Southern blot procedures. Preliminary cloning of the provirus and cellular flanking sequences was into the EcoRI site of λ WES. λ B. Subsequent subcloning was into appropriate sites of pBR322. Nucleic acid sequencing protocols described by Maxim and Gilbert for the chemical cleavage of terminally radiolabeled oligonucleotides were employed. Site-specific mutagenesis mediated through the controlled resectional activity of the exonuclease BAL-31 was designed and performed by the author on subclones of raf-containing fragments. The biological activity of these mutants (transforming activity) was determined by DNA transfection of NIH/3T3 cells using a modification of the calcium phosphate precipitation procedures of Graham and Van der Eb. RNAs were extracted from cultured cells utilizing phenol heated to 65 C and poly-A-containing transcripts were selected by dt-cellulose chromatography. Analysis of RNAs was either quantitative (dot blotting samples on to nitrocellulose membranes followed by hybridization with a nick-translated ^{32}P-labeled probe) or qualitative (electrophoretic separation of RNA species, their transfer to DBM-paper and detection through hybridization with specific, radiolabeled probes). Synthetic polypeptides were purchased from Peninsula Laboratories, Inc. (San Carlos, CA), covalently attached to a carrier protein (KLH) and inoculated

into rabbits in collaboration with Dr. Stephen Oroszlan. Metabolic labeling and electrophoretic separation of viral-specific proteins were performed by Dr. Alan Schultz. A Drosophila melanogaster genomic library made in Charon 4A was obtained from Dr. Tip Benyajati (LBI) and screened for raf oncogene-containing phage by conventional methods after reducing the stringency of hybrid selection. Data was analyzed with the aid of the computer facilities at the Frederick Cancer Research Facility.

Major Findings:

1. Characterization of v-raf, a new oncogene. A novel, acutely transforming replication-defective retrovirus (3611-MSV) was isolated and characterized. DNA sequence determination of the acquired oncogene (v-raf) and its flanking viral elements has provided essential information pertaining to its acquisition, composition, and mechanism of action. Until the analysis of 3611-MSV, the recombinant parent of transforming retroviruses was unknown. The parent of 3611-MSV was an ecotropic murine type C virus; that is, no prerequisite recombinational events need occur prior to the transduction of the cellular oncogene. 3611-MSV acquired its transforming sequences by the substitution of 2.39 kb of viral structural information with 1.49 nucleotides of cellular oncogene. Interestingly, this oncogene adoption occurred in a w that precluded its expression (it entered into a non-translatable reading frame). Thus, the oncogenic potential of this recombinant was carried cryptically in the virus stock until a nucleotide deletion shifted the transforming sequences into the proper reading frame. Deletion mutants, constructed using BAL-31 exonuclease, (see figure 1) indicated that the active portion of the transforming gene was contained within 890 nucleotides, 35 nucleotides from its 5' point of acquisition. Two polyproteins were detected in transformed cells, both represented the translation product of the molecularly fused viral gag gene and the raf oncogene, terminating at an amber codon within the cellular sequences. The larger protein (90 kilodaltons) contains a leader peptide, is glycosylated, and is intimately associated with cytoskeletal elements. The smaller protein (75 kilodaltons) is uniquely modified at its amino terminus by the post-translational addition of the fatty acid myristate. This is believed to be the active form of the polyprotein. To determine the intracellular localization of the v-raf protein (or c-raf protein from normal cells) three polypeptides were synthesized, coupled to KLH and used to immunize rabbits. The reactive antisera which was obtained has not been tested yet. The amino acid sequence, predicted from the nucleic acid sequence of v-raf, revealed a close relationship between the members of the tyrosine kinase-oncogene superfamily and v-raf (see figure 2). The fact that the later enzymatic activity is not demonstratable in 3611-MSV-transformed cells suggests that tyrosine phosphorylation is not essential for oncogenicity. Family comparisons now allow for the prediction of which sequences are related to the kinase activity and which are necessary for transformation. Of a unifying interest is the observation that one of these latter sequences is nearly identical to the presumed active site of the ras family of oncogenes.

Like many oncogenic sequences, raf-related RNA is found in uninfected cells. The role of these sequences in human malignancy is not understood. Preliminary experiments indicate that cells derived from human lung carcinomas express large amounts of raf-related RNA (>1000 copies/cell). The qualitative analysis of these RNAs is in progress.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of the novel retroviral transduced oncogene v-raf has resulted in the re-evaluation of the mode of action of the tyrosine kinase-oncogene superfamily. This will result in a fresh assault on the mechanisms of carcinogenesis mediated through these highly conserved sequences. Ultimately, the nature of the biochemical functions which drive a cell to cancerous growth will be discerned.

Proposed Course:

The expression of raf-related RNA sequences in human malignancies and the relationship of this potential cellular oncogene to neoplastic transformation will be investigated. Clones derived from cDNA copies of "normal" and "transformed" c-raf RNA will be analyzed for transforming activity by transfection. If an activated c-raf gene is transforming, its sequence will be compared with that of its "normal" counterpart.

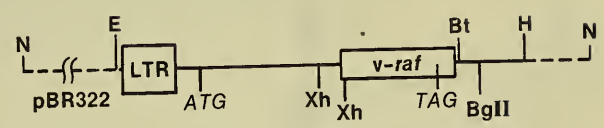
Experimental approaches directed toward uncovering a mechanism of carcinogenesis will be facilitated by employing the genetic tools available in Drosophila and yeast biology. The latter system holds the most promise since genes may be shuttled in and out of yeast by homologous recombination. Thus, the environment of a specific gene is not altered as a consequence of manipulation. v-raf-related sequences are presently being cloned from Drosophila. Similar technologies will be used to obtain the homolog in yeast.

Publications:

Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr. and Stephenson, J. R.: Structure and biological activity of v-raf, a novel oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA (In Press)

Figure 1

Bal-31 Resection of v-*raf*



Transformation	Clone #	Region Deleted
+	E-H	
+	3-1	
+	3-5	
+	7-5	
+	4-12	
–	4-6	
–	4-7	
–	7-1	
–	A1,5,8	
–	A17,23	
–	A3,16,21	
+	X-X	

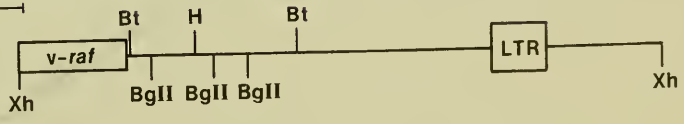
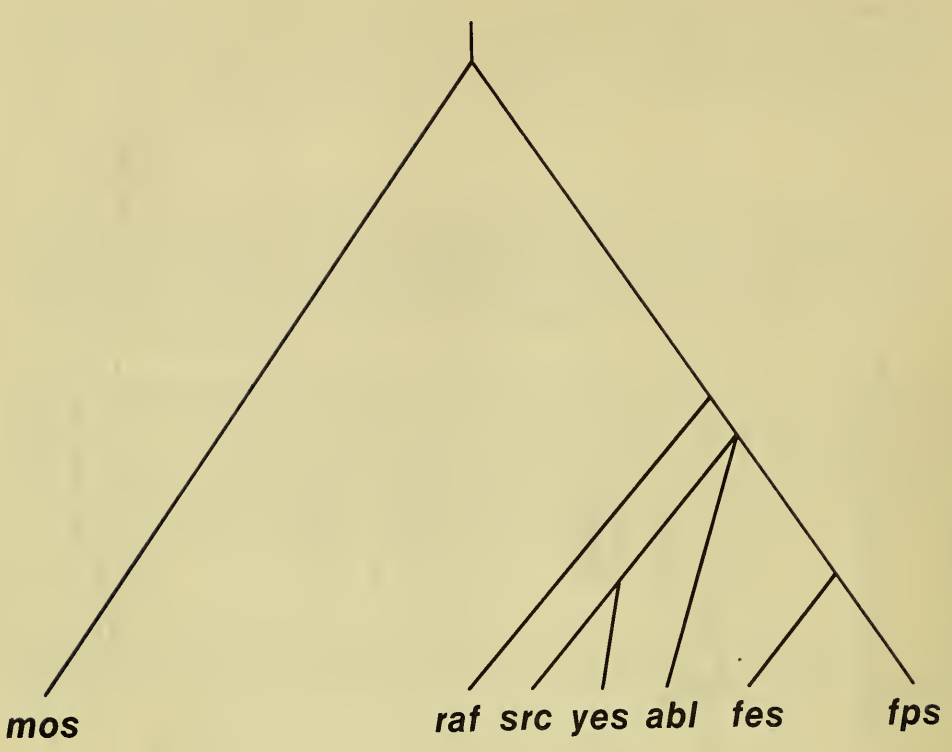


Figure 2



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