

ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL CANCER INSTITUTE,
Division of Cancer Cause and Prevention
Volume I

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ANNUAL REPORT
DIVISION OF CANCER CAUSE AND PREVENTION

NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

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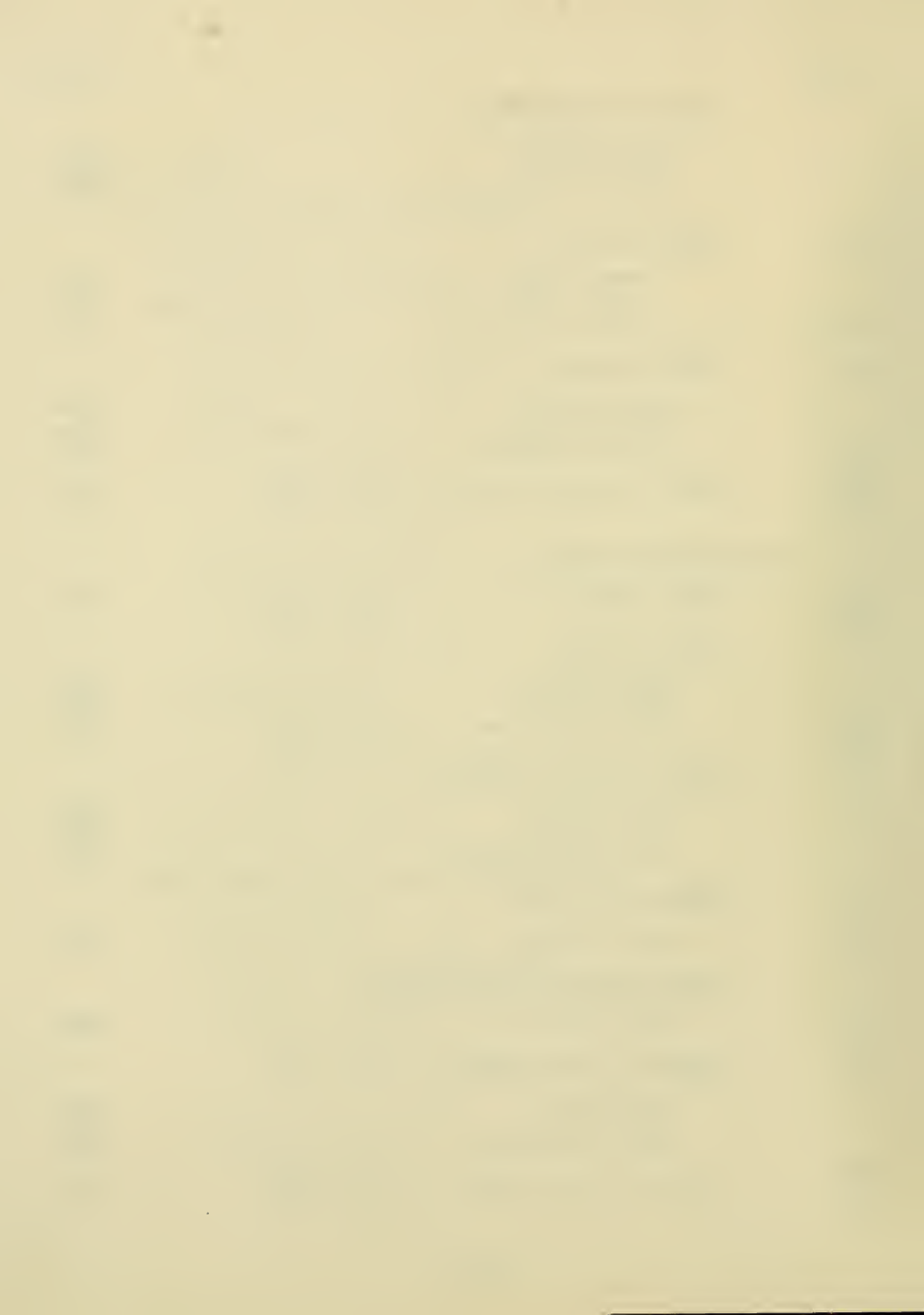
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ANNUAL REPORT

DIVISION OF CANCER CAUSE AND PREVENTION

Richard H. Adamson, Ph.D., Director

October 1, 1981 through September 30, 1982

OVERVIEW

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 this year; the organizational components of this program are now the Biometry Branch, the Clinical Epidemiology Branch and the Environmental Epidemiology Branch. The Experimental Oncology Section of the Laboratory of Cellular and Molecular Biology was site visited and is in the process of being transferred to the Division of Cancer Biology and Diagnosis. Also, an effort has been made 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 19.3% and that for Field Studies and Statistics was 16.3% of the total DCCP budget. The current organizational chart for DCCP is shown in Figure 1.

The extramural component, 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 the Division completed the movement of the Laboratory of Experimental Pathology and a new laboratory, the Laboratory of Comparative Carcinogenesis, to the Government-owned facilities at the Frederick Cancer Research Facility (FCRF).

During the coming year, the Division proposes to consolidate the Laboratory of Cellular and Molecular Biology in Building 37 and to move the Laboratory of Chemoprevention to Building 41. 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 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 three 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 last year 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. It is anticipated that the next cycle of site visits will begin in the Spring 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 mechanisms of biological and chemical prevention of carcinogenesis and the role of tumor promoters, hormones and other cofactors in human cancer causation.

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 and an increased use of RFAs to stimulate research activity in high priority areas.

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

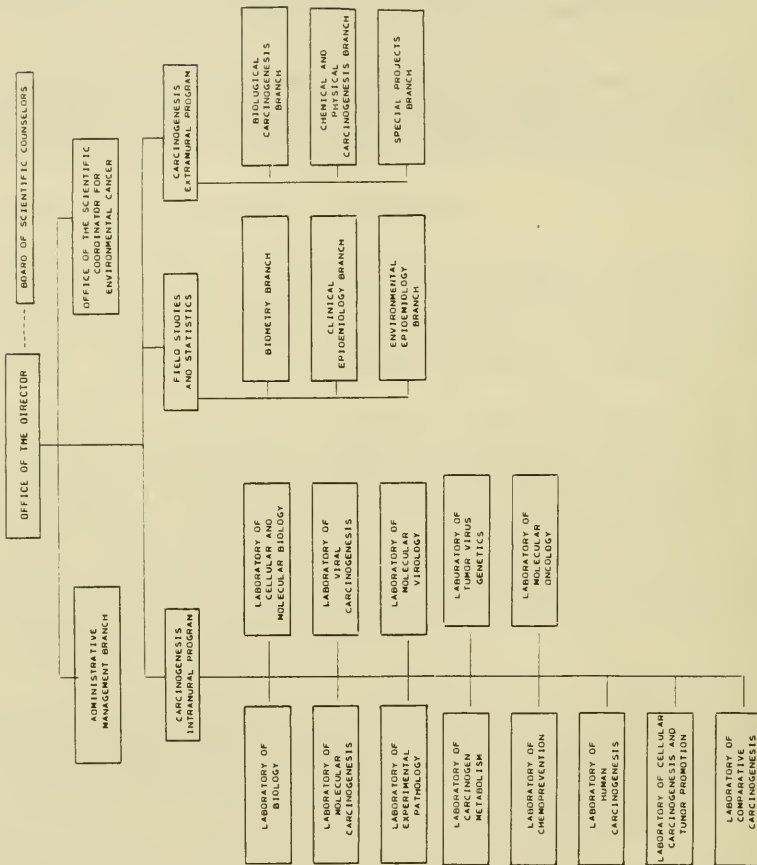


Table 1

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 1982 Estimate

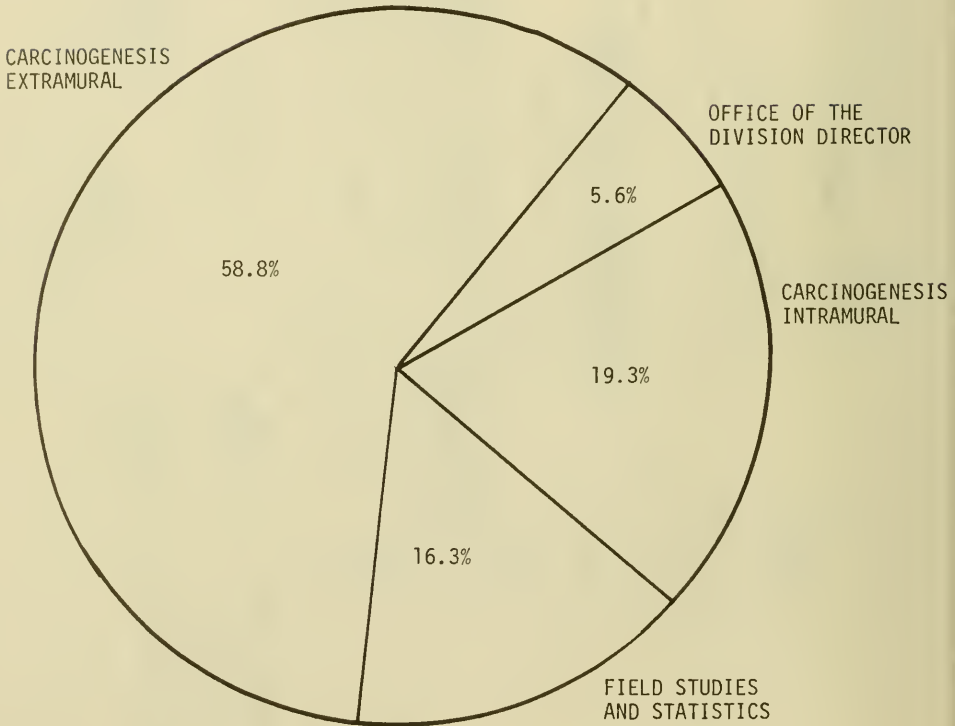
	<u>Office of the Division Director</u>	<u>Carcinogenesis Intramural</u>	<u>Carcinogenesis Extramural</u>	<u>Field Studies and Statistics</u>	<u>Total</u>
Intramural	3,388	26,809	1,603	8,661	40,461
Contracts	7,447	12,556	16,415	24,626	61,044
CREG/RFA			5,937		5,937
Cooperative Agreements	446				446
Preventive Oncology			607		607
Research Project Grants			95,209		95,209
Total	11,281	39,365	119,771	33,287	203,704

Figure 2

NATIONAL CANCER INSTITUTE
DIVISION OF CANCER CAUSE AND PREVENTION

Current Distribution of Funds

FY 1982 Estimate



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 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 transformation 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 in the genes of normal cells and 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 vitamins and trace elements 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

During the past several years, a revolution has occurred in terms of our ability to acquire knowledge of the biological processes responsible for cancer. These remarkable achievements have occurred as a result of technological breakthroughs in research disciplines that are generally referred to as recombinant DNA and hybridoma-monoclonal antibody technology. These combined technologies have provided a first glimpse of dominant cellular genes responsible for human cancer and sensitive immunological reagents both for detecting the products of these genes in tumor cells and assessing their influence on cellular targets. These technologies have accelerated the rate at which we are acquiring a new and greater understanding of the mechanisms of oncogenesis. This new information can soon be expected to have a profound impact on clinical diagnostic procedures.

The study of cancer viruses has provided a direct means for investigating cellular genes with oncogenic potential. New methodologies have made it possible to isolate these dominant transforming genes from human tumor cells. This approach has been most productive in identifying the genetic elements involved in cancer. It is also evident that the methodologies developed from the study of cancer viruses are being recruited by all investigators studying the molecular basis of neoplastic disease. The following describes how retroviruses have provided new insight into the cancer process.

The study of retroviruses has shown that they are unique among animal viruses in their mode of transmission and in the intimate association that has evolved between these agents and their hosts. Retroviruses are common to a large number and wide variety of vertebrate species. Certain members of this group, the acute transforming retroviruses, arose by recombination of viral genes with cellular genes. The acquisition of cellular genes is associated with the ability of the viruses to induce rapid neoplastic disease in newborn animals. Since the acquired sequences are associated with the acute oncogenic potential of these viruses, the acquired cellular genes have been termed onc (for oncogenic) genes.

To date more than two dozen such viruses have been isolated and characterized and more than 18 unique onc genes have been identified in both avian and mammalian species. These onc genes are transduced from the normal cellular genome and indeed they are representatives of a family of genes that are highly conserved in vertebrates (and in some cases even invertebrates). The onc genes are usually found in low or single copy number per haploid genome and most of these genes are expressed in normal cells at very low levels. As part of the viral genome, the onc gene is expressed at high levels and it is this gene dosage effect which has been correlated with the transforming activity of the virus. Since there are approximately 18 such genes that are found in the normal cell, the question

raised immediately is whether any of these onc genes are responsible for human neoplastic disease.

During the past year there has been an intensive investigation in many laboratories directed specifically at answering this question. Several human genes, homologous to onc genes of retroviruses, were molecularly cloned and their structures analyzed in detail. Furthermore, the complete nucleotide sequence of some human onc genes has been determined. More importantly, the expression of these genes in various human cells has been examined and preliminary investigations indicate that RNA transcripts corresponding to specific onc genes can be identified in certain human neoplastic cells. These analyses have revealed that some onc genes are expressed at modest levels in most cell types; some are rarely expressed; some are expressed chiefly in specific kinds of tumors; and some appear to be expressed in tumor cells of certain lineage or differentiation states. Several of the onc genes expressed in human tumors were originally identified as part of the viral onc genes expressed in avian retroviruses. It is possible that this expression may be related to the disease phenotype of the particular tumor cell.

Until a few years ago, it was unknown whether normal cell genes had oncogenic potential. It has been shown that certain normal cell onc genes could be activated by transcription control elements derived from retroviruses. While it is expected that the cellular onc gene homolog in the animal from which the acute transforming retrovirus was isolated will have oncogenic potential, it is unknown whether the conserved onc genes found in other animal species will also have oncogenic potential.

One of the most innovative applications of the new technologies has been the preparation of chemically synthesized peptides corresponding to open reading frames in the nucleic acid sequence of a variety of genes. This has been especially useful for the onc genes where it has been virtually impossible to obtain antibody against specific onc gene transforming proteins. By this methodology, it has been possible within the past year to generate antibodies against five different onc genes. Obviously, in the future, these antibodies could serve as diagnostic reagents. They are immensely useful at the present time for characterizing the molecular properties of the transforming proteins in vitro.

The true parents of the acute transforming retroviruses, the leukemia retroviruses, cause a variety of neoplastic diseases in animals after long latent periods. When these viruses infect a large population of cells, they can integrate at many loci in host chromosomes. The stable integrated form of the virus (called the provirus) is bracketed by transcription control elements which are virus-specific and are generated during the integration process. During the past two years, it has been demonstrated in several instances that these viruses cause neoplastic disease by integrating at loci in the host chromosome adjacent to the same cellular onc genes that have been discussed above. This specific integration results in elevated levels of expression of specific cellular onc genes that are now regulated by the viral transcription control element. The long latent period required for neoplastic disease induced by these viruses can thus be associated with specific integration in the host chromosome adjacent to a cellular onc gene.

Although it is not clear that all leukemia viruses cause neoplastic disease by a similar mechanism, the model itself is important for several reasons. First

it indicates that elevated levels of specific cellular genes with oncogenic potential can result in neoplastic transformation. The same effect can be mediated by alteration (e.g., genetic insult) of regulated cellular transcription control elements. It is known, for example, that certain neoplastic diseases show a high correlation with chromosome translocation. It is conceivable that these translocations result in alteration of normal transcription control elements and result in the elevated expression of cellular genes with oncogenic potential.

Human DNA was analyzed for the presence of sequences homologous to the transforming gene of Moloney murine sarcoma virus. A fragment of DNA was identified and cloned which contained a region of continuous homology to the murine sarcoma virus. It was present as a single copy in human DNA and was assigned to human chromosome #8. In a similar study, a fragment of human DNA homologous to the primate cell-derived transforming gene of simian sarcoma virus was detected and was assigned to human chromosome #22.

A considerable amount of information is becoming available about transcription control elements. It has been known for some time that certain nucleotide sequences which bracket structural genes, called consensus sequences, are associated with the initiation and termination of messenger RNA transcripts. However, it appears that for at least some genes a region termed the enhancer or activator sequence also has to be present for these transcriptional control signals to be utilized. Enhancer sequences have been shown to play a role in the expression of retrovirus onc genes. It is not clear how activators or enhancers function. Apparently, they can be located in a number of different positions surrounding the structural gene. Comparison of the limited sequence data that is currently available has failed to show any significant sequence homology between the known enhancer elements.

Analogous sequences are likely to be associated with the control of expression of cellular onc genes. It is possible that mutations in gene enhancer or activator sequences of cellular onc genes could be responsible for expression of a transforming phenotype.

The mouse mammary tumor virus (MMTV) has been used in studies of mammalian transcriptional control mechanisms. Earlier observations showed that the rate of MMTV RNA synthesis was regulated by glucocorticoid hormones and that the recognition signals for this stimulation were part of the genomic structure of MMTV. During the last year identification and localization of the steroid target site in the LTR was accomplished. A new gene (pLTR) has been discovered within the LTR of the MMTV. The presence of this gene has now been confirmed in exogenous C3H-S MMTV and in two endogenous proviruses of C3H. A pLTR message encoding the information for this gene is uniquely expressed in preneoplastic mammary tissue induced in Balb/c animals by the chemical carcinogen DMBA. The role of this gene in mammary carcinogenesis and/or MMTV regulation is under investigation.

One of the most provocative developments which has occurred during the past two years has been the direct isolation of dominant transforming genes from human tumor cell lines. A biological assay referred to as transfection was used early in tumor virology to demonstrate that host genomic DNA containing dominant viral onc genes could be transferred to normal cells. By using this technology dominant transforming genes have been transferred from a variety of tumor cell lines, including human. To date, it has been possible to transfer dominant

transforming genes from human lung cancer, colon cancer, bladder cancer, and breast cancer cell lines as genomic DNA into recipient cells. Utilizing techniques of recombinant DNA, it has also been possible to identify and isolate (in certain instances) these dominant transforming genes from the transformed recipient cell and to show that these isolated genes are of human origin. In the molecularly cloned state the genes have high transforming activity.

Some of these genes (for example, genes associated with human bladder, lung and colon cancers) are related to a specific family of one of the acute transforming retroviral onc genes. We now have the capacity to compare normal human genes that have oncogenic potential to their activated, tumor-forming counterparts, and we will be able to identify the genetic alterations that have occurred in these normal human genes which have resulted in an expression of neoplastic disease. The correlation of a human onc gene with one of the known acute transforming viral onc genes provides an enormous leap in our understanding of human neoplastic disease because the study of tumor viruses has provided an understanding of how viral onc genes can cause cellular transformation.

In another research area, monoclonal antibodies that are reactive with the major envelope and internal structural proteins of mouse mammary tumor viruses (MMTVs) have been developed. Different monoclonal cell lines were shown to be reactive with type-specific, group-specific, and interspecies determinants of MMTVs. The monoclonals to type-specific determinants were used to demonstrate that differences exist between each of six MMTVs derived from different strains of laboratory mice. The monoclonals generated have been used to demonstrate the diversity of expression of individual antigenic determinants in primary mammary tumors of various mouse strains and species. The phenomenon of diversity of expression of a defined antigen among mammary tumors, within a given tumor mass, has striking parallels to that observed in human breast cancers using monoclonal antibodies to human tumor-associated antigens.

Eleven monoclonal antibodies reactive with human mammary tumor cells have been generated and characterized. The monoclonals could be placed into five major groups. Some of the monoclonals demonstrated a "pancarcinoma" activity reacting with the surface of some nonbreast carcinomas, but none of the 11 monoclonals reacted with the cell surface of melanomas, sarcomas, various hematopoietic malignancies, and numerous apparently normal cell lines. The monoclonal antibodies developed to date react with approximately 85 percent of human mammary tumors; studies are in progress to generate additional monoclonal antibodies to the thus far unreactive mammary carcinomas.

Antigenic variation was observed in the expression of specific tumor-associated antigens within individual mammary tumor masses using monoclonal antibodies. This diversity was also observed in human mammary tumor cell lines grown *in vivo* and *in vitro*. Preliminary results indicate that exposure of mammary tumor cells to certain compounds may enhance the cell surface expression of some tumor-associated antigens. Monoclonal antibodies to human mammary tumor metastases were tested for reactivity to antigens, and studies are in progress to develop RIAs with several of them.

Studies to localize human mammary tumors using radioactively labeled monoclonal antibodies were also performed. ^{125}I -IgG and its fragments were injected into athymic mice bearing human tumor transplants. The radiolabeled antibody localized in mammary tumors within 24 hours, whereas no localization was observed in

mice bearing melanomas or when radiolabeled normal murine IgG was injected into human mammary tumor-bearing mice. The ability of the radiolabeled antibody to localize in mammary tumors was sufficient to give high quality gamma scans of tumor-bearing mice. Several monoclonal antibodies are now being labeled with other radioactive isotopes that may be more appropriate for clinical studies.

The study of hormone-like control factors that have profound influence on the genetic and phenotypic expression of cells is another promising area of research. A family of transforming growth factors (TGFs) related to, but distinct from, epidermal growth factor (EGF) has been partially purified from the conditioned medium of human tumor cells as well as from extracts of the tumor cells themselves. These biologically active peptides produce at least some of their effects by interacting with EGF-specific membrane receptors. This action can be blocked by very small amounts of retinoids (vitamin A derivatives) and another class of inhibitors, pseudodipeptides.

TGF from medium conditioned by a human metastatic melanoma cell line, mouse sarcoma growth factor and rat TGFs were purified and their amino acid composition determined. In addition, the presence of another class of factors, the tumor inhibitory factors (TIFs), has been detected as products of some human tumor cells. These factors inhibit the growth of some human tumors and stimulate normal human and rat fibroblasts to proliferate in monolayer cultures.

TGFs were detected in the urine of normal, pregnant and tumor-bearing humans. TGF of relatively high molecular weight (30,000-35,000) was found in the urine of 18 of 22 cancer patients, but in only 5 of 22 nonmalignant controls. The pattern of expression of high molecular weight TGF in the urine of cancer patients has led to an ongoing evaluation of diagnostic, prognostic and therapeutic applications. Additional studies performed during the past year on TGFs are described under Chemical Carcinogenesis.

CHEMICAL CARCINOGENESIS

Studies on the mechanisms of chemical carcinogenesis continue to receive increased programmatic emphasis. Scientists are working on the hypothesis that chemical carcinogenesis is a complex process with distinct phases, progressing from a long latency period to multiple stages of preneoplastic to neoplastic change. In the animal host, many systems may be required to activate a chemical to its carcinogenic form; any minor modification in chemical structure could lead to profound changes in a metabolic pathway. Current evidence indicates that the carcinogenic insult may be an early event involving a structural change in the DNA of certain cells--a point mutation or perhaps more generalized damage to DNA--as a precursor to transformation to a cancer cell. Significant interactions may occur epigenetically in the cytoplasm or at the cell surface. Nevertheless, it is possible that the carcinogen-bound or damaged DNA can be repaired and that failure or inability to repair this damage may be linked to cancer. The resulting increased expression of a gene or increased transcription of a gene product which in some way regulates or controls cell growth and differentiation may be responsible for maintaining the transformed state. Finally, there may be other factors required for transformation to malignancy. For example, tumor promoters, hormones or other growth factors may enhance or accelerate the expression of neoplastic potential in the latent or initiated cell. Understanding the mode of action of these promoters is an important goal in carcinogenesis research.

Currently, laboratory investigations seek to understand how exogenous carcinogens and endogenous factors are processed by enzymatic mechanisms. In an attempt to study the genetic basis of human variability in carcinogen and drug metabolism, monoclonal antibodies to four different forms of cytochrome P450, the major receptor enzyme for drugs, carcinogens, and environmental chemicals, were prepared. Using these antibodies, it has been possible to define antigenic site relatedness for different enzymatic functions of P450s and to identify and quantify the amount of a specific enzyme activity in a tissue dependent on specific P450s. This system represents a new approach for phenotyping and mapping the cytochrome P450s responsible for specific metabolic reactions and thus may be useful in determining the relationship of P450 phenotype to individual differences in drug metabolism and carcinogen susceptibility.

As another approach to identify the genetic factors involved in carcinogenesis, human cancer-prone genetic diseases are being studied with special attention to 1) identifying groups of people with an increased susceptibility to environmental carcinogenesis; 2) correlation of cellular hypersensitivity with clinical abnormalities; and 3) the molecular basis for the cellular hypersensitivity. Patients with xeroderma pigmentosum (XP), ataxia telangiectasia (AT), and other diseases susceptible to ultraviolet light (dysplastic nevus syndrome) were studied. Detailed examinations of the clinical features of affected individuals were made. A registry of XP patients is being established. Cultures of skin and blood also are being established and the effects on cell survival, mutagenesis, and DNA damaging agents are being examined. An attempt to clone the genes responsible for UV sensitivity in XP cells is in progress. These studies may give insights into mechanisms of cancer induction and suggest modes of cancer prophylaxis. In addition, these diseases serve as models for studies of human environmental carcinogenesis.

A unique methodology has been developed which makes possible a study of the interaction of carcinogens with DNA. Antibodies were developed in rabbits against the two major guanine adducts formed *in vivo* and *in vitro* by the interaction of the carcinogen 2-acetylaminofluorene (AAF) with nucleic acids. Antisera have also been developed against *cis*-dichlorodiammine platinum-DNA (*cis*-DDP-DNA) as well as against DNA substituted with 7,8-diol 9,10 epoxide of benzo[a]pyrene (BPDE), with *trans* (7R)-benzo[a]pyrene-N²-deoxyguanosine (BPdG) as the major antigen. These antisera, which are specific for adducts, have been used to develop highly sensitive quantitative immunoassays for monitoring carcinogen binding and for morphological localization of binding sites.

Using this methodology, the persistence and removal of AAF adducts have 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 cell culture, immunofluorescence studies with anti-BPdG antibodies have shown that bound benzo[a]pyrene 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. The BPdG antibody has also been used in immunoassays to screen DNA obtained in lung tissue from cancer patients and controls. Several positive samples have been obtained in lung tissue from cancer patients and in one case from DNA obtained from circulating lymphocytes. Exposure histories are currently being assessed for the patients studied. 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 *cis*-DDP-DNA immunoassay has been used

to measure platinum bound to DNA of ascites tumor cells recovered from mice receiving cis-DDP therapy. This capability forms the background to apply immunoassays to monitor platinum binding to DNA of cancer patients receiving chemotherapy.

Highly sensitive and accurate methods for the detection of mutations caused by chemical carcinogens have been developed. A plasmid was constructed using recombinant DNA technology, which consisted of two well-defined genetic functions. The plasmid was then modified by reaction with the diol-epoxide of benzo[a]pyrene. By exposure of the plasmid to the carcinogen before the plasmid was introduced into the bacterial cell, problems of toxicity to the cell were minimized. A mutation frequency of two percent (exceptionally high) was observed. The mutated plasmid DNA was recovered from the cells permitting a determination of changes in the DNA sequence. It was found that the mutations caused by BP diol-epoxide were not located at random but tended to cluster in regions of identical nucleotide sequence. The selection of the "hot spot" may depend on specific DNA conformations of an unknown nature.

Another system developed for studies on metabolism and mutagenicity of chemical carcinogens involves the use of intact hepatocytes to examine both metabolic processing and the genotoxic potential of selected chemical carcinogens. By coinoculating rat hepatocytes with Salmonella tester strains (Salmonella/hepatocyte system), it is possible to evaluate the role of both metabolic activation and detoxification in determining the mutagenic potential of known or suspected chemical carcinogens. Measuring mutation frequency in the Salmonella strains (target cells) and genotoxicity in the hepatocyte (host cell) by the number of alkali labile sites in DNA, it is possible to determine, for aromatic amides such as AAF, those metabolic pathways responsible for mutations in the bacteria as well as those causing genetic damage in the host cell. Furthermore, by substituting a variety of cell types (including human cell lines) for the hepatocyte, the capacity of these cells to metabolically activate and/or inactivate chemical carcinogens can be determined.

Gene cloning and gene transfer techniques have been applied to investigations on malignant transformation by chemicals. When chemically transformed human fibroblasts and the untransformed parental cells were compared by two-dimensional gel electrophoresis of their proteins, one new polypeptide was recognized in the proteins from a transformed line and was identified as a product of mutated beta-actin gene. Following isolation of mRNA of this protein and cloning cDNA complementary to the actin mRNA, a recombinant containing human beta-actin cDNA was derived. This finding provided the first molecular evidence for the occurrence of a mutation in chemically transformed cells. This mutated beta-actin gene has been found to be further mutated in subclones of the transformed cells. Variations in expression of the mutated beta-actin gene were accompanied by corresponding changes in the malignant potential of the subclones. The expression of this mutated beta-actin was also associated with the expression of the in vitro transformed phenotypes in hybrid cells formed between the transformed and normal parent human fibroblasts. These observations suggest that the mutated beta-actin has an important role in the expression of the transformed phenotypes.

Studies have shown that alternating purine-pyrimidine sequences in DNA can form a left-handed helix called Z-DNA. The presence of such sequences in natural genomes would have great implications in the regulation of gene expression and in the processes of cell differentiation and carcinogenesis. Various eukaryotic

genomes have been surveyed for potential Z-DNA forming sequences. A high copy number of a dT-dG alternating sequence was found in several eukaryotic genomes, including yeast and human. Two different cloned human actin genes contained the sequences (dT-dG)₂₅ and (dT-dG)₁₅. A comparison of the nucleotide sequence of the dT-dG alternating region and its flanking region in various genes indicated that the repeated element consists only of (dT-dG)_n (designated the Z1-element). Another purine-pyrimidine alternating sequence, (dT-dC), was found to be moderately repeated in some eukaryotic genomes, but not detected in others. (The repeated element with dG-dC alternating sequence is designated the Z2-element.) These results provide direct evidence for the general and abundant occurrence of potential Z-DNA forming sequences in eukaryotic genomes. Experiments are now underway to test the hypothesis that Z-DNA sequences may be regulatory elements for gene expression and even a key element in carcinogenesis.

A number of studies have focused on the factors which control the growth and differentiation of normal cells and their malignant counterparts. One such factor is a family of polypeptides, the transforming growth factors (TGFs). These TGFs are acid-stable, low molecular weight materials which can be isolated from a variety of epithelial and mesenchymal tumors of murine, avian and human origin, caused by chemicals or viruses, or of spontaneous origin.

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 and its ability to induce colony formation in soft agar is not enhanced by EGF. In contrast TGF β does not compete for binding to EGF receptors, but it requires the presence of a polypeptide that can bind to the EGF receptor for expression of its biological activity. Using TGF α and TGF β isolated from the acid-ethanol extract of murine sarcoma virus-transformed mouse cells, it has been shown that either TGF α or EGF can function to potentiate the ability of TGF β to form large colonies in soft agar; neither TGF α or EGF by themselves have significant ability to induce formation of large colonies. Experiments using chemically modified analogues of EGF show that the ability of these analogues to potentiate the colony-forming activity of TGF β can also be correlated with their ability to compete with native EGF for receptor binding. These results can be generalized to all TGF β s isolated thus far from both neoplastic and non-neoplastic tissues of murine, bovine, or human genomes. Similarly, TGF from human melanoma cells has the same properties described here for TGF α from virally transformed mouse cells. TGF β represents the major TGF of tissues when assayed in the presence of EGF. It is currently being purified from virally transformed mouse cells grown in culture, from mouse kidney, and from bovine salivary gland and kidney. TGF β s from mouse and bovine tissues have been purified over 1000-fold; at this stage of purification, the various TGF β s appear to be similar to each other and different from either TGF α or EGF. Their chemical characterization in terms of their amino acid composition and sequence is in progress.

Since TGF β has been found in all tissues examined thus far, including blood platelets, it can be assumed that these TGFs have a normal physiological role, perhaps in tissue repair. Experiments designed to test the effects of bovine TGF β on wound healing have shown that TGF β stimulates tissue repair in rats bearing subcutaneous wound healing chambers. These experiments represent the first demonstration of in vivo activity of a TGF.

Human TGFs and possible controlling factors are being purified from normal human placenta and from two human tumor cell lines. The TGF from human placenta has been purified and three regions of transforming activity obtained by HPLC. One region appears to be homogeneous and is now undergoing further characterization using amino acid analysis and sequencing techniques. In addition, an inhibitor of cell proliferation has been detected in extracts of normal human placenta and a human tumor cell line. This factor appears to be nonprotein in nature and has an apparent molecular weight of 2000; it is currently being purified for characterization by mass spectroscopy and nuclear magnetic resonance techniques.

The serial passage and clonal growth of human bronchial and esophageal epithelial cells in serum-free medium and without feeder cells has provided new opportunities for the study of carcinogenesis and differentiation in these human cells. Such studies also permit related studies on metabolism of chemical carcinogens, DNA damage by carcinogens and transformation of epithelial cells. It has been found that serum, platelet-derived growth factor, or calcium ions (< 1 mM), induce terminal squamous differentiation in normal bronchial epithelial cells, but not in carcinoma cells which continue to grow and in some cases grow at a faster rate. Comparative studies of normal and malignant cells also revealed a striking difference in their pattern of cytoskeleton proteins including keratins and their production of polypeptide hormones. In addition, marked differences in the pattern of keratins were noted between adenocarcinomas and squamous cell carcinomas.

The cytotoxicity, mitogenicity, and differentiating effects of chemical and physical agents from several classes of carcinogens and cocarcinogens are being tested in human bronchial epithelial cells. Some of these compounds were mitogenic, whereas others induced differentiation. For example, teleocidin induced rapid differentiation with a corresponding decrease in ornithine decarboxylase activity. 2,3,7,8-Tetrachlorodibenzo-p-dioxin, on the other hand, was mitogenic and increased ornithine decarboxylase and aryl hydrocarbon hydroxylase activities. Further, the degree of activity change was specific for each compound. These data are important for designing initiation-promotion studies in long-term in vitro carcinogenesis experiments.

Cultured bronchial epithelial tissues and cells are also being used to study the mechanism responsible for the cocarcinogenic effect of asbestos and tobacco. 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. Various fiber types were found to have different effects on human bronchial epithelial cells. Chrysolite 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. Mesothelial cells from lung pleura were more sensitive than epithelial cells to the cytotoxic effects of asbestos.

DNA damage caused by chemical and physical carcinogens and DNA repair has also been evaluated in human bronchial epithelial cells. These cells are able to repair single strand breaks (SSB) in DNA damaged by X-radiation, UV-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines. The number of SSB and the rate of their removal are nearly equal in both normal epithelial and fibroblastic cells. Asbestos fibers cause neither DNA-protein

cross links nor a detectable increase in SSB even in the presence of the combination of arabinofuranosyl cytosine and hydroxyurea, which inhibit the polymerase step during excision repair and thus enhance the sensitivity of the assay by allowing SSB to accumulate.

In another study, human bronchial cells were exposed to X-rays, then incubated with or without the presence of formaldehyde, and the repair of DNA was measured. The presence of formaldehyde significantly inhibited the repair of the X-ray-induced SSB correlating with the potentiation of cytotoxicity in human cells and mutation frequency in Chinese hamster V-79 cells by the combinations of the agents.

Tumor promoters are a group of chemicals of particular interest since they are capable of enhancing or accelerating the expression of neoplastic potential in latent neoplastic cells. Many tumor promoters differ from classical carcinogens in that they do not covalently interact with macromolecules, are not mutagenic, may be tissue specific, and can induce many transient phenotypic changes in target cells at extremely low exposure levels. Understanding their mode of action is an important goal in carcinogenesis research since the promotion phase of carcinogenesis appears to be reversible. Skin carcinogenesis by classic carcinogens in the mouse is associated with an alteration in differentiation which allows initiated cells to proliferate under conditions where normal cells must terminally differentiate.

Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. The finding that basal cells are heterogenous in response to phorbol esters, in that some cells are induced to differentiate while others are stimulated to proliferate, may form the cellular basis for selection. Putative initiated cells cannot be stimulated to differentiate by phorbol esters thus providing a mechanism for their clonal expansion if they were a subpopulation of a large normal cell mass. Retinoids prevent the terminal differentiation induced by phorbol esters, which could play a role in their anti-promoting activity. Studies on the progression of benign to malignant tumors in vivo suggest 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.

The availability of cell surface macromolecules, such as the epidermal growth factor (EGF) receptor, is greatly enhanced in fibroblasts cultured in the presence of retinoic acid, while phorbol esters decrease the number of available receptors. Antagonism between retinoids and phorbol esters was also found at the level of the biosynthesis of procollagen $\alpha 1$ and $\alpha 2$; again for this molecule retinoids enhanced its synthesis, specifically the incorporation of mannose, whereas tumor promoters inhibited such incorporation. A third biochemical target of the antagonistic action of retinoids and tumor promoters appears to be the cell surface glycoprotein, fibronectin. It appears that retinoids induce retention of this molecule at the cell surface while tumor promoters cause its shedding into the medium. The emerging suggestion is that the retinoids are necessary to maintain the functional expression of the cell surface in a variety of cells and that phorbol esters directly interfere with this function.

The high evolutionary conservation of the phorbol ester receptor argues for the presence of an endogenous compound which normally interacts at the binding site. Since brain contains the highest level of receptors, it was screened for potential inhibitors of binding. A major heat-stable low molecular weight inhibitor was found. It was identified as ascorbic acid and shown to act irreversibly through a free radical mechanism to cause lipid peroxidation. Its action can be blocked by free radical inhibitors, and efforts to identify other inhibitors are in progress.

Lymphokines are biologically active hormone-like substances produced by stimulated lymphocytes. Lymphotoxin, a lymphokine produced by mitogen or antigen-stimulated lymphocytes, inhibits tumor cell growth and is anticarcinogenic. Its anticarcinogenic effect is on both promoted and nonpromoted hamster cell transformation. There are differences in cell sensitivity to lymphotoxin of these two types or stages of carcinogenesis, suggesting that lymphotoxin acts by different mechanisms to inhibit transformation. In vitro, lymphotoxin has a persistent anticarcinogenic effect, regardless of whether added prior to or after TPA-promoted UV-initiated transformation or even before any carcinogenic insult. It also induces synthesis of high molecular weight (approximately 200,000 daltons) cell surface membrane glycoproteins in nontransformed cells, whereas it inhibits this glycoprotein synthesis in tumor cells. These membrane changes occur within hours following binding of lymphotoxin to the cell; they are the first identifiable biochemical alterations that result from lymphotoxin interaction with mammalian cells and they correlate with its anticarcinogenic and tumor cell growth inhibitory activities. Studies in vivo have shown that lymphotoxin injected into guinea pigs retards the growth of transplanted tumor cells. Moreover, lymphotoxin injected into pregnant hamsters simultaneously with either diethylnitrosamine or with ⁹⁹technetium decreased the frequency of transformed cells subsequently isolated from embryos. Thus, lymphotoxin is anticarcinogenic and inhibits tumor cell growth under both in vivo and in vitro conditions.

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 order of magnitude. The induction of rapidly fatal gestational choriocarcinoma in pregnant monkeys given ENU suggests a correlation between choriocarcinoma and pediatric neoplasia that may exist in human populations if environmental carcinogens play a role in causation of these tumors in man. 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.

The development of hepatocellular neoplasia in a patas monkey given diethylnitrosamine (DEN) transplacentally, followed by phenobarbital beginning 4 years after birth, provisionally suggests that (1) DEN, like ENU, is a transplacental carcinogen in this nonhuman primate; (2) the transformed hepatocytes which result from prenatal exposure to DEN may persist, latent, for years after exposure to the carcinogen has ceased; and (3) phenobarbital may be a promoter of hepatocellular neoplasia in this species as it is in rats and mice.

FIELD STUDIES AND STATISTICS

Epidemiologic investigations into the environmental and host determinants of human cancer have been given special emphasis. By compiling statistics on cancer incidence, mortality and survival, and by pursuing multidisciplinary projects that combine epidemiologic and experimental approaches, considerable new information on the etiology of cancer has been generated. To provide a systematic means of identifying risk factors, findings are grouped and reported under categories such as incidence/mortality studies, occupational studies, nutritional studies, family studies, and lifestyle studies.

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. This year NCI Monograph No. 57 presented detailed data on cancer incidence and mortality for the period 1973-77, enabling a comparison of cancer incidence and mortality among various ethnic groups and geographic areas. The continued surveillance of cancer patterns is important for identifying emergent problems and changing risks that suggest environmental hazards. Special attention is now being given to the analysis of survival statistics utilizing data available on patients diagnosed from 1973 through 1979, and a summary report is being prepared on survival results for white and black patients by geographic area. At the recommendation of the DCCP Board of Scientific Counselors, efforts were made this year to evaluate alternative mechanisms of achieving follow-up for collection of survival data and to improve the systematic recording and monitoring of costs in each SEER registry with the objective of reducing expenses and developing an effective cost-accounting system.

Since nonmelanoma skin cancer is not covered by the cancer registries, a special incidence survey was carried out in 1977-78 in various areas of the country, and the results were published this year in a monograph. In the past, a county-by-county survey of cancer mortality in the United States (1950-69) identified geographic peculiarities that have provided etiologic clues for further study and updated maps of cancer mortality for 1970-75 are now being completed for the more common tumors. In addition, a volume of U.S. cancer mortality statistics (1950-75) was published with emphasis on temporal trends for various cancers, through displays of two- and three-dimensional graphs over time by age, sex, and race. Also published this year was an atlas illustrating the geographic patterns of nonneoplastic diseases in the U.S. that may share etiologic factors with cancer. Several Institutes are pursuing the leads generated by these maps.

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 during World War II as the explanation for the high rates of lung cancer in coastal areas, use of smokeless tobacco for the elevated rates of mouth cancer among women in rural southern areas, and nutritional deficiencies and alcohol consumption for the high rates of esophageal cancer among black men in urban areas.

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; and lung cancer in shipyard workers and other groups exposed to asbestos, in steel and foundry workers exposed to 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 exposed to formaldehyde during manufacturing and usage is under way. Considerable effort was devoted to working with other Government agencies to utilize and develop record systems that may help in screening occupational groups at high risk of cancer.

Radiation studies received further emphasis as pressure mounts 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 treatment regimens were shown to be 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. A survey of breast cancer among atomic bomb survivors revealed elevated risks among women exposed at younger ages, particularly at 10-19 years; and for the first time, women exposed under age 10 showed a dose-related excess risk. A follow-up study of women receiving multiple chest fluoroscopies during pneumothorax treatment of tuberculosis revealed that repeated low radiation doses increased the risk of breast cancer, particularly from exposures during early adult life. No excess of other types of cancer were seen among fluoroscopically examined men or women. In a study of women treated with radioactive iodine for hyperthyroidism, there was a slight excess of cancer of the thyroid and other organs with high ^{131}I exposure, but the findings are preliminary due to the small numbers of cases.

Drug studies were expanded to evaluate the effects of estrogenic compounds, which now appear to be related to the risk of breast cancer. In several groups of cancer patients treated with alkylating agents, there was a substantially elevated risk of acute nonlymphocytic leukemia.

Nutritional studies were intensified this year to clarify the role of dietary components in cancer etiology. Field studies are taking advantage of 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. To evaluate the relationship between bladder cancer and saccharin, a case-control interview study of 3,000 cases and 6,000 controls revealed no overall excess risk for persons who had ever had artificial sweeteners, but subgroup analyses provided some evidence consistent with a weak carcinogenic or promoting effect. In case-control studies the role of dietary fat was suggested for breast cancer and a deficiency of fruits and vegetables containing vitamin A and C was suggested for oral cancer.

Family studies, enhanced by collaborative ties with laboratory investigators and a computer-based data resource, have resulted in the delineation of familial cancer syndromes and leads to mechanisms of host susceptibility. Of special interest has been the identification of the dysplastic nevus syndrome as a marker of susceptibility to melanoma, enabling early detection and treatment of this potentially lethal cancer. Educational videotapes were developed to acquaint high-risk patients and health professionals with the syndrome and opportunities

for prevention. Studies of neurofibromatosis have helped clarify the risks of various cancers associated with this hereditary syndrome. Several reports were prepared on familial syndromes associated with sarcoma and other neoplasms, with carotid body tumors, with smoking-related respiratory cancer, with retinoblastoma and other neoplasms including pineal tumors, and with kidney cancer and polymastia. The Inter-Institute Medical Genetics Clinic, codirected by one staff member, provides a multidisciplinary setting for studying families and patients prone to cancer. The value of alert clinical observations was demonstrated repeatedly by the detection of cancer-prone families and other high-risk groups.

Environmental pollutants were evaluated through epidemiologic studies that integrated appropriate environmental and body measurements, with special emphasis on a case-control study of bladder cancer in relation to the level of halogenated hydrocarbons in the drinking water.

Infectious agents received substantial attention as the program became involved in collaborative studies to evaluate the role of a newly discovered human retrovirus in the origins of T-cell leukemia. In addition, recent clusters of Kaposi's sarcoma and opportunistic infections in homosexual males prompted studies to evaluate the role of sexually transmitted viruses, use of amyl and butyl nitrite, and other exposures.

REPORTS ON INTERNATIONAL AGREEMENTS AND INFORMATION EXCHANGE ACTIVITIES

(Fiscal Year 1982)

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 viral 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 the Sixth Soviet-American Program Review Meeting on the Problem, "Malignant Neoplasia," took place at the NCI, Bethesda, MD, USA, during the period of September 13-22, 1981. During the course of the meeting, the results of the six subareas were presented to the Joint Committee. After reviewing the progress, the members proposed to combine or discontinue some activities. With respect to carcinogenesis, "Tumor Virology" and "Somatic Cell Genetics" were combined to form one more inclusive area, "Carcinogenesis." Studies concerned with basic research in viral and chemical carcinogenesis are included in this group. Other areas of cooperation, not within the present mission of DCCP, are described in the report of OIA, OD, NCI.

As on previous occasions, the members of the Joint Committee expressed the desirability of continuing (a) the exchange of biological materials for joint research; (b) the exchange of working scientists; (c) the exchange of information of mutual interest; (d) publishing scientific communications jointly as exemplified by the U.S.-U.S.S.R. monographs, and, in particular, on a monograph that would highlight ten years of cooperation between the two countries.

Two scientist exchanges under the aegis of the former "Tumor Virology" subarea took place in this fiscal year:

SCIENTIST EXCHANGES

U.S.S.R. to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	(Duration)	Title of Research
Dr. S. Klimenko Ivanowsky Inst. (Moscow)	Dr. W. Haseltine Sidney Farber Cancer Center (Boston)	6 months	Molecular Biology of Retroviruses

U.S. to U.S.S.R.:

<u>Applicant (Laboratory)</u>	<u>Recipient (Laboratory)</u>	<u>(Duration)</u>	<u>Title of Research</u>
Dr. M. Howett Penn State Univ. (Hershey)	Dr. B. Lapin Institute for Exp. Pathology (Sukhumi)	2 months	Modulation of Virus Expression in Baboon Lymphomas

In general, the accessibility to a large colony of baboons remains the major contribution to the U.S. These animals have been a valuable source for (1) isolating primate retroviruses, (2) isolating primate herpesviruses and determining their role in the development of certain lymphoproliferative diseases and (3) identifying genetic factors resulting from inbreeding. The results of many of these studies have been published in U.S. and Soviet journals. Eight studies involving 38 monkeys are projected for continuation: 32 Old World monkeys (10 baboons, nine rhesus monkeys, six stump-tailed macaques, two pigtailed macaques, five cynomolgous monkeys) and six New World monkeys (five owl monkeys, one white-lipped marmoset). These animals are being held for tumor development and are bled several times per year. In January 1982 one of the stump-tailed macaques, inoculated with material from a leukemic baboon in 1974, died of a lymphoproliferative disease; follow-up studies are in progress.

This year the new working group on "Carcinogenesis" was organized; the members include Dr. Snorri Thorgeirsson (Chairman), Dr. Stuart Aaronson, Dr. Mark Pearson (Frederick Cancer Research Center), Dr. Warren Nichols (Institute for Medical Research, Camden, New Jersey) who chaired the disbanded "Somatic Cell Genetics" group. A meeting of members of this working group is expected to occur in Moscow this fall.

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 any nonclinical research relating to understanding the process of 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.

On March 8-9, 1982, the Chairman and one member from each of the U.S. and French working groups met in Bethesda. The purpose of the meeting was to review the progress of the cooperation since its reorganization in November 1980. To summarize, the discussions focused on science policies of the new French government as they relate to INSERM, support for cancer research under special emphasis programs (contracts) by INSERM, support for cancer research by the NCI under a reduced Federal budget, and suggestions for restructuring of the cooperation, e.g., reducing the numbers of working groups, and minimizing need for international travel.

The following statements summarize the major recommendations of the Joint Committee. Both sides should (1) continue to stimulate and support the exchange of scientists, (2) exchange information about the qualifications of NCI and INSERM grantees and their research projects on an informal basis, (3) explore other possibilities for obtaining supplemental funding to support collaborative research, (4) identify promising research areas for new collaborations and (5) consider possible ways of restructuring the present Basic and Clinical Cancer Research Groups with the goal of limiting membership to a few individuals who primarily set policy and review applications for exchange scientists.

SCIENTIST EXCHANGES

U.S. to France:

Applicant (Laboratory)	Recipient (Laboratory)	(Duration)	Title of Research
Dr. J. Lapeyre Univ. of Texas (Houston)	Dr. M. Leng CNRS (Orleans)	3 months	Effect of Histones on Nucleic Acid Binding
Dr. C. Moore Univ. of N.C. (Chapel Hill)	Dr. M. Yaniv Institut Pasteur (Paris)	6 months	Structure and Biological Function Topoisomerase- DNA Complexes
Dr. E. Kohen Papanicolaou Inst. (Miami)	Dr. G. Moreno Laboratoire de Biophysique (Paris)	Travel only	Understanding the Role of Photosensitizers as Mutagens and Carcinogens
Dr. S. Schmid NCTR (Little Rock)	Dr. M. Daune CRNS (Strasbourg)	3 months	Repair and Mutagenesis of Plasmid DNA Modified by Carcinogenic Compounds
Dr. G. Greene Univ. Chicago	Dr. P. Chambon CRNS (Strasbourg)	3 months	Estradiol Receptors and Hormone-Dependent Tumors
Dr. H. Richard- Foy Universite de Paris Sud (Paris)	Dr. G. Hager NCI (Bethesda)	6 months	Study of Regulation of Expression of MMTV Genes by Glucocorticoids
Dr. J. Lemonne Institut Pasteur (Paris)	Dr. M. Essex Harvard School Public Health (Boston)	6 months	Hepatitis Virus Antigens Associated with Liver Cancer
Dr. B. Sola Hopital Cochin	Dr. E. Scolnick NCI (Bethesda)	6 months	Studies on a Variant of Friend Virus

Applicant (Laboratory)	Recipient (Laboratory)	(Duration)	Title of Research
Dr. G. de The CNRS (Lyon)	Dr. M. Essex Harvard School Public Health (Boston)	3 months	Antiviral Interventions in NPC, Liver Carcinoma, & Leukemias of Man

U.S.-Japan Agreement. This year marks the third in the second five-year program of this agreement. 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. Under the new program coordinator for the U.S., seminars were held on "Carcinogenesis and Gene Expression in Liver Cell Culture" and "Intestinal Metaplasia and Stomach Cancer."

The present organization consists of four broad program areas: Etiology, Cancer Biology and Diagnosis, Cancer Treatment, and Interdisciplinary Research. 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. N. Shimizu Univ. Ariz. (Tucson)	Dr. T. Sugimura National Cancer Center (Tokyo)	3 months	Chemical Carcinogenesis
Dr. W. Troll NYU (New York)	Dr. T. Sugimura National Cancer Center (Tokyo)	1 week	Free Oxygen Radicals in Tumor Promotion

Japan to U.S.:

Dr. K. Fuginaga Univ. Sapporo (Sapporo)	Dr. M. Green St. Louis Univ. (St. Louis)	2 months	Mutants of Adenoviruses
Dr. K. Hirai Tokai Univ. (Isehara)	Dr. M. Nonoyama Showa Univ. (Tampa)	1 month	Viral Genomes in Marek's Disease Tumor Cells
Dr. A. Ishimoto Kyoto Univ. (Kyoto)	Dr. J. Gautsch Scripps (LaJolla)	1 month	B-tropic and N-tropic Activity in Xenotropic Viruses

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. A. Matsukage Aichi Cancer Inst. (Nagoya)	Dr. T. Kakefuda NCI (Bethesda)	3 months	DNA Polymerases and Interactions with Carcinogens
Dr. T. Sekiya National Cancer Center (Tokyo)	Dr. G. Vande Woude NCI (Bethesda)	2 weeks	Limits of <u>v-onc</u> Genes for Studies on Human <u>onc</u> Genes

U.S.-Italy Agreement. Research pertinent to this Division is included in the Cancer Prevention Program. Although the program includes most areas of basic research relating to carcinogenesis, the early activities of this program have been concerned primarily with projects in cancer epidemiology. This year a workshop on Occupational Cancer was held at Portofino, Italy, on October 20-23, 1981. Three occupational groups of binational interest were studied: leather and tannery workers, benzene-exposed workers and shipyard employees. The participants focused on issues relating to the design, conduct and analysis of studies to evaluate the risk of cancer among these groups.

SCIENTISTS EXCHANGES

U.S. to Italy:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. R. Monson Harvard School Public Health (Boston)	Dr. L. Santi Univ. Roma (Rome)	2 months	Epidemiologic Methods for Studies on Occupational Cancers
Dr. C. Cesarone Inst. Oncology (Genoa)	Dr. F. Becker U. Texas (Houston)	3 months	Alterations in DNA by Carcinogenic Agents

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.

SCIENTIST EXCHANGES

Germany to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. R. Beyme Inst. Documenta- tion, Information, Statistics (Heidelberg)	Dr. J. Fraumeni NCI (Bethesda)	3 months	Determination of Cancer Risks in Certain Occupations
Dr. D. Komitkowski Inst. Exp. Path. (Heidelberg)	Dr. H. Stewart NCI (Bethesda)	2 months	Registry of Experimental Cancers

U.S.-Peoples Republic of China. This 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 epidemiology of esophageal, nasopharyngeal and hepatocellular carcinomas. Members of this Division have already begun collaborative projects to establish culture conditions for human explants of these organs. This year substantial progress was made in establishing culture conditions and methods of assessment for human liver explants and hepatic epithelial cells; metabolism of carcinogens, including studies on DNA damage and repair; biochemical and immunochemical epidemiology of individuals at high risk of developing liver cell cancer; and in vitro transformation of human hepatic epithelial cells. Similar studies are being done for human esophageal cells from fetal sources.

SCIENTIST EXCHANGES

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. C. Harris Dr. H. Atrup NCI (Bethesda)	Dr. Chu-Chieh Cancer Institute (Beijing)	2 weeks (each)	Risk Factors in Esophageal and Hepatic Cancer
Dr. Chu-Chieh Dr. Tsung-Tang Cancer Institute (Beijing)	Dr. C. Harris NCI (Bethesda)	1 month	Risk Factors in Esophageal and Hepatic Cancer

ANNUAL REPORT OF THE
OFFICE OF THE SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER
NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

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 Environmental Protection Agency (EPA) and National Institute for Occupational Safety 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. Actually, since the inception of this office in 1972, it was realized that the coordination activities and efforts were fulfilling 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 appraisal of occurrence, frequency and concentration of such environmental carcinogens, close working arrangements are required and collaboration is continuing with EPA, NIOSH, Food and Drug Administration (FDA), Occupational Safety and Health Administration (OSHA), U. S. Department of Agriculture (USDA), Department of Defense (DOD), U.S. Department of Interior (USDI) and many other agencies or subagencies. Data and information from these sources are transmitted to 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 ninth year (64th meeting to be held in July 1982), 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 Office of Management and Budget (OMB) and the U. S. Congress in 1977. This office also provides the primary interface with the National Toxicology Program (NTP) regarding chemical selection for carcinogenesis testing and the subsequent reporting to industry and trade associations on results of testing these chemicals for carcinogenicity.

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 provided by staff and by contract (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.

Other information resource contracts are supported from this office such as the one with the International Agency for Research on Cancer (IARC), Lyon, France, in connection with the monograph bulletin listing laboratories, worldwide, which are involved in carcinogenicity bioassays. Another resource contract is that with Technical Resources, Inc., Bethesda, Md., involving 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 Center 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). The large 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 the BOA (Basic Ordering 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 which, in January 1983, will celebrate its tenth anniversary. This group consists of 28 agencies or sub-agencies 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 October 1, 1982 there will have been 65 meetings. Topics of meetings held so far this year are as follows: 1) Registries on Environmental Cancer, Tumors in Lower Animals, and Environmental Pathology; 2) National Academy of Sciences Report on Health Effects of Nitrate, Nitrite and N-Nitrosamines; 3) Occupational Carcinogenesis; 4) Gene-Tox Program; and 5) Information Systems. 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.

The aforementioned IARC program on Monographs and Survey Bulletin requires some staff time since we are involved in three working group meetings per year and also make logistical arrangements with our contractor, SRI, to supply essential information for IARC publications. Within this reporting period Volumes 26, 27, 28 and 29 have been issued. The Survey Bulletin, Volume 10, will probably be issued next reporting period (18-month intervals in publication).

This office has continued an active role on the Task Force on Environmental Cancer and Heart and Lung Disease. In addition to participating in all Working Group activities, one of our members serves on the Task Force Planning Group which reviews and plans the activities of the Working Group meetings including preparation of the Fifth Annual Report to Congress. This staff member also serves as Chairperson and coordinator for the Project Group on Exposure and Metabolic Mechanisms. The Journal of Environmental Science and Health has devoted an entire issue to publishing 20 "Position Papers" which were used at the Project Group's "Workshop on Exposure to Environmental Agents, Their Metabolism and Mechanisms of Toxicity" and this collection of position papers will accompany the Fifth Annual Report to Congress.

The most significant workshop, mentioned in last year's report, was the Second Annual NCI/EPA/NIOSH Collaborative Workshop on Environmental and Occupational Cancer held in September, 1981. However, considerable work was expended in this reporting period on the preparation of the Proceedings of this Workshop. These Proceedings, improved in format and numbering 799 pages, were published in May, 1982.

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. These organizations have been involved in processes of chemical nomination and class studies in order to take full advantage of their expertise. A trade union has been similarly involved in a large class study of dyes in concert with the concerned trade association.

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 interfaced between the institute's epidemiologists and industrial organizations to effect collaborating projects. One large successful study has been initiated and four more are in the very preliminary stages.

In one continuing contact, this office has provided 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 50 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 Pollutants, Drugs and Medical Devices and Cosmetics

Several reports have been written this year resulting from tasks performed under various contracts as follows:

- 1) "Organic Air Pollutants: Carcinogens, Mutagens and Promoters" - This is a government report containing data on organic air pollutants that were evaluated for carcinogenicity and mutagenicity in the published literature. This report was compiled by SRI under contract with NCI and contains data on 25 carcinogens, 20 suspect carcinogens, 15 tumor promoters/cocarcinogens and 50 mutagens.
- 2) "Genotoxic Assessment of Selected Drugs and Medical Procedures" - This two-volume report was prepared under a BOA with Tracor-Jitco, Inc., and contains IARC-style monographs for 109 drugs and 15 medical procedures. The monographs are grouped according to their use and summary tables for each class contain data on test results for in vitro and in vivo carcinogenicity including human epidemiology. In addition, estimates of exposure and metabolism were provided as new information to monographs prepared previously and new information on published IARC monographs was also included.
- 3) A second BOA task involves the preparation of IARC-style monographs concerning "The Potential Carcinogenicity of Cosmetic Ingredients." These monographs are being prepared by Tracor-Jitco, Inc., and this document will be completed at the end of FY82. The contractor will also update 12 monographs prepared by SRI, who completed the first phase of this project in FY81.

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 200 chemicals 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.

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 to 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

As a follow-up of the Workshop on the Collaborative Programs of NCI/EPA and NCI/NIOSH, work was completed and a rather comprehensive Proceedings of 799 pages was published in May 1982 with 350 copies distributed. Beyond our initial distribution list, numerous requests have been received for this important document. In some respects this documentation reveals the progress and achievements of these significant collaborative projects and work in the field of environmental and occupational carcinogenesis.

Work on the preparation of the PHS-149 report, "Survey of Compounds Which Have Been Tested for Carcinogenic Activity" was completed for the years 1974-75 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 are being 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 200 chemicals 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.

An extensive compendium on "Genotoxic Assessment of Selected Drugs and Medical Procedures" was prepared in monograph style (Parts I & II). Final review of these two volumes will be made by an authoritative review body, with an added section on carcinogenic assessment (section 4.3) similar in format to IARC monographs.

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 carcinogenic water contaminants and atmospheric pollutants, 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.

Publications

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. Science and Health. (In press)

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Kelsey, M. I., Kraybill, H. F., Helmes, C. T., Sigman, C. C.: A data base of organic pollutants that have been evaluated for carcinogenicity and mutagenicity. In Cooper, J. A. and Malek, D. (Eds.): Residential Solid Fuels, Environmental Impact and Solution. Portland, Oregon, Oregon Graduate Center, 1982, pp. 577-605.

Kelsey, M. I.: In vitro effect of bile acids. In Autrup H. and Williams, G. M. (Eds.): Experimental Colon Carcinogenesis. CRC Press Inc. (In press)

Kelsey, M. I., Pienta, R. J.: Transformation of hamster embryo cells by neutral sterols and bile acids. Toxicology Letters 9: No. 177-182, 1981.

Kraybill, H. F.: Effect of processing on nutritive value of food: Irradiation. In Rechcigl, M. (Ed.): Handbook of Nutritive Value of Processed Food. Volume 1, Food for Human Use. CRC Press, pp. 181-208, 1982.

Kraybill, H. F.: Multimedia exposure and concerns for a holistic approach toward assessment of risk for environmental carcinogens. J. Environ. Science and Health. (In press)

Kraybill, H. F.: Nutritional impacts on cancer risk. In Proceedings of the Toxicology Forum, 1982 Annual Winter Meeting, Arlington, Virginia, pp. 145-165, 1982.

Proceedings of the Second NCI/EPA/NIOSH Collaborative Workshop: Progress on Joint Environmental and Occupational Cancer Studies (September, 1981). Editors: H. F. Kraybill, I. C. Blackwood and N. B. Freas. National Cancer Institute, Bethesda, Maryland, April 1982. GPO 1982-361-132-561.

CONTRACT NARRATIVES

OFFICE OF THE SCIENTIFIC COORDINATOR FOR ENVIRONMENTAL CANCER

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AMERICAN HEALTH FOUNDATION (N01-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 (ACN) has been shown to be quite toxic in these systems, but this has been overcome in cocultivation studies of rat liver hepatocytes (activation) with Chinese hamster ovary (CHO) cells (target cells). Thus far, data indicate that ACN induces Sister Chromatid Exchange (SCE); other monomers are under study.

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 occurrences of environmental carcinogens and will be important information to include in our other contract (N01-CP-05633-01) concerning the carcinogenicity of drugs and medical procedures.

Proposed Course: The contractor will examine the properties of ACN and other monomers and polymers toward CHO cells using HPC as an activation system. End points which will be studied include DNA repair, SCE and cytotoxicity. Other activation systems such as mouse and hamster HPC may be examined.

Date Contract Initiated: September 1, 1981

Current Annual Level: \$99,037 (pass-through money from FDA)

ARTHUR D. LITTLE, INC. (N01-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. A secondary objective is the incorporation of an intact rat hepatocyte 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 efforts at using intact rat hepatocytes as the source of metabolic activation have generally proven to be less than satisfactory. There are severe restrictions as a result of cell toxicity to the C3H cells, and the validity of the results, whether positive or negative, is questionable. The contractor has determined, as did the contractor of the parallel contract, that cell synchronization is of minimal value in enhancing sensitivity but that splitting the cultures, at a suitable interval after treatment, does appear to increase the number of Type III foci.

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: The final year of this contract will be devoted primarily to testing coded samples, in parallel with a similar contract, and utilizing, for both, defined protocols, so that an adequate evaluation of the assay system can be made by the end of the fifth and final contract year.

Date Contract Initiated: September 30, 1978

Current Annual Level: \$245,000

CENTERS FOR DISEASE CONTROL (Y01-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 regarding to the incidence of cancer.

Major Findings: The annual update of the cohort registry has been completed. A great deal of effort has been placed on developing a comparative high exposure/low exposure clinical study. Considerable attention was devoted to working with CDC staff on the protocol, documents, procedures and laboratory testing aspects of the study--which will encompass 390 subjects. Two pilot clinics were successfully conducted in February 1982. Manuscripts submitted for publication were "Serial PBB Levels, PCB Levels and Clinical Chemistries in Michigan's PBB Cohort" and "Partitioning of PBB in Human Tissues, Fluids and Feces." The contract was

peer reviewed (comments and recommendations not yet received) in April by an ad hoc committee of the Committee to Coordinate Environmental and Related Programs.

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: Cohort update will be continued in its sixth year. The high exposure/low exposure clinical study will be actively pursued.

Date Contract Initiated: April 8, 1976

Current Annual Level: \$165,000

ENERGY, DEPARTMENT OF (N01-CP-90202) (Subcontractor: Associated Universities, Brookhaven National Laboratory,

Title: Project to Coordinate Information and Technological Transfer in Environmental Mutagens and Carcinogens

Contractor's Project Director: Dr. Alexander Hollaender

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

Objectives: The National Cancer Institute, in collaboration with EPA, NIEHS and DOE, supports training centers for instruction of national and international scientists, through leadership of Dr. Hollaender, on procedures and information and data bases in the area of mutagenesis and carcinogenesis.

Major Findings: While this project was continued with no additional funding, for all practical purposes the monitoring and supervision of this project was terminated with final rendition of reports.

Significance to Biomedical Research and the Program of the Institute: This training program prepares a cadre of trained professionals in this specialized area which can relate to program managers and science administrators at NCI. This has both a direct and indirect spinoff to contract and grants projects supported by NCI.

Proposed Course: This project was terminated March 1982.

Date Contract Initiated: April 1, 1979

Current Annual Level: No funds allocated in FY82

ENVIRONMENTAL PROTECTION AGENCY (Y01-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: In response to requests from OMB and the U. S. Congress, NCI entered into a collaborative agreement with EPA to jointly conduct projects on environmental cancer (experimental, epidemiological and information resources).

Major Findings: As to information resource, one project has resulted in the annual publication of a comprehensive report on "Chemicals Found in Human Biological Media: A Data Base." This is a data base made available to Federal agencies and state health departments. The other projects in the experimental and epidemiology areas have shown some important breakthroughs on the role of environmental chemicals in cancer induction. In one project, a Gulf Breeze, Florida laboratory, in studying tumorigenesis in aquatic animals, has shown that one species, the mullet, handles the carcinogen BAP the same as the rodent. Trifluralin given to fish produces an unusual spinal defect which may be a preneoplastic lesion.

Significance to Biomedical Research and the Program of the Institute: Until this program was mandated, it was not feasible to really emphasize adequately the significance of studying, extensively, environmental chemicals in various media (air, water, and diet) and how these impact on human cancer. These projects provide for some visibility in this area. This emphasis provides NCI with some important data on environmental cancer, as well as supporting the mission and program initiatives of EPA in environmental health and regulation and control of environmental insults. Studies on water carcinogens, UV radiation, hexachlorobenzene, asbestos, and other atmospheric carcinogenic contaminants are examples of problems addressed. This program is being followed with much interest by the U. S. Congress.

Proposed Course: Many of the projects are planned for 3-5 years. At our Annual Workshop, suggestions for new areas to be covered were considered. The EPA projects are slanted more to experimental studies, while NCI projects are epidemiological, experimental and information resource. An effort will be made to maintain some representative balance and to keep an equal distribution of projects and funding.

Date Contract Initiated: June 22, 1978

Current Annual Level: \$3,090,045 (\$1,890,045 to NCI and \$1,200,000 to EPA)

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (N01-CP-15751)

Title: Program on Evaluation of Carcinogenic Risk of Chemicals to Man

Contractor's Project Director(s): Dr. Lorenzo Tomatis and Mr. Julian Wilbourn

Program Director: Dr. H. F. Kraybill

Objective: The IARC prepares monographs on environmental chemicals relevant to evaluation of carcinogenic risk of these chemicals to man. Implementation of the IARC Monograph Program involves three steps: 1) the collection of data relevant to the assessment of carcinogenic risk of these chemicals to which humans may be exposed, 2) the critical analysis and evaluation of these data by IARC staff and international working groups of experts in chemical carcinogenesis and epidemiology, and 3) the editing and preparation of authoritative monographs and distribution of these monographs assembled in volumes. NCI receives 400 copies of these volumes (about 3 times per year) for distribution. Ancillary to this project, another project is supported to collect data on testing of chemicals for carcinogenicity in laboratories around the world including the USA (NCI and NTP). This data is compiled into an Information Survey Bulletin. This Bulletin is prepared and distributed every 18 months.

Major Findings: To date, 29 volumes of the monographs have been issued. Also issued this year was Volume 9 of the Information Survey Bulletin. Both of these efforts are indispensable since they are widely used reference sources which are authoritative and quoted by all scientists in this field.

Significance to Biomedical Research and the Program of the Institute: As an information and data resource, the IARC Monographs have become an invaluable reference to provide instantaneous evaluations and judgements regarding appraisal of carcinogenicity for about 587 chemicals, with the list expanding. The chemicals are selected on the basis of use and structure class. IARC also publishes supplements on methodology and recently compiled a list of chemicals which are carcinogenic to man and experimental animals. The IARC is now publishing monographs on chemical processes such as woodworking, leather working and rubber processing with attention to epidemiological studies. The Information Bulletin is a useful reference source for laboratory workers engaged in chemical carcinogenesis.

Proposed Course: The contractor will continue on this cost-sharing project (USA contributes 60-70%; the remaining funds from come member countries) and will convene advisory working groups 3 times a year in connection with monograph preparation. IARC staff will continue to compile data for the Information Survey Bulletin. A cooperative agreement for 3 years has been set up rather than continue with the usual contract which was extended for only one year as a JNCP contract.

Date Contract Initiated: May 15, 1974

Current Annual Level: \$287,960 (seven-month extension to facilitate negotiation of cooperative agreement)

LAWRENCE JOHNSON ASSOCIATES (N01-CP-15746)

Title: Support of Second Workshop on NCI/EPA and NCI/NIOSH Collaborative Programs on Environmental and Occupational Carcinogenesis

Contractor's Project Director: Ms. Gana Browning

Project Officer (NCI): Ms. I. C. Blackwood

Objectives: The objective of the Second Annual NCI/EPA/NIOSH Workshop on Collaborative Projects on Environmental and Occupational Cancer was to report on progress and decide on future direction of projects carried out under two interagency agreements between the National Cancer Institute and the Environmental Protection Agency and between the National Cancer Institute and the National Institute for Occupational Safety and Health, respectively. The purpose of this contract is to provide conference support and assistance in editing the proceedings resulting from this workshop.

Major Findings: The proceedings of this Workshop have been published and distributed to approximately 350 people.

Significance to Biomedical Research and the Program of the Institute: This workshop provides a forum for the exchange of findings and information developed from the various projects sponsored under the two interagency agreements on environmental and occupational cancer. The results of these studies have potential applicability for protecting not only workers but the general population from exposure to carcinogens both in the workplace and the environment.

Proposed Course: This was a one-year contract which expired in April 1982.

Date Contract Initiated: March 31, 1981

Current Annual Level: One year contract, therefore, no funds for FY82.

MICROBIOLOGICAL ASSOCIATES (N01-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: There are indications from the rerunning of selected coded compounds that the S-9 activation system does make a contribution to the sensitivity of the C3H 10T-1/2 assay. In addition, the utilization of the splitting of the culture at a proper interval after treatment (Phase II) does enhance the development of Type III foci and thus the sensitivity of the compound. Cell synchronization has not proven to be a significant factor in detecting positive compounds.

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: The final year of this contract will be devoted primarily to testing coded samples, in parallel with a similar contract, and utilizing, for both, defined protocols, so that an adequate evaluation of the assay system can be made by the end of the fifth and last contract year.

Date Contract Initiated: September 30, 1978

Current Annual Level: \$245,000

MICROBIOLOGICAL ASSOCIATES (N01-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, 49 compounds have been reported upon and 23 compounds remain to be tested.

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 the next year as candidate compounds are identified by a variety of mechanisms and organizations. The Project Officer will coordinate 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: \$306,371

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 91 distinct projects have been initiated; 29 are still in progress, and it is anticipated that of that number 13 should be completed by the end of FY 82. Many of the projects were reported on in the combined NCI/EPA/NIOSH Collaborative Workshop held in September 1981.

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: The ongoing projects will be continued as dictated by their objectives and workscopes following a review to be held in August 1982 by a combined ad hoc review group of senior staff members of both Institutes. New projects will be begun as a result of initiation by the staff of either organization, relevance and technical review by the ad hoc review group and approval by the DCCP Board of Scientific Counselors. Because of budget restrictions, each Institute participant will be held, for FY 83, to a total expenditure (continuing and new projects) of 1.2 million dollars.

Date Contract Initiated: September 23, 1976

Current Annual Level: \$2,600,000

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 writing, 37 compounds have been reported upon and 19 compounds remain to be tested.

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 the next year as candidate compounds are identified by a variety of mechanisms and organizations. The Project Officer will coordinate 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 (N01-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. Tucker Helmes

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

Objectives: This project is an important information and data resource. The project has five tasks as follows: 1) Support to NCI Chemical Selection Working Group; 2) Support to IARC for monograph preparation; 3) Updating and expanding data base on exposures and data base on mutagens, carcinogens, co-carcinogens and promoters; 4) Data processing services and support; and 5) Overall support to DCCP and Office of Environmental Cancer on resource research documents on environmental cancer. In addition, a task was added to give support to the National Library of Medicine.

Major Findings: This project is in the third year of a planned five-year project. It provides information and data on summary sheets which are used in NCI's chemical selection process for chemicals to be tested in the National Toxicology Program (NTP). The contractor furnishes production, exposure and chemical data on chemicals that are entered into the IARC monographs. SRI has completed a series of Class Studies (use and structure class), which will be published, that serve

as a basis for chemical selection. SRI has prepared reports on carcinogens in air and water which are utilized in the carcinogenesis program and as a basis for selection of environmental chemicals to be tested. This contract project has shifted in emphasis as a predominant resource to assist CSWG to perform special studies and resource needs regarding data on environmental carcinogens and publication of compendiums on carcinogens which serve as resource/reference documents.

Another significant development in this project was the availability, in hard copy, of a data base on carcinogens, mutagens, cocarcinogens and promoters. This data base will be accessible for "on line" search at DCRT. This year a compendium was made available entitled "Bioassay Summary Reports," which are abstracts on over 200 chemicals published in NCI/NTP Technical Reports.

Proposed Course: The SRI project will continue to support needs on chemical selection process, but at a reduced effort of 20 summary sheets per year (about one-half production of last year). Under Task V, more subtasks have been outlined for preparation of special reports on environmental carcinogens which can be used by NCI and DCCP as authoritative reference sources.

Significance to Biomedical Research and the Program of the Institute: This project continues to be one of the most important resources for NCI, NTP and other agencies for information on environmental chemicals. The reference documents prepared have been most useful to NCI in the carcinogenesis testing and research programs. The resource is used in preparation of lists of chemicals for carcinogenesis testing and research under the Biomedical Research and Training Act.

Date Contract Initiated: September 30, 1979

Current Annual Level: \$872,100

TECHNICAL RESOURCES, INC. (N01-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 project provides a resource for preparation of PHS-149, for the missing years. This survey is made from literature worldwide.

Major Findings: Since there was a lapse in publication of this widely used resource report, a previous contractor produced a report for 1978. The current contract, instituted in April 1981, will produce, over a 3 year period, Volumes for 1974-75, 1976-77 and 1979-80. In the future a contract will be secured to keep this publication current.

Significance to Biomedical Research and the Program of the Institute: This report provides the information resource for staff at NCI, academia and other research organizations setting forth data gleaned from the world literature (about

650 journals) on chemicals bioassayed for carcinogenicity. This report, first published in 1951, has come to be recognized as an indispensable standard reference source for workers in the field of carcinogenesis. NCI distributes these surveys and the Superintendent of Documents (GPO) retains some for sale.

Proposed Course: The first camera-ready PHS-149 report was made available in April 1982, but printing has been held up temporarily due to a moratorium on printing imposed by the government.

Date Contract Initiated: March 31, 1981

Current Annual Level: \$268,220

TRACOR-JITCO, INC. (N01-CP-05633-01)

Title: Carcinogenicity of Drugs and Medical Procedures

Contractor's Project Director: Dr. Harold Seifried

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

Objectives: The project provides resource information on the chemical and biological activity of drugs and medical procedures.

Major Findings: The contractor has prepared and delivered its final report "Genotoxic Assessment of Selected Drugs and Medical Procedures," which contains monographs on 109 drugs and 15 medical procedures. This document will now be reviewed and evaluated by an "Expert Panel" and will be published as a Government report.

Significance to Biomedical Research and the Program of the Institute: The bound report of monographs dealing with the potential carcinogenicity of drugs and medical procedures 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: Contract completed in May 1982.

Date Contract Initiated: May 18, 1981

Current Annual Level: \$99,988

TRACOR-JITCO, INC. (N01-CP-05633-02)

Title: Carcinogenicity of Cosmetics

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 is preparing approximately 50-75 IARC-style monographs on currently used cosmetic ingredients that were approved.

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: The project is operating in its second year with a new contractor and Dr. Seifried has begun to prepare monographs on those cosmetic ingredients which have published in vitro and/or in vivo carcinogenic activity. In addition to the new monographs, the contractor will update the monographs prepared last year by the other contractor. As with the drug monographs, this report, which is scheduled to be completed in late September, 1982, will be reviewed by an outside Steering Committee and eventually will be evaluated by an "Expert Panel" as explained previously.

Date Contract Initiated: September 30, 1981

Current Annual Level: \$99,322

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Registry of Experimental Cancers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Harold L. Stewart	NIH Scientist Emeritus	DCCP	NCI
Other	Bernard Sass	Veterinary Medical Officer	DCCP	NCI
	Margaret K. Deringer	Guest Scientist	DCCP	NCI
	Cornelia Hoch-Ligeti	Guest Scientist	DCCP	NCI
	Carel F. Hollander	Guest Scientist	DCCP	NCI
	Annabel G. Liebelt	Biologist-IPA Fellow	DCCP	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Office of the Director

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

4 1/2

PROFESSIONAL:

2 1/2

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

The Registry has acquired a total of 2129 (150 since the 1981 report) single or group accessions from investigators outside the NCI. Approximately 57,800 (2800 since the 1981 report) records have been coded.

Thirty 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 2 months.

Project Description

Objectives: 1) The storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents) and 2) the use of such 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 and 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 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 2129 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 *Pracomys (Mastomys) Natalensis*," "Malignant Schwannomas of Rats," "Harderian Gland Tumors of Mice," and "Renal Tumors of Rats." The last three Study Sets have been completed since the 1981 Annual Report. These Study Sets, with descriptive material, are loaned to investigators who request them. Forty-four loans of up to two months have been made this year.

Investigators come to the Registry for study and consultation. There have been single or multiple consultations with 30 individuals since the 1981 report. One foreign investigator has been a guest at the Registry for 2 months.

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 standardization 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 the weekly slide conferences held in Room 1D16 of the Landow Building.

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. The report entitled, "Histologic Typing of Liver Tumors of the Rat" was published in January 1980 (JNCI 64: 177-206, 1980). During the period from the date of publication until April 2, 1982 the Registry has received 8518 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 (WHO) 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, who 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:

Henry, C.J., Billups, L.H., Avery, M.D., Rude, D.R., Dansie, D.R., Lopez, A., Sass, B., Whitmire, C.E. and Kouri, R.E.: Lung cancer model system using 3-methylcholanthrene in inbred strains of mice. Cancer Res. 41: 5027-5032, 1981.

Hoch-Ligeti, C., Congdon, C.C., Deringer, M.K., and Stewart, H.L.: Primary tumors of the spleen in guinea pigs. Toxicol. Pathol. 9: 9-16, 1981.

Komitowski, D., Sass, B. and Laub, W.: Rat mammary tumor classification: Notes on comparative aspects. JNCI 68: 147-156, 1982.

Kouri, R.E., Billups, L.H., Rude, T.H., Whitmire, C.E., Sass, B. and Henry, C.J.: Correlation of inducibility of aryl hydrocarbon hydroxylase with susceptibility of 3-methylcholanthrene-induced lung cancer. Cancer Letters 9: 277-284, 1980.

Sass, B.: Mixed mesenchymal tumours of the mouse uterus. Lab. Anim. 15: 365-369, 1981.

CARCINOGENESIS INTRAMURAL PROGRAM
ANNUAL REPORT OF THE LABORATORY OF BIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

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, 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 occurring during carcinogenesis.

New mammalian cell transformation models are being developed for identifying genetic factors involved in cell transformation, for determining the relationship between differentiation and transformation, and for determining the crucial processes of transformation. The current investigations utilize human fibroblast cells from cell strains and freshly derived fetal material. We have demonstrated that the sensitivity of cells to transformation can be increased by a metabolic block by specific amino acid deficiencies. Consequently, it is now possible to obtain a carcinogen dose-dependent transformation frequency as indicated by the ability of the transformed cells to form colonies in agar. Carcinogenic agents, both chemical and physical, that are effective in inducing transformation do so at the same concentrations as those used for animal cells; the transformation frequencies obtained approximate those observed in well-established experimental models. These studies should lead to a rapid, reliable, and quantitative system for the assay of neoplastic transformation in human cells. Because carcinomas become predominant in humans between the second and third decades, transformation models utilizing epithelial cells must be developed. These would provide specific biological markers associated with differentiation, be relevant to the process of carcinogenesis, and provide a basis for studying at the cellular level, the intervention or prevention of organ specific cancer.

The number of chromosome translocations and markers identified in human leukemias, lymphomas, and solid tumors far exceed those that have resulted from experimental manipulation of cells that lead to malignancy. Suspending the cells from surgical

human cancer specimens directly into medium with agar for the selection of tumor cells capable of forming colonies resulted in tumor clones with a variety of chromosome markers. This contrasts with the chromosome analysis of human cells transformed with known carcinogenic chemicals. These experimentally developed transformed cell lines have diploid stem lines and rarely exhibit any abnormal chromosomes. Furthermore, in all experimentally derived lines the constitutive heterochromatin distribution was also normal and no deviation of normal nucleolar organized stained regions was observed. Consequently, the experimentally derived transformed lines might not yet possess the full range of characteristics associated with human cancer.

We have transformed rat mammary epithelial cell in vitro by 7,12-dimethylbenz[a]-anthracene (DMBA), the first demonstration of transformation of mammary cells in vitro. The tumors derived from these cells are carcinomas which contain estrogen receptors. These encouraging results dictate that the same protocol should be successful in transforming human mammary epithelial cells.

The cytogenetic characteristic of DMBA transformation of rat fibroblast cells is involvement of the first two chromosomes of the rat complement. Premetaphase banding analysis of transformed mammary cells showed a change in chromosome balance, trisomy or monosomy for a number of chromosomes. A marker derived from chromosome three was found in all transformed mammary cell lines examined; therefore, the marker is considered a nonrandom change associated with mammary epithelial cell transformation. This chromosome may have particular significance since it is often associated with the nucleolar organizing function.

The importance of DNA metabolism to carcinogenesis is validated by studies involving transformation by environmental agents including alkylating chemicals. Coordination of DNA repair with in vitro transformation studies indicates that increased transformation frequency resulting from interaction of two carcinogens cannot be due to either decreased excision or post-replication repair since the amount of unrepaired DNA in the combination is the same as that obtained by single treatment. Furthermore, the studies showed that insulted cells may continue to survive and replicate with persisting DNA damage. Because the gaps in the damaged DNA are subsequently filled by an unknown process, the nature of the genetic information inserted in a DNA opposite altered bases is of paramount importance. It has been hypothesized that slow removal of O^6 -methylguanine is the cause for carcinogenesis induced by methylating agents. With a series of alkylating agents and concentrations that induce equivalent transformation frequencies in Syrian hamster embryo cells, a similar number of O^6 -methylguanines were found. Furthermore, hamster embryo cells have only a limited capacity to remove any specific lesions. Therefore, O^6 -methylguanine appears to be the lesion responsible for initiating carcinogenesis induced by methylating agents. The importance of DNA replication to carcinogenesis is also clear from studies utilizing density-dependent inhibition of replication cultures for transformation and bisulfite, a nonmutagenic agent that induces transformation. After carcinogen treatment of confluent cell cultures, only a small population of the cells go through a complete "S" phase and subsequent mitosis. The transformed colonies observed subsequent to reseeding are derived from this population. Kinetic studies demonstrate that only these cells become transformed even though both dividing and nondividing cells have survived carcinogen treatment. Failure to subculture the cells for an additional interval eliminates the transformation frequency.

Thus, DNA synthesis is temporally related to an early step in carcinogenesis. Bisulfite, an effective transforming agent, has no effect on excision or post-replication repair, on sister chromatid exchanges or chromosome aberrations. However, bisulfite did inhibit overall DNA replication, apparently by inhibiting a fraction of the replicons from operating. Results suggest that bisulfite produces transformation by a nonmutagenic type event. To further study the nature of the transformed state, current experiments involve transfection with DNA extracts from transformed cells. In the first part of this study, DNA sequences responsible for the induction and maintenance of the transformed state are being isolated and characterized. Transformed foci have been isolated from mouse cells transfected with DNA derived from tumorigenic hamster cell lines obtained after carcinogen treatment. DNA extracted from these foci have been used successfully for a second round of transfection. These have been reisolated and the DNA purified for further characterization. Concurrently, middle repeat sequences of hamster cell DNA are being isolated for use as a probe for identifying the hamster DNA sequences in the transformed foci.

Lymphokines are biologically active hormone-like substances produced by stimulated lymphocytes. Lymphotoxin, a lymphokine produced by mitogen or antigen-stimulated lymphocytes, inhibits tumor cell growth and is anticarcinogenic. The anticarcinogenic and tumor cell growth inhibitory activities of lymphotoxin operate through diverse mechanisms. Colony formation assays demonstrate that lymphotoxin inhibition of tumor cell growth is predominantly cytostatic (reversible) since cell proliferation resumes after removal of the hormone. The indirect tumor cell growth cytolytic activity of lymphotoxin may be an important part of the anticarcinogenic activity spectrum of this lymphokine because lymphotoxin's direct tumor cell growth inhibitory activity is often 10-fold or more less effective per unit activity than is the anticarcinogenic activity of the hormone. If, however, the tumor-producing cells were treated with lymphotoxin followed by natural killer cells or macrophages, the cytolytic destruction increased compared to cytolysis, obtained by natural killer cells or macrophages alone. Thus, lymphotoxin may act through an amplification mechanism for the control of cancer development and be effective at early and later stages in carcinogenesis.

Lymphotoxin has an anticarcinogenic effect on both promoted and nonpromoted hamster cell transformation. Furthermore, there are differences in cell sensitivity to lymphotoxin of these two types or stages of carcinogenesis suggesting that lymphotoxin acts by different mechanisms to inhibit transformation. Phytohemagglutinin (PHA) also inhibits promoted transformation; however, its mechanism differs from that of lymphotoxin. PHA has no effect on transformation induced by UV irradiation alone but causes a dose-dependent decrease in 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-promoted, UV-initiated transformation. The PHA inhibition of TPA-promoted transformation is reversible because PHA is effective only if present with, but not before, TPA. Lymphotoxin, on the other hand, has a persistent anticarcinogenic effect, regardless of whether added prior to or after TPA or even before any carcinogenic insult. The receptors for TPA and PHA are functionally discrete and may be in close proximity or even coexist on the same glycoprotein. Promoted transformation can be inhibited by several interventive measures. Lymphotoxin alters the physiological state of the cell, causing a change in the cellular response to TPA. Steric hindrance of TPA binding due to the presence of lymphotoxin or of PHA on the cell surface could also inhibit TPA-promoted transformation. Lymphotoxin also induces synthesis of high

molecular weight (approximating 200,000 daltons) cell surface membrane glycoproteins in nontransformed cells, whereas it inhibits this glycoprotein synthesis in tumor cells. Moreover, these membrane changes occur within hours following binding of lymphotoxin to the cell and coincide with the kinetics of the anticarcinogenic action of the hormone. These are the first identifiable biochemical alterations that result from lymphotoxin interaction with mammalian cells and correlate with the anticarcinogenic and tumor cell growth inhibitory activities of the hormone.

The biphasic inhibition of carcinogenesis and tumor cell growth frequently observed *in vivo* and *in vitro* with respect to the number of normal lymphocytes may reflect immunomodulation of lymphotoxin by the lymphocytes or other cells in the leukocyte populations. This biphasic inhibition of carcinogenesis and tumor cell growth is eliminated and becomes proportional to leukocyte number if the lectin and lymphotoxin-stimulator, phytohemagglutinin, is also present or if lymphotoxin itself is added to either the admixture of lymphocytes and cells undergoing carcinogenesis or to lymphocytes and tumor cells. Such modulation of natural lymphoid cell cytotoxicity may be one means by which the individual host or developing cancer cell circumvents and escapes any inherent homeostatic mechanisms for the prevention and control of cancer.

Anticarcinogenic and tumor cell growth inhibitory activities of lymphotoxin have been defined by *in vitro* carcinogenesis models. *In vitro* systems allow the study of interactions during carcinogenesis unencumbered by host homeostatic or other influences. Lymphotoxin's prevention of transformation is relevant only if it exerts antineoplastic activity *in vivo*. Lymphotoxin injected into guinea pigs retards the growth of transplanted tumor cells. Moreover, lymphotoxin injected simultaneously with either diethylnitrosamine or with the gamma emitting radio-nuclide, ⁹⁹Technetium, into pregnant hamsters decreased the frequency of transformed cells subsequently isolated from embryos. Thus, *in vivo* lymphotoxin is anticarcinogenic and inhibits tumor cell growth.

The multifaceted but coordinated plan of the Laboratory of Biology which involves separating and analyzing biological steps responsible for neoplasia will lead to an understanding of the underlying biochemical and molecular basis of carcinogenesis. Quantitative transformation systems using human cells from diverse organs are being developed and used to study the modulation of carcinogenesis in terms of somatic and cell surface changes. The ability to focus on target cells and to probe the changes induced by chemical or physical carcinogens during carcinogenesis is relevant to understanding the development of cancer in humans.

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

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

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.A. DiPaolo	Chief	LB NCI
OTHER:	J. Doniger	Expert Scientist	LB NCI
	C.H. Evans	Chief, Tumor Biology Section	LB NCI
	N.C. Popescu	Senior Staff Fellow	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:

5.5

PROFESSIONAL:

3.5

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Stages leading to neoplasia of human and hamster cells are being dissected to determine their biological importance in chemically or physically induced carcinogenesis. After synchronization by metabolic block, cells derived from a carcinogen-treated standard human cell strain show dose-related colony growth in agar. These colonies form multifoci that invade chick embryonic epidermis. Promoted and nonpromoted hamster cell transformations have a differential sensitivity to lymphotoxin. Furthermore, lymphotoxin is inhibitory even prior to carcinogen insult. Promotion is also inhibited by phytohemagglutinin (PHA) but only in the presence of a promoter. Lymphotoxin induces a new physiological state, whereas PHA hinders promoter binding to the cell surface. Although a positive correlation exists between the induction of sister chromatid exchanges, cytotoxicity and transformation, promotion is not associated with chromatid alterations. DNA metabolism studies indicate that O6-methylguanine is the lesion responsible for the initiation of carcinogenesis induced by methylating agents. Finally, transfection studies indicate that DNA sequences induced by carcinogen treatment are responsible for the initiation and maintenance of the transformed state.

Project Description

Objectives: The overall approach to problems in carcinogenesis will be to continue to investigate the modulation of transformation of human and other cells, vital aspects in the etiology and prevention of cancer. Whereas cell biology was emphasized in the past, molecular biological approaches are increasingly important in the elucidation of the objectives of the laboratory. Our 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 chromosome 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 phenomena *in vitro*. Such procedures are required to determine whether or not the transformation observed is due to the direct or indirect effect of the carcinogen and in order to study the early events associated with *in vitro* transformation. Cells are derived from freshly isolated cells from animals and humans 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 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 for detection of acute carcinogen-induced damage include sister chromatid exchange (SCE), chromosome aberrations and micronuclei identification are utilized. 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: The concept of transforming fresh human foreskin cells and standard human embryonic cell strains by inhibiting DNA synthesis with a selective metabolic block originated in this laboratory. Nonmalignant normal human cells grow with a relatively long doubling time and have subpopulations capable of different scheduled and/or unscheduled DNA repair synthesis. Such proliferating populations repair over 90% of the damage caused by chemical carcinogens in approximately 6 hrs and only rarely transform. The synchronization of cells in the G₁ phase of the cell cycle and the use of substances, primarily hormones,

in the G₁ phase of the cell cycle and the use of substances, primarily hormones, to increase DNA synthesis when added during the early S phase increases the transformation frequency significantly. Relationship between dose of carcinogen and transformation is indicated by the acquisition of anchorage independent growth. Colony growth in agar indicates an inductive phenomenon and implies dose-dependency. Since the expression of a transformed phenotype after carcinogen insult requires cell multiplication in mass cultures, a precise measure of the relationship of the number of cells treated with carcinogens to the colony frequency in agar is not obtained. Nevertheless, the frequency of colony growth of human cells exposed to carcinogen and then placed in soft agar may approach that of similarly treated rodent cells (UV 10⁻³). Cells derived from agar which are dispersed and grown usually produce tumors after either intracranial or subcutaneous induction. In both cases the growth is limited. In the brain the cells distort the architecture of the brain and cause neurological abnormalities; after a short time death results. Histological examinations of brain section shows undifferentiated fibrosarcomas. A similar tumor type is also found when the cells are recovered from subcutaneous injection. In both cases, however, limitation of tumor growth suggests that nude mice are not the ideal host for demonstrating neoplasia of experimentally transformed human cells. Since a similar situation often occurs upon injection of tumors derived from humans, different xenobiotic systems are being explored. We are optimistic about our current results utilizing chick embryonic skin in vitro as a substrate for demonstrating the neoplasia of human cells. When human cells from control and treated populations are seeded on the dermal side of chick embryonic skin, only the transformed cells form multifoci that invade the epidermis. After 72 hrs growth, the skin can be fixed and prepared for histological examination. Invasiveness, an unequivocal property of malignancy, is not observed when experimentally derived transformed human cells are injected subcutaneously into nude mice.

Growth factor supplements enhance the growth of a variety of cells and can be substituted for the complex and unknown variables associated with feeder cells or serum. For example, human endothelial cells produce a factor that can be used to replace feeder cells in the growth of hybridoma cells for monoclonal antibody production. Human endothelial cell factor, however, is not available commercially. Endothelial cell growth supplement (ECGS), available as an extract of bovine neural tissue, promotes the growth of human endothelial and epithelial cells, mouse BALB/3T3 fibroblast cells, and colony size of hamster embryo cell strains. The addition of 50 or 100 µg of ECGS/ml medium can substitute for X-irradiated feeder cells in a quantitative 7-day Syrian hamster embryo cell (HEC) colony assay for transformation. The transformation and normal colony frequencies in the presence of ECGS were similar to those obtained with feeder cells. A positive dose-response relationship occurred in transformation with carcinogens. With ECGS, a series of complex operations can be accomplished such as UV irradiation and subsequent treatments with the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and an anticarcinogenic agent such as a lymphokine. Thus, substituting ECGS for irradiated feeder cells can eliminate a potential complex source of variability in the study of cocarcinogenesis and anticarcinogenesis.

The HEC system for transformation is unique in that the transformation frequency is independent of the number of cells at risk. Furthermore, dose-dependent relationships are consistent with a zero threshold response. Nevertheless,

transformation frequency initiated by a relatively low carcinogen concentration can be enhanced by subsequent treatment with noncarcinogens referred to as promoting agents. Such enhancement may be up to 50-fold greater when the initiating agent is a relatively low dose of either a chemical or physical carcinogen. This enhanced transformation as well as the transformation associated with relatively high concentrations of carcinogens is reduced by a hamster-derived lymphotoxin, a lymphokine. Linear regression lymphotoxin dose response curves obtained under the conditions of promoted and nonpromoted transformation show differences in cell sensitivity and suggest different mechanisms of action. The lymphotoxin inhibits the transformation frequency when given prior to any carcinogenic insult during the initiation stage between the carcinogen and TPA or when added during the promotion stage in the presence of TPA. Phytohemagglutinin (PHA) also inhibits TPA-promoted transformation. We conclude that PHA and lymphotoxin effect the biological activity of TPA by diverse mechanisms. PHA has no effect on transformation induced by irradiation of hamster embryo cells but, like lymphotoxin, causes a dose-dependent decrease in TPA-promoted transformation. As demonstrated by the ineffectiveness of concanavalin A, the PHA effect is not a general lectin phenomenon. The PHA inhibition of TPA-promoted transformation is considered reversible because PHA is effective only if present with TPA, whereas lymphotoxin has a persistent effect regardless of when added. The terminal galactose of the oligosaccharide that is the major determinant for lymphotoxin cannot be considered the major determinant for TPA binding on the cell because galactose alone does not inhibit TPA. The two receptors for TPA and PHA are, therefore, functionally discrete and may be in close proximity or even coexist on the same glycoprotein. Several interventive measures can inhibit promoted transformation. Lymphotoxin alters the physiological state of the cell, causing a change in the cellular response to TPA. Steric hindrance of TPA binding due to the presence of lymphotoxin or of PHA on the cell surface could also inhibit TPA-promoted transformation. In the final analysis, the mechanisms by which PHA or lymphotoxin inhibits TPA promotion are obviously different. Whereas either will inhibit promoted transformation when given during TPA treatment, only lymphotoxin results in inhibition when lymphotoxin or PHA are removed prior to initiation and promotion. The induction of high molecular weight glycoproteins in lymphotoxin-treated HEC suggests lymphotoxin may induce a new cellular physiological state that is responsible for its anti-carcinogenic action. A second mechanism may involve steric hindrance of TPA binding due to the presence of lymphotoxin or PHA on the cell surface. Somatic cell changes during the various steps and stages of carcinogenesis can be determined by analysis of the difference in sensitivity to lymphotoxin during stages of transformation, initiation and promotion.

Specific chromosome translocations have been identified in certain human leukemias, lymphomas, or solid tumors. The utilization of refined chromosome banding methods also permitted the detection of subtle chromosome defects in leukemias with an apparent normal chromosome constitution. Chromosome analyses of *in vitro* human material exposed to known cancer agents are essential for determining the role of chromosome changes in human neoplasia. Eight different transformed cell lines obtained after forskin treatment with β -propiolactone (β -P1), aflatoxin B₁ (Afb₁), 4 nitroquinolone N-oxide, β -propanesultone (β -PS), ethyl methylsulfoxide (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were examined. All the lines had a diploid stemline containing cells with a normal diploid number and

cells with either hypo or hyperdiploid number. The distribution of abnormal chromosomes in cell lines range from no abnormal chromosomes to as many as 5 abnormal chromosomes. Two lines transformed by EMS and one by β -PS had no abnormal chromosomes. In fact, one of the two EMS transformed lines was truly normal in terms of abnormal chromosomes or numerical deviations. Except for an abnormal chromosome derived from chromosome 11 in lines transformed β -P and AFB₁, no common abnormality was found in lines transformed by different carcinogens. The identified abnormal chromosomes were derived from chromosomes 1, 6, 7, 9, 11, 18, and X. In all lines the constitutive heterochromatin distribution was normal and no deviation of the normal nucleolar organized stained regions was observed.

The identification of specific characteristics for malignancy is important to the detection of neoplasia and to the understanding of the nature of the cancer cell. The acquisition of uncontrolled growth characteristics by neoplastic cells is not accompanied by an increase in number of sister chromatid exchanges (SCE). Human and experimentally induced cancer do not exhibit a distinctively higher SCE frequency than normal cells. However, SCE induction by alkylating agents is an indicator of malignancy. The dose response curves of SCE induced by MNNG were higher in malignant cells originating from human, Syrian hamster, Chinese hamster and mouse than the normal counterparts. The obvious explanation for the results obtained with logarithmically growing cells is the higher rate of cell division and DNA synthesis of malignant cells compared to normal cells. Experiments with G₁-arrested cells, however, demonstrated that nonreplicating malignant cells also respond with a higher SCE frequency than normal cells. Thus, malignant cells can be distinguished from normal cells by their increased SCE response to MNNG. This response is due to a decreased repair capacity associated with the acquisition of malignancy as malignant cells are deficient in removal of O⁶-methylguanine induced by MNNG. This deficiency is independent of species or whether the malignant cells were transformed by a chemical or viral agent.

Rat and human neoplasia chromosome alterations tend to cluster on certain chromosomes. Whether the causative agent is a chemical carcinogen or an oncogenic virus the first three chromosomes of the rat complement are preferentially involved in structural or numerical alterations. The first two chromosomes have regions with increased vulnerability to 7-12-dimethylbenzo(a)anthracene (DMBA) acute damage. Analysis of rat fibrosarcomas induced by in vitro transformation of rat embryo cells or by in vivo injection of DMBA showed that chromosomes 1, 2, and 3 were preferentially involved in various rearrangements. Rat mammary epithelial cells transformed by DMBA alone or DMBA and TPA treatment were analyzed cytogenetically by the high resolution banding method. All cell lines had hyperdiploid chromosome modes and chromosome markers. Prometaphase banding analysis showed that the involvement of certain chromosomes numbers 4, 9, 14 and X were frequently trisomic, whereas 8, 15 and 16 were monosomic. One line was also trisomic for chromosome 2. Some cells contained as many as 6 abnormal chromosomes. Chromosomes 1, 2, 3, and 8 participated in the formation of the majority of the markers. A marker derived from chromosome 3 was found in all three lines and is considered a nonrandom change associated with mammary epithelial cell transformation. The involvement of this chromosome may have a distinctive significance because of its association with the nucleolar organizing function. Mammary epithelial or fibroblast cells transformed by DMBA have involvement of the same chromosomes.

Thus, chromosome alterations associated with neoplastic transformation are independent of the origin of the target cells.

The ability of chemical or physical carcinogens to damage DNA and to induce SCE correlates with in vitro neoplastic transformation. A positive correlation was found between the induction of SCE, cytotoxicity and morphologic transformation by split doses of carcinogen using exponentially growing HEC. Compared to a single dose, a split dose of N-acetoxy-2-acetylaminofluorene (AcAAF) caused an enhancement of SCE response when the two exposures were separated by 2-7 hrs but not if separated by 24 hr. Split and single doses of mitomycin C (MMC) induced the same number of SCE. AcAAF and MMC induced long lived lesions responsible for SCE formation. In contrast MNNG, methyl methasulfonate (MMS) and ultraviolet light (UV) irradiation-split doses reduced the SCE frequency as compared to a single treatment. This reduction was most pronounced when 24 hrs separated the two exposures. The split dose protocol was also used in density-dependent inhibition of replication or G₁-arrested cultures. Growth-inhibited cultures have been useful for studying the mechanism(s) of chemically induced transformation and is analogous to the static growth state of the majority of human tissues. MNNG-induced lesions responsible for SCE formation persisted for 24 hrs. No difference in SCE frequency induced by a single 0.2 µg MNNG/ml or split doses separated 24 hrs was detected. Split doses of MNNG separated by shorter time intervals caused a significant enhancement in SCE frequency. This enhancement is due to cell kinetic changes caused by MNNG. With both culture conditions, less than 5% of the cells synthesize DNA; however, a larger fraction of the population (depending on the dose of carcinogen) escape from G₁ arrest and resume DNA replication. SCE formation is closely associated with replication of DNA. Thus, the first exposure to MNNG induces DNA replication in a fraction of the cells, and the second will affect the cells in the more susceptible cell cycle phase required for SCE formation. These data demonstrate that split doses of a chemical or a physical carcinogen may have an additive, enhancing, or reducing effect on SCE formation on diploid HEC depending upon the agent used. AcAAF and MMC which cause large side chain alkylation of DNA had a sustained influence on SCE frequency. MNNG and MMS which cause small modifications in DNA bases and UV light that induces pyrimidine dimers had a transient effect on SCE frequency. The effect on SCE is reflected on cell lethality and induction of neoplastic cell transformation. SCE induction by split doses of carcinogen is related to the type of carcinogen-induced DNA lesion, the ability of target cell to repair the carcinogen-induced DNA damage, changes in cell cycle kinetics and growth conditions during carcinogen insult.

DNA repair and metabolism are considered relevant to carcinogenesis but the underlying processes are still obscure. Slow removal of O⁶-methylguanine has been the basis for the hypothesis that this lesion is responsible for carcinogenesis induced by methylating agents. A positive correlation exists between the persistence of O⁶-methylguanine 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 kidney and then liver. The Syrian hamster embryo system allows for a quantitative comparison between induction and repair of methylated DNA lesions and the induction of transformation and lethality by a variety of different methylating carcinogens. We have found

that concentrations of MNNG or MMS that induce equivalent transformation frequencies in HEC induce a similar number of O⁶-methylguanine. Furthermore, HEC has a limited capacity to remove these O⁶-methyl lesions. Therefore, there are data that confirm the hypothesis that O⁶-methylguanine is the lesion responsible for the initiation of carcinogenesis induced by methylating agents.

After MNNG treatment of density-inhibited post-confluent (DDIR) HEC cultures, only a small population of the cells incorporate [³H]thymidine within the next 10-20 hrs. The transformed colonies, observed subsequent to reseeding, are derived from the population incorporating [³H]thymidine. This study demonstrates that MNNG-induced thymidine incorporation resulted from semi-conservative DNA synthesis as analyzed by DNA density gradients. Furthermore, cell cycle distributions at selected times after treatment indicates that the MNNG responsive population was released from the density dependent G₁ block, proceeded through S and became blocked again at G₂ or M. These results indicate that DNA synthesis is temporally related to an early step in carcinogenesis.

Studies with bacterial cells have demonstrated that pyrimidine dimers (the major UV photoproduct) are the lesions primarily responsible for biological effects induced by UV. These studies have been facilitated by the fact that exposure of bacterial cells to visible light specifically removes pyrimidine dimers from the DNA but all the other UV-induced lesions remain. This process is called photoreactivation. Photoreactivation is difficult, if not impossible, to demonstrate in many mammalian systems including Syrian hamster and human. However, photoreactivation can be easily accomplished in marsupial cells. Therefore, opossum cells are treated with carcinogens in order to obtain neoplastic transformation to firmly establish the role of pyrimidine dimers in carcinogenesis. Photoreactivation coupled with cell cycle kinetics to interfere with transformation will be used to dissect underlying molecular processes in the initiation of carcinogenesis. We have demonstrated that fetal opossum cells can efficiently photoreactivate pyrimidine dimers from their DNA. Furthermore, these cells also have the ability to excise pyrimidine dimers in the dark as well as human cells.

A continuing effort is being made to ascertain the interaction between DNA and nonmutagenic agents which induce transformation. Bisulfite is a compound that warrants extensive study for its effects at the cellular level because it is a food and pharmaceutical additive and it is a ubiquitous pollutant in the form of SO₂. It was recently reported that bisulfite, at neutral pH, does not act as a mutagen in either bacterial or mammalian systems but does enhance UV-induced mutation in both model systems. Therefore, bisulfite was tested in the hamster transformation system to determine if it is cocarcinogenic with UV. The results indicated that while there is no synergism for transformation with bisulfite plus UV, bisulfite alone is an effective transformation agent. The effect of bisulfite on DNA metabolism was also investigated. Bisulfite does not induce any excision or post-replication repair responses and does not effect UV-induced repair. Furthermore, neither sister chromatid exchanges or chromosomal aberrations were induced by bisulfite. However, it does inhibit overall DNA replication apparently by inhibiting a fraction of the replicons from operating. These results suggest that bisulfite induces transformation by an epigenetic rather than genetic mechanism. In further studies, bisulfite-induced tumorigenic cell lines are being isolated to determine (1) if any permanent chromosomal changes

are associated with bisulfite carcinogenesis, or (2) if new DNA sequences were formed which can transform 3T3 cells by transfection.

The nature of the DNA sequence of daughter strands replicated from carcinogen-damaged parental DNA is critical to understanding carcinogenesis. Therefore, DNA sequences responsible for induction and maintenance of the transformed state are being isolated and characterized. Transformed foci have been isolated from cells transfected with DNA derived from six Syrian hamster embryo tumorigenic cell lines, five originally treated with 3-methylcholanthrene (MCA) and one with 1 benzo(a)pyrene. Transformed foci are not observed if the 3T3 cells are transfected with DNA from normal HEC. DNA preparations isolated from MCA DNA transformed foci were used for a second round of transfection with 3T3 cells. In all cases, transformed foci were observed again. These were reisolated and the DNA is being purified for further characterization. Concurrently, middle repeat sequences of Syrian hamster cell DNA are being isolated to be used as a probe for identifying the Syrian hamster sequences in the DNA from the 3T3 transformed foci. The identification of Syrian hamster sequences in the DNA of 3T3 transformed cells will allow for isolation of those sequences responsible for the transformed phenotype. These sequences will be used to determine how specific carcinogens affect somatic DNA changes.

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 cellular approach with human and animal cells will make it possible to study how to block, reverse, or eliminate transformed cells. In the process, biologically valid techniques for identifying potential carcinogens relevant to humans will also be developed.

Proposed Course: The goal of this project is to establish conditions and methods for in vitro quantitative study of chemical transformation to determine the underlying biochemical and molecular processes responsible for the somatic changes resulting in malignancy.

1. Development of rapid assay systems for chemical carcinogens suitable for studying the modulation of transformation.
2. Define the conditions required for the quantitative transformation of human fetal cells.
3. Determine ways to increase or decrease the susceptibility of primary cell lines or cell strains to chemical transformation.
4. Determine whether in vitro transformation is accompanied by the appearance of tumor-specific antigens.
5. Identification and isolation of preneoplastic cell subpopulations by fluorescence-activated cell sorting.

6. Determine the role of DNA repair, in enhancing neoplastic transformation with radiation and alkylating compounds.
7. Transformation of epithelial-like cells which result in the formation of carcinomas when the cells are transplanted into animals.
8. Analysis of variations in populations of somatic cells and their correlation with genetic changes.
9. Isolation and characterization of DNA sequences responsible for carcinogenesis induced by chemical or physical agents.

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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Immunobiology of Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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4

PROFESSIONAL:

3.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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. 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 and by macrophages resulting from lymphotoxin-target cell interaction. The direct acting anticarcinogenic activity of lymphotoxin is more potent than the tumor cell inhibitory activity but through indirect mechanisms the lymphokine may be an effective homeostatic mechanism for control of carcinogenesis at its later stages of development.

Project Description

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 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 and by macrophages. Thus, lymphotoxin is also through indirect mechanisms, a cytolytic lymphokine with the potential to irreversibly inhibit tumor cell growth as well as prevent carcinogenesis.

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 one hundred thirty 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 or to macrophages, then there can be increased cytolytic destruction of the tumor cells compared to cytolysis resulting from natural killer cell or macrophage 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 antigen or mitogen stimulation. 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 eighteen hour 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 alteration 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. We have previously demonstrated that there appears to be negative feedback control of lymphotoxin secretion. Additional observations this past year 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 eliminate 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 that result from lymphotoxin interaction with mammalian cells and 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 at a time similar to when the anticarcinogenic action of the hormone appears to take place. 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 cytotoxicity. For these reasons, it is very important to understand not only the mechanisms of carcinogenesis but also to understand the mechanisms 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. Among their advantages, *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 this year. 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}Tc 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*. Intraperitoneal administration of phytohemagglutinin, or the antigens, keyhole limpet hemocyanin or sheep erythrocytes, furthermore, 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*. 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 cytotoxic 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.

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October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cell Surface in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
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TOTAL MANYEARS:

2

PROFESSIONAL:

1

OTHER:

1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Expression of cell surface associated properties during chemical carcinogenesis is being investigated in animal and human in vitro model systems. The purpose is to identify cellular characteristics useful in isolating preneoplastic cell populations for study and for 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. Continuing studies include protease stimulation of lymphotoxin production, onco-fetal antigen expression during carcinogenesis and relationship of cell surface properties to the developmental state of the cells.

Project Description

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 are 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. These 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 in plasmin, thrombin, kinin and complement activation as well as the production of inhibitory molecules that function in the local post-synthetic control of proteolytic activity.

In the Syrian hamster the progression to neoplastic transformation is telescoped occurring within weeks, whereas in the guinea pig it may 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, did 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. 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 it was found that 18 hr treatment of full term embryo cells with 3-methylcholanthrene induced the expression of at least one cell surface neoantigen by a portion of the treated cells. It has also determined that the neoantigen(s) expressed were common to several neoplastic cell lines and to mid-gestation embryo cells and that expression of the neoantigen(s) preceded neoplastic transformation. These data indicate that neoantigen expression may be a property of preneoplastic cells and that the antigens may provide a means of recognizing premalignant cell populations. Monoclonal antibody reagents have been produced to mid-gestation hamster embryo cells and are being evaluated.

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. This work is being done using the guinea pig carcinogenesis model with its extended stages of carcinogenesis and the Syrian hamster model of carcinogenesis where the same progression occurs over a more condensed period. Relevant studies with human cells will be performed whenever possible to extend these findings to human cancer as rapidly as possible.

These studies will determine the importance of specific tumor cell surface and intracellular factors in carcinogenesis and in enabling progressive in vivo growth of tumor cells. Identification of these factors and the mechanisms 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: Investigations will seek to identify interrelationships among the properties identified as being quantitatively related to tumor development and/or carcinogenesis and will focus on those properties most promising in

regard to being characteristically expressed during preneoplastic development. Experiments seeking relationships among protease activity, developmental antigen expression, lymphotoxin production and sustained skin reactivity and experiments relating expression of cell surface determinants to lymphotoxin sensitivity and cellular differentiation are underway. The library of monoclonal reagents recognizing developmental antigens will be expanded for this phase of the project and characterization of the antigens recognized on human and animal cells and their expression during carcinogenesis will continue. Investigations will continue to focus on mechanisms through which cells acquire their ability to form progressively growing tumors, the expression of aspects of these mechanisms during carcinogenesis and the means of identifying preneoplastic cells and arresting their further development. It is intended that these approaches will identify means of preventing neoplastic disease through elimination of preneoplastic cells and will permit selection of preneoplastic cells for study of altered genetic regulatory mechanisms.

Publications:

1. McCabe, R. P., and Evans, C. H.: Plasminogen activator, fibronectin, lymphotoxin sensitivity and natural skin reactivity relationships to guinea pig cell tumorigenicity. J. Natl. Cancer Inst. 68: 329-335, 1982.
2. McCabe, R. P., and Evans, C. H.: The regulatory role of extracellular proteases in tumor growth. Survey of Immunologic Research (In press).
3. Ohanian, S.H., McCabe, R. P., and Evans, C. H.: Immunogenicity of guinea pig cells transformed in culture by chemical carcinogens. J. Natl Cancer Inst. 67: 1363-1368, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Normal and Malignantly Transformed Cell Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECTPI: J. P. Fuhrer Expert Scientist LB NCI
OTHER: 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:

2

PROFESSIONAL:

1.25

OTHER:

0.75

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Interactions between cells, such as cell-cell recognition, adhesion, and alignment, depend upon highly organized, genetically programmed and timed associations of molecules recessed within the cell surface membrane or exposed at the membrane outer or inner surfaces. Abnormal interactions between malignant cells and host effector mechanisms may result from compositional and/or structural abnormalities in the molecules that comprise this dynamic internal and external membrane architecture. Computer-facilitated analyses of one and two dimensional IEF/SDS electrophoresis gels are used to quantitate and characterize the proteins and glycoproteins of normal and transformed cell membranes. HPLC and mass spectrometric analyses identify changes that occur in monoclonal antibody immunoprecipitated membrane molecules in response to the treatment of cells with chemical and physical carcinogens, cocarcinogens, modulators, lymphokines, and other immunobiological effectors. These studies have demonstrated that the lymphokine, lymphotoxin, stimulates the synthesis of high MW membrane glycoproteins in normal hamster embryo cells and simultaneously prevents them from becoming malignantly transformed. Conversely, lymphotoxin decreases synthesis of high MW glycoproteins in malignantly transformed hamster cells and inhibits their growth.

Project Description

Objectives: The primary objectives of this project are to identify changes in the molecular composition and/or 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 interact with normal and transformed cells in order to effectuate control over 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 growing in culture are metabolically labeled with radioactive protein and carbohydrate precursors or externally labeled with ^{125}I or $^3\text{H NaBH}_4$. Membranes or membrane extracts are prepared from labeled cells and are analyzed for relative 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 are currently limited to those derived from data describing the effects of lymphotoxin on the membranes of normal and transformed cells. Lymphotoxin is a lymphokine, nonantibody mediator of cellular immunity that possesses the ability to inhibit carcinogenesis and modulate the growth of tumor cells.

1. Normal hamster embryo fibroblasts (HEC) respond to lymphotoxin treatment by an increase in the synthesis of high molecular weight glycoproteins. This increase is evident after 24 hours of lymphotoxin treatment and is proportional to the dose of lymphotoxin used. This is the first demonstration of a lymphotoxin-induced biophysicochemical alteration in normal cells that may be related to the anticarcinogenic and tumor inhibitory activities of this lymphokine.
2. A 210,000 MW glycoprotein increased in the membranes of nontumorigenic cells within 9 hours after the initiation of lymphotoxin treatment and underwent a rapid increase after 17 hours of lymphotoxin treatment. This membrane glycoprotein change, induced by lymphotoxin, occurs in close temporal association with lymphotoxin-mediated development of cellular resistance to carcinogen-induced transformation to the neoplastic state.
3. Lymphotoxin treatment results in decreased incorporation of glucosamine into high molecular weight glycoproteins in lymphotoxin sensitive transformed HEC. This response is also dependent on the dose of lymphotoxin used and has been demonstrated in two different transformed HEC cell lines. It is qualitatively and quantitatively the opposite effect of that observed in lymphotoxin-treated nontransformed cells.
4. The differential synthesis of high MW glycoproteins in normal lymphotoxin-treated cells and decrease in glycoprotein presence in transformed HEC occurs without significant changes in detectable protein composition of the membranes. This is of considerable interest as glycopeptides are an integral part of cell surface antigens and mono- and oligosaccharides constitute determinant features of cell membrane glycoproteins and glycolipids, some of which are known to be receptors for host immunobiological and other physiological regulatory interactions.

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. The preparation of

monoclonal antibodies to normal, fetal and transformed cell antigens and to lymphotoxin currently in progress will be expanded into a major production effort for carcinogenesis-related hybridomas since monoclonal antibodies will be needed in later stages of the project for the sorting of cell populations, analyses of antigen distribution and turnover, isolation of transformed cell surface antigens for structural comparison to cross-reacting normal and fetal antigens, and for the isolation and structural analysis of lymphotoxin receptors. This biochemical and immunochemical approach, combined with computer-facilitated quantitation, will permit intensive analyses of the mechanisms of carcinogenesis and of the responses of cells to host effectors.

Publications:

1. 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. (In press).

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Carcinogen-induced Transformation of Mammary Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. W. Greiner	Staff Fellow	LB, NCI
OTHER:	C. H. Evans	Chief, Tumor Biology Section	LB, NCI
	J. A. DiPaolo	Chief	LB, NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

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SECTION

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TOTAL MANYEARS:

2

PROFESSIONAL:

1.25

OTHER:

0.75

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An in vitro model system has been developed for the study of the interrelationships between differentiation and carcinogenesis in hormonally responsive mammary epithelial cells. The epithelial cells lining the ducts and alveoli of the mammary glands of Sprague-Dawley rats are separated from stromal and fat cells by enzymatic digestion followed by density gradient centrifugation. These epithelial cells are susceptible to chemical carcinogens and proliferate when added to a hormone-supplemented medium. Whereas normal mammary epithelial cells have a finite life span of approximately 2-3 weeks, after carcinogen treatment cells can be subpassaged and cultured for three months. Carcinogen-treated mammary epithelial cells acquire anchorage-independent growth and form adenocarcinomas when injected into nude mice. Transformed cells retain differentiated features including keratin and mucin secretion. They also continue to possess estrogen receptors as indicated by their susceptibility to the cytotoxic action of tamoxifen. The extended latent period for malignant transformation and retention of differentiated function provide an epithelial model system for study of differentiation and carcinogenesis.

Project Description

Objective: The purposes of this project are 1) to elucidate the cellular and molecular events involved in the induction and development of breast cancer; 2) to relate carcinogenesis and differentiated function in hormonally responsive mammary epithelial cells; 3) to develop markers for carcinogen-induced transformation of mammary epithelial cells; and 4) to identify regimens which can inhibit, reverse or retard the carcinogenic process in these target cells. The development of an in vitro model for the study of carcinogen-induced transformation of the epithelial cells derived from hormonally responsive Sprague-Dawley rat mammary glands is of paramount importance.

Methods Employed: While the fat and stromal cells constitute the majority of cells in the mammary glands, the biological function (i.e., lactation) of the endocrine-sensitive target organ is a property of the epithelial cells which line the ducts and alveoli. These cells are the presumed target cells for carcinogens for both the rat and human. Techniques have been developed for the routine isolation of epithelial cells from the rat mammary fat pad. The abdominal and inguinal fat pads are excised, enzymatically digested and the fat cells separated by differential centrifugation. Fibroblasts are separated from multicellular epithelial aggregates by gradient centrifugation. The epithelial nature of the isolated cells has been established by the presence of high-affinity steroid receptors, casein and β -lactalbumin production, presence of desmosomes and microvilli and the ability to form normal mammary architecture and carry out normal physiological functions when transplanted back into the cleared rat mammary fat pad. A hormone-supplemented medium has been developed for the mammary epithelial cell. Primary cultures of mammary epithelial cells with the enzymatic capacity for oxidative metabolism are exposed to chemical carcinogens. Neoplastic transformation is assessed by changes in the growth capabilities of these cells in vitro, induction of morphological changes, acquisition of anchorage-independent growth and the tumorigenicity of carcinogen-treated cells by injecting into homozygous athymic nude mice and/or into the cleared fat pad of immunosuppressed weanling Sprague-Dawley rats.

Major Findings: Isolated rat mammary epithelial cells can be routinely grown in a hormone-supplemented medium consisting of cortisol, progesterone, 17 β -estradiol, insulin and prolactin added at physiological concentrations. The cells can be maintained in primary culture for 2-3 weeks until they cease to divide. During this time in vitro rat mammary epithelial cells form multicellular hemicysts, often referred to as domes, which are considered an in vitro characteristic of epithelial cells. Carcinogen treatment of mammary epithelial cells evokes certain phenotypic changes which are not observed in the untreated, control cells. Rat mammary epithelial cells treated with 7,12-dimethylbenz[a]anthracene (DMBA) can be subpassaged after 2-3 weeks in culture. Furthermore, the carcinogen-treated cells have been maintained in vitro for up to 3 months during which time dome formation persists, indicating the differentiated epithelial characteristics of the cells. Therefore, carcinogen treatment imparts upon the mammary epithelial cells an extended life span not seen in control cells. In addition, carcinogen-treated cells also acquire the ability to form colonies in agar, a phenotypic property often associated with neoplastic transformation. Cells subsequently produce adenocarcinomas when injected into homozygous athymic nude mice.

Transformed cells retain differentiated features including keratin and mucin production. They also continue to possess estrogen receptors as indicated by their susceptibility to the cytotoxic action of tamoxifen. Transformation in this epithelial model thus develops through a series of steps or stages permitting analysis of the transformation process and differentiation at multiple points during the development of carcinogenesis.

Significance to Biomedical Research and the Program of the Institute: Breast cancer is the most common neoplasm in women of the Western world and the pathogenesis of the disease remains unknown. The cause and growth of breast cancer has a strong genetic component. A major portion of that component may involve hormonal stimulation. Endocrine influences during early menarche, as well as late menopause, are associated with an increased risk of breast cancer. On the other hand, an early first, full-term pregnancy often protects against breast cancer. Specific markers (i.e., receptors) for endocrine-dependent growth are used for selecting breast cancer patients most likely to respond to endocrine intervention therapy.

To date, the best species in which to study hormonally influenced breast cancer is the rat. DMBA-induced mammary adenocarcinoma formation is under genetic control, i.e., varies from 100% in Sprague-Dawley to 0-10% in Long-Evans rats. Moreover, the induction by DMBA of mammary tumors in the Sprague-Dawley rats is strictly influenced by the endocrine state of the animal. Specifically, pregnancy and lactation impart a protection against DMBA-induced tumors, as do the removal of the ovaries and/or pituitary prior to carcinogen treatment. The ablation of endocrine organs during the growth of rat mammary tumors results in tumor regression.

Therefore, the similarities of genetic factors and requirements for continued endocrine stimulation establish a basis for using the rat as a prototype for studying human mammary carcinogenesis. The development of an in vitro transformation system for mammary epithelial cells provides an opportunity 1) to determine the mechanisms whereby chemical carcinogens interrupt normal mammary epithelial differentiation that results in tumor formation, 2) to determine the involvement of hormones in differentiation and carcinogenesis at the target cell level, 3) to develop specific markers for the transformation of mammary epithelial cells, and 4) to initiate concomitant study of in vitro transformation of human breast cells to establish a basis for extrapolation of data between the two species.

Proposed Course: Investigations will continue to define the requirements for in vitro transformation rat mammary epithelial cells to serve as a model for the development of a human breast epithelial cell culture system and the concomitant study of differentiation and carcinogenesis.

Publications:

None

ANNUAL REPORT OF THE LABORATORY OF CARCINOGEN METABOLISM

NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

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 modifier of cellular differentiation may inhibit and/or promote the neoplastic process.

Research in the Office of the Chief has involved establishing several new projects to study the mechanism of chemical carcinogenesis. These projects employ, in addition to classical biochemical methodologies, 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 technique allows the simultaneous separation of hundreds of polypeptides in a single polyacrylamide gel. We have acquired, and are further developing, a computer-based system to analyze autoradiograms produced from these gels. This system automatically finds and measures the amount of radioactivity in any polypeptide resolved by these electrophoretograms. Other semi-automatic programs match the spot patterns found in different gels and can identify spots which are missing or vary significantly between electrophoretograms. Still other programs link together a series of gels which may constitute an experiment and allow the investigator to quantitatively follow the synthesis of almost every protein throughout that experiment, or throughout a series of related experiments.

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. The main focus of our research is on rat hepatoma cell lines at different stages of differentiation, human promyelocytic leukemia cell line (HL60), and a variety of undifferentiated human B-cell lymphoma cell lines of both American and African origin. The results obtained so far include construction of cDNA libraries for several hepatoma cell lines and characterization of changes in transcriptional levels of α -feto-protein and albumin genes after in vitro treatment of the hepatoma lines with sodium butyrate and dexamethasone. Also, partial cloning of the genes for γ -glutamyltranspeptidase and epoxide hydrolase from rat liver has been achieved. The results from studies with the HL60 include identification and quantitation of

cellular proteins that are associated with differentiation of this cell line along either the granulocyte (induced by dimethylformamide) or macrophage (induced by TPA) pathways.

The undifferentiated human B-cell lymphoma lines are derived chiefly from childhood tumors originating from gut-associated lymphoid tissue. The cell lines, representing early to intermediate stages of B lymphocyte differentiation, are surface IgG positive and contain a 8, 14 translocation (i.e., contain a 14_q+ chromosome). Since increased expression of the proto-oncogene, c-myc, has been observed in virally induced bursal lymphomas in the chicken, we have been especially interested in examining the expression of c-myc in our human cell lines. The possible modulation of proto-oncogene expression by Epstein-Barr Virus (EBV) or the 8,14 translocation is also being investigated. We have found increased (approximately 5-fold) levels of a human c-myc-specific RNA (2.6kb) in several of the tumor lines as compared to EBV-transformed cord blood lymphocytes and to a fully differentiated human tissue (liver). Another oncogene, c-mos, located on chromosome 14, was examined in our lymphoma lines and no increase in the expression of human c-mos RNA was observed.

Considerable progress has been made in identifying and characterizing endogenous and exogenous factors that may control initiation, promotion and progression of chemically induced murine hepatomas. The modulating effects of blood flow and oxygen tension on hepatoma formation and development has been of particular interest. Diverting the blood from the splanchnic area directly into the inferior vena cava, by performing porta-caval shunt operations, resulted in changes in the liver similar to those produced by hepatocarcinogens in vivo such as expression of γ -glutamyltranspeptidase activity and lack of glucose-6-phosphatase activity. The effect of porta-caval shunt on promotion of initiated hepatocytes is presently being investigated.

Studies on metabolism and mutagenicity of chemical carcinogens have been focused on the use of intact cells to examine both metabolic processing and the genotoxic potential of selective chemical carcinogens. By coincubating intact rat hepatocytes with Salmonella tester strains (Salmonella/hepatocyte system), we have been able to evaluate the role of both metabolic activation and detoxification in determining the mutagenic potential of known or suspected chemical carcinogens. Measuring mutation frequency in the Salmonella strains (target cells) and genotoxicity in the hepatocyte (host cell) by the number of alkaline labile sites in DNA, has enabled us to determine, for aromatic amides such as 2-acetylaminofluorene (AAF), those metabolic pathways responsible for mutations in the bacteria as well as those causing genetic damage in the host cell. Furthermore, by substituting a variety of cell types (including human cell lines) for the hepatocyte, we can now evaluate the capacity of these cells to metabolically activate and/or inactivate chemical carcinogens. Progress has also been made in our studies on regulation and multiplicity of cytochrome P-450 dependent monooxygenases in rat liver. Using AAF as a probe for studying both N- and C-hydroxylations, and employing kinetic analysis of these reactions, we have been able to demonstrate that more than one form of cytochrome P-450 is involved in each of the C-hydroxylations, whereas only one form catalyzes the N-hydroxylation. Similar results were obtained from studies on AAF metabolism in human livers.

Studies on conformation changes in DNA and RNA induced by chemical carcinogens have recently been initiated. The results obtained so far include observation in solution of A- and B-form of DNA conformation in electric field. In the

absence of an electric field, DNA conformation in solution possesses a coiled coil-like tertiary structure. Electrophoresis of circular DNA reveals that organic solvents and alkylated ammonium ions unwind, whereas metallic and ammonium ions as well as polyamines wind the DNA helix; and potential energy calculations indicate that there are four stable conformations for dinucleoside phosphates in solution.

The research activities in the Analytical Chemistry Section for the past year have focused on two main areas: 1. N-Nitroso Compounds. a) Studies of nitrosamine-forming mechanisms have concentrated on thallium (I) anti-methanediazotate, a reagent with lower reactivity toward "magic ethyl" and related powerful electrophiles other than alkanediazotates, reacts with certain alkyl halides with unusual stereoselectivity. In addition to some synthetic utility, the results suggest a new mechanism of electrophilic participation in nitrosamine-forming reactions--X-coordination of halide by the soft thallium center during the assisted solvolysis of alkanediazotates. b) Considerable progress has been made on proving the generality of our previously discovered aluminum-nickel alloy method as an efficient, reliable means of quantitatively converting most carcinogens containing N-N or N-O bonds to innocuous products in one economical step. The reaction proceeds smoothly in every solvent tested thus far except acetone. Not only all nitrosamines, but also all hydrazines, nitramines, azo and azoxy compounds, triazenes, nitrosamides, tetrazenes and N-hydroxy derivatives have proven amenable to destruction in this way. The procedure has been endorsed by the International Agency for Research on Cancer as a recommended means of decontaminating laboratory wastes containing nitrosamines. c) The first crystallographic confirmation of a unidentate iron-nitrate complex was identified, titanium (III) chloride was found to convert nitrosamines to the corresponding hydrazines in good yield, and the literature concerning the stereochemistry of thialdine has been corrected

2. Mass Spectrometry and Nuclear Magnetic Resonance. (a) Our studies are continuing to elucidate the fundamental mass spectral fragmentation and rearrangement mechanisms that give rise to the observed mass spectra of dialkyl nitrosamines, using stable isotope labeled compounds and metastable defocusing techniques. In addition, these studies have provided model systems for characterizing the radical cation behavior of this class of carcinogens. b) Application of the two spectral techniques has been useful in characterizing the relative stereochemistry of the trihydroxytetrahydrobenzo(a)pyrene derivatives and the chemical reactivity and conformation of the tetrahydrobenzo(a)pyrene ring system. c) Synthetic studies are being initiated to construct several methylated and arylaminated oligodeoxynucleotides with the aim of studying by NMR the conformation changes these moieties elicit in the parent compounds. We also plan to study the utility of mass spectrometry in the structure characterization of these adducts. d) Collaborative studies are being continued in the structural analysis of bioactive materials of interest in cancer cause and prevention. Mass spectrometry is being applied for the confirmation of amino acid phenylthiohydantoin derivatives resulting from sequencing studies on transforming growth factors. Low and high resolution mass spectral studies have contributed to the structural determination of Fredericamycin A, an antitumor antibiotic of novel skeletal type.

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 inter-agency, 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.

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PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04493-05 LCM

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Modifications of the Bioenergetics Pathways in Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ann E. Kaplan	Chemist	LCM	NCI
OTHERS:	Mary J. Wilson	Chemist	LCC	NCI
	Barry Bunow	Expert	LAS	CR

COOPERATING UNITS (if any) Dr. H. Amos, Dept. Micro., Harvard Med. School, Boston, MA; Dr. S.A. Margolis, Natl. Bureau of Standards; Dr. M.S. Cassidy, Dept. of Physiol. George Washington Univ. Med. Ctr., Washington, D.C., Dr. M. Freeman, Genetics Screening & Counseling Center, Denton, TX

LAB/BRANCH

Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:

1.1

PROFESSIONAL:

1.1

OTHER:

0

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(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Increased export of lactic acid, observed with cell transformation, may arise both from the modification of lactate dehydrogenase (LDH) and from mitochondrial defects leading to progressive impairment of the bioenergetic system. Comparing a nitrosomethylurea-transformant, NMU-3, with its control, TRL 12-13: (1) At confluence, lactic acid formation in NMU-3 accounts for the equivalent of 75% of the glucose taken up from the medium. (2) Kinetic properties of LDH in NMU-3 serve to facilitate the formation of lactic acid. (3) A shift in molecular forms of LDH with transformation shows an increase in an alkaline type in NMU-3. (4) A similar shift in molecular distribution takes place in NIL-PY vs NIL, the former, a viral (polyoma) transformant. (5) Treatment with alkaline (serine) phosphatase appears to alter the TRL form of LDH to a form similar to NMU-3. This suggests that post-translational events are important for normal feedback control of LDH in TRL cells. (6) Ultrastructural studies identify a loss of 1/3 mitochondria per cell profile in NMU-3, along with marked defects in cristae and matrices. However, cell shape and size are unchanged, suggesting that the intracellular cytoskeletal structures are still unaffected. (7) Membrane transport mechanisms for lactic acid appear unaltered in NMU-3.

Project Description

Objectives: The rate of export of lactic acid increases in neoplastic cells of both clinical and experimental origin. In patients, this leads to an increase in serum lactic acid, representing an incomplete utilization of chemical energy. The loss of available energy from metabolites leads to cachexia and death.

After almost 60 years of investigation, the underlying causes of the incomplete oxidation of metabolites in neoplastic cells remain unclear. The objectives of this laboratory are to apply current biochemical and biophysical tools 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 pathways in tumor cells will permit the development of new means of preventing or suppressing the growth of tumor cells.

With this objective in mind, we developed three main research areas to study the question of 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 (export and import) of lactic acid, and (3) morphological changes. We have begun to identify cellular and molecular modifications appearing with transformation. These investigations were carried out with rat epithelial cell lines, TRL, established from 10-day-old rat hepatocytes, and a transformant, NMU-3, developed from this line by exposure in vitro to nitrosomethylurea. Since the NMU-3 cell slowly produces carcinomas in vivo, the most common type of tumor identified in cancer patients, we consider these cell lines to be useful as models for molecular changes in neoplastic cells.

Methods Employed: Physico-chemical methods employed in this study of control and chemically transformed hepatocytes include: (1) scanning and transmission electron microscopy; (2) growth of cells on surfaces with different architectural and molecular properties; (3) direct intracellular spectrophotometric measurements of changes in pH with lactic acid synthesis, export and import, through absorption changes in 6-carboxyfluorescein; (4) stopped-flow kinetic measurements of LDH reacting with pyruvate, followed by parametric analysis of the data; (5) separation of molecular subtypes of LDH by gel electrophoresis and isoelectric focusing, including identification of changes in these molecular types following exposure to alkaline phosphatase, a serine phosphatase; (6) development of new methods of purifying LDH, including HPLC, to identify molecular changes with transformation; (7) through collaboration with Dr. H. Amos, we applied some of the above methods to evaluate molecular modifications in LDH from virally transformed chick embryo fibroblasts, NIL-PY vs. their control line, NIL.

Major Findings: (1) Lactate dehydrogenase (LDH). We stabilized the LDH extracts from TRL and NMU-3 cells, and from NIL and NIL-PY cells to prevent hydrolysis by proteolytic enzymes. This provided very reproducible enzyme preparations for comparison of molecular modifications resulting from both chemical and viral transformation.

We previously demonstrated modified kinetic behavior which facilitates lactate formation with LDH from NMU-3. Modifications in molecular structure of LDH which appear in this preparation include (a) the altered proportions of LDH isozymes (4 and 5), and (b) the shift in isoelectric points of the subtypes of LDH from acidic in TRL to alkaline in NMU-3.

In our studies with NIL and NIL-PY we found that only one isozyme appears in both cell lines, but in NIL-PY it is more reactive with respect to lactate formation. This was also observed with the NMU-3 type LDH. Thus we find that enzymes from two different types of transformed cells show qualitative changes in their behavior with lactate as well as with pyruvate.

In studies of LDH by isoelectric focusing, LDH from NIL-PY also shows more alkaline forms of the enzyme relative to NIL. Here again the enzyme from virally transformed cells shows modifications parallel with those of the chemically transformed enzyme.

These isoelectric modifications of LDH with transformation (acidic to alkaline) prompted us to study the effect of alkaline (serine) phosphatase (AP) on LDH from TRL and NMU-3. First we find that incubation of both LDH preparations with AP at 37°C does not affect their enzyme activity. However, the isozymes of the TRL preparation are modified following exposure to AP and now appear with the properties of the NMU-3 enzyme. The latter shows no change with exposure to AP. These results suggest that the enzymatically "controlled" behavior of LDH from TRL may be related to post-translational modifications of the enzyme. These modifications may even take place at the catalytic site of this enzyme because serine is located there and is critical for LDH activity. We have not yet determined whether the enzyme modifications (from TRL-type to NMU-type) result from pretranslational modifications of DNA or mRNA or are the result of hydrolytic activity following the release of a protein kinase in the transformed cell.

(2) Lactic acid synthesis, transport and intracellular pH. (a) Compared with TRL 12-13, the NMU-3 cell produces three times as much lactic acid and causes the pH of the growth medium to drop below 7. The increased synthesis of lactic acid parallels the increased and modified activity of LDH. By confluence, 25 mM lactate accumulates in the medium. If fresh medium, free of lactic acid, is added at this time, NMU-3 will continue to export lactate at three times the rate of its control cell. During growth to confluence, three-fourths of the glucose available to NMU-3 is re-exported as lactic acid, representing a very inefficient use of energy available from carbohydrate. (b) Because these two cell lines grow as monolayers with similar cell shape and size, we developed a method to introduce the dye, 6-carboxyfluorescein, into the cells to serve as an intracellular indicator of pH by split beam and dual wavelength spectrophotometric analysis. Here again, the TRL cell remains above pH 7, whereas the NMU-3 cell may reach an internal pH of 6.6 as lactate first accumulates and then moves across the plasma membrane with H⁺ ions. The kinetics of this export function were analyzed by dual wavelength changes in the absorption of 6-carboxyfluorescein. When the cells are exposed to the bioflavonoid, quercetin, which inhibits the export of the acid, intracellular pH drops in both cells with the accumulation of acid. (c) In studying membrane transport through spectrophotometric analysis, we find the plasma membrane unaltered in kinetics with respect to lactate transport. Furthermore, when lactate is added to fresh medium with quercetin-inhibited cells, acid import appears to be the same

in both cell lines. Thus the physiological properties of the plasma membrane appear to be unaltered in NMU-3 with respect to both the export and import of lactic acid even though the ultrastructure of the membrane appears modified (see below).

(3) Ultrastructural changes in TRL 12-13 and NMU-3. (a) Cell size and shape appear unchanged with transformation by light microscopy and by scanning and transmission electron microscopy. This suggests that cytoskeletal proteins associated with cell size and shape are not altered in NMU-3. However, fibronectin is absent in NMU-3 according to results of both scanning and electron microscopy. Since microtubular structure and actin aggregation both depend upon energy transfer, the results suggest that these requirements are still being met in NMU-3 even though increased lactic acid production indicates that the bioenergetic system is altered. (b) In growing TRL 12-13 and NMU-3 on flat surfaces and on two types of microspheres, unexpected changes in cell shape appeared with both microspheres. Whereas the hepatocyte lines grow to look like squamous cells on flat surfaces, on both microspheres they assume the cuboidal appearance characteristic of hepatocytes. We do not know if the altered shape of the cells on microspheres is due to the physical architecture of the surface or to chemical properties of the two microspheres. Here again, cell size and shape are consistent for both the TRL 12-13 and NMU-3 cells, with no apparent change due to transformation. However, even under these growth conditions, which seem more favorable for both cell lines, NMU-3 cells do not produce fibronectin. (c) In the TRL 12-13 cell ultrastructural results show cytoplasmic organelles, polar distribution of free ribosomes vs. rough endoplasmic reticulum in close juxtaposition with the mitochondria, Golgi, GERL, and nucleus. The TRL 12-13 cell, like the hepatocyte in situ, seems to be actively synthesizing protein for export. In contrast, the NMU-3 cell, although of the same size and shape, differs in all other respects: it has thin linear membranes, lacks vesicles, and is deficient in all organelles. This cell gives the appearance of synthesizing self-protein with only fragmentary evidence of RER that is dissociated from mitochondria. The mitochondria are fewer in number by 35 percent, and are very defective in cristae and matrices, giving the impression that this altered morphology contributes to the overall defect in the energy balance of these cells.

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. One relates to the observation of defective mitochondria and suggests that these are very fragile in structure, as is true in other transformed cells. They utilize glutamate, but not three-carbon units from glycolysis, for oxidative phosphorylation. The fragility of the mitochondria, as well as this atypical enzymology, suggests that methods might be developed to destroy neoplastic cells through their unique pathway for energy production.

The second source of increased lactic acid output comes from the molecular modification of LDH with its altered kinetic behavior, which facilitates the output of lactate. In the case of the latter, the same types of molecular modifications of LDH appear in both chemically and virally transformed cells, even though the NMU-3 and NIL-PY cells differ in tumor type (carcinoma vs sarcoma). Membrane transport of lactic acid is the same in TRL 12-13 and NMU-3. For this reason it does not serve as a contributing factor in increased lactic acid output. The

morphological and molecular differences identified through these studies point to the significant differences between TRL and NMU-3 cells which invite the development of specific inhibitors to destroy the transformed cell.

Proposed Course: Having moved the laboratory to the NCI Frederick Cancer Research Facility, opportunities are developing to extend this program through new collaborations. With respect to LDH, we plan to purify the enzyme from both cell lines and, through collaborative investigation, identify the structure of the protein and the post-translational subunits suggested by the results with alkaline phosphatase. We hope to do this work with the enzymes from both the TRL and NMU-3 cell lines, as well, and to complete kinetic studies with purified enzymes. The studies with lactate formation and transport are essentially completed and manuscripts are being prepared for publication. Further ultrastructural studies are planned to identify the integrity of the cytoskeleton through antibody and fluorescent tag binding. This will be a collaborative effort. New studies are planned to identify enzymatic defects in the mitochondria of the NMU-3 cell. The collaborative study of changes in LDH in a human subject due to loss of a fragment from chromosome 12 will be completed.

Publications:

Kaplan, A.E., Yamaguchi, M.K., Tralka, T.S. and Hanna, C.H.: Ultrastructural differences between control and nitrosomethylurea-transformed cells of rat hepatocyte origin. Exp. Cell Res. 138: 251-260, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 04510-09 LCM

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Environmental and Genetic Factors in Digestive System Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard S. Yamamoto	Chemist	LCM	NCI
OTHERS:	Nabil M. El-Torkey	Visiting Fellow	LCM	NCI
	Preston H. Grantham	Chemist	LCM	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Carcinogen Metabolism

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINDRS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves studies on the possible sex difference in response to the colon carcinogens, 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) and studies on mutagenicity of urine after feeding chemical carcinogens. In the female F344 strain rats, DMH produced few colon carcinomas; however, AOM induced colon tumors and also kidney tumors. Male F344 rats were susceptible to tumor induction after treatment with both DMH and AOM at low levels and with short latent period. Carcinogens and their metabolites in the urine were tested for mutagenicity using the Salmonella typhimurium system. Modification of the urinary mutagenicity was tested with an inhibitor of carcinogenesis such as acetanilide, tyrosine and selenium.

Project Description

Objectives: To test the various urinary metabolites of carcinogens and related non-carcinogens for mutagenic activity by the *Salmonella typhimurium* system, and to determine the interrelationships of hormonal factors in intestinal carcinogenesis.

Methods Employed: Male and female rats were injected with dimethylhydrazine (DMH) and azoxymethane (AOM). The males were castrated and implanted with estrogen pellets before, during or after the period of administration of AOM. The females were ovariectomized and implanted with estrogens and treated similarly. After a specified time, the rats were killed and examined for tumors. Urines were collected periodically and tested for mutagenic metabolites. Carcinogens or non-carcinogens are administered to rats. Urines from these rats are collected and assayed for mutagenic activity with the addition of β -glucuronidase, sulfatase, deacetylase and/or S-9 (an Arachlor-induced activated liver fraction). Usually sufficient activity is found in individual rat urines to allow quantitation and comparison within and between groups. A periodic collection is necessary in all cases where multiple administration is necessary. Urine with high mutagenic activity can be set aside for separation and analysis.

Major Findings: 1. Female F344 rats were less susceptible to DMH. However, with AOM female rats resisted tumor formation by having few tumors appearing later in life, with additional kidney adenocarcinoma formation. Male F344 rats were very susceptible to DMH and AOM carcinogenesis, but failed to produce kidney tumors. Castrated male rats, like female rats, resisted colon carcinogenesis, but did not develop kidney tumors. Castrated male rats responded to 17- β -estradiol (E-2) by rapidly producing kidney tumors following treatment with AOM. Ovariectomized females responded similarly to intact females when they were treated with diethylstilbestrol (DES), whereas intact females succumbed rapidly to DES implantation.

2. None of the metabolites of diaminoanisole (DAA) in the urine was directly mutagenic, but could be activated by S-9 alone or S-9 with β -glucuronidase. Mutagenicity of the urine of DAA-treated rats increased with each injection. With DAA, rats on a tyrosine diet excreted urine with greater mutagenicity than rats on a control diet. Tyrosine, a precursor of thyroid hormones, seems to occupy the receptor site and prevent DAA from causing thyroid carcinogenesis, by forcing a greater excretion of DAA metabolites. Acetanilide and selenium, both compounds inhibit FAA carcinogenesis, caused an increase in the activatable mutagens, but with different mechanism. Alcohol together with DAA did not increase the activatable mutagens; in fact there might have been a slight decrease.

Significance to Biomedical Research and the Program of the Institute: The high morbidity and increasing mortality from intestinal cancer is a major health problem in the United States. The sex difference in mortality as noted in the latest NCI Monograph shows a steady increase in males as compared to leveling in females. The development of a model where the target organ is changed by hormonal manipulation is still important in studying carcinogenesis.

Proposed Course: This project is being terminated.

Publications:

Laqueur, G.L., Matsumoto, H. and Yamamoto, R.S.: Comparison of the carcinogenicity of methylazoxymethanol- β -D-glucosiduronic acid in conventional and germfree Sprague-Dawley rats. J. Natl. Cancer Inst. 67: 1053-1055, 1981.

Nawata, H., Yamamoto, R.S. and Poirier, L.A.: Changes in polyamine levels and ornithine decarboxylase activity during hamster kidney carcinogenesis induced by 17- β -estradiol. Carcinogenesis 2: 1207-1211, 1981.

Nawata, H., Yamamoto, R.S. and Poirier, L.A.: An inverse correlation between uterine and ovarian levels of ornithine decarboxylase and S-adenosylmethionine decarboxylase in the rat. Proc. Soc. Exp. Biol. Med. 167: 563-566, 1981.

Nawata, H., Yamamoto, R.S. and Poirier, L.A.: Tissue distribution of ornithine decarboxylase and S-adenosylmethionine decarboxylase in male and female rats. Res. 2: 659-663, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04542-10 LCM

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Chemistry of N-Nitroso Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Larry Keefer Chief, Anal. Chemistry Section LCM NCI
OTHER: Shui-mei Wang Visiting Fellow LCM NCI

COOPERATING UNITS (if any) J. Fanning & J. Resce, Clemson U.; C. Day, Crystallitics; G. Lunn & E.B. Sansone, FCRF; A. Croisy, Institut Curie; T. Hansen, Drexel U.; S.-J. Uhm, Korea Inst. Sci. Tech.; E. Reist, SRI Interntl.; A. Likhachev, V. Anisimov, A. Ovsyannikov & S. Revskoy, Petrov Inst. (Leningrad)

LAB/BRANCH

Laboratory of Carcinogen Metabolism

SECTION

Analytical Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.9

PROFESSIONAL:

1.9

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINDRS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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

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. In our studies of nitrosamine formation mechanisms, most of the recent effort has focused on the chemistry of thallium (I) anti-methanediazotate. This novel synthon is different from other alkanediazotates in two important respects: it is surprisingly unreactive toward most alkylating agents, including "magic ethyl," one of the most potent ethylating species known; it is very reactive toward methyl iodide, displaying unusual stereoselectivity in a nitrosamine-forming pathway which leads to near exclusion of the isomeric azoxymethane as a product. These results have been tentatively rationalized in terms of soft acid assistance by thallium of reaction between the methanediazotate anion and an alkylating center attached to a soft leaving group. The reaction has been used to synthetic advantage, e.g., in the stereoselective preparation of the conformationally unfavorable Z-methylbenzyl nitrosamine.
2. Major effort has been placed on development of chemical degradation methods useful in the decontamination and disposal of carcinogenic laboratory waste. In collaboration with Drs. E. B. Sansone and G. Lunn of the Frederick Cancer Research Facility's Environmental Control and Research Laboratory, a reductive procedure has been introduced in which nitrosamines are quantitatively cleaved to the corresponding (presumably innocuous) amines simply by adding aluminum-nickel alloy powder to them in the presence of aqueous alkali. The procedure has given reliable results with every nitrosamine studied and has worked in all solvents, except acetone, which reacts preferentially with the reducing agent produced *in situ*. The method is also fully applicable to the carcinogenic hydrazines, and preliminary work suggests that all other carcinogen types containing N-N or N-O bonds, including nitrosamides, nitramines, azo and azoxy compounds, triazenes, tetrazenes and N-hydroxy species, will also be amenable to safe, economical, rapid, one-step degradation under these conditions. The International Agency for Research on Cancer has very recently recommended the procedure as the method of choice for a variety of nitrosamine decontamination problems commonly encountered in the carcinogenesis laboratory.
3. In a biological investigation conducted at the N.N. Petrov Research Institute of Oncology in collaboration with Dr. A. Likhachev and coworkers, the effects of aging on the fate of methyl(acetoxymethyl)nitrosamine in rats were studied.

Among other findings, the liver of older rats had a greater capacity to excise O^6 -methylguanine from DNA than that of young rats, while in colon and ileum, O^6 -methylguanine excision was higher in younger than in older animals.

4. Three findings from our basic chemistry research program are also noteworthy. (a) A crystalline dimer of Fe(salen)-ONO₂ has been found to be the first crystallographically verified example of a unidentate iron-nitrate complex yet reported. The structural relationship between this compound and a powdery N-nitrosating agent which we have also isolated and which has the same empirical formula, is now being investigated. (b) The crystal structure of thialdine, a heterocyclic base first described by Wöhler and Liebig in 1849, has been determined, leading to a correction of the literature concerning the configuration of this molecule. (c) The reduction of nitrosamines using titanous chloride has been found to produce hydrazines in relatively pure form, and may be a reaction of some synthetic utility.

Publications:

Gaffield, W., Lundin, R. and Keefer, L.K.: Chiroptical properties of N-nitrosopyrrolidines and N-nitrosamino acids: Implications for the nitrosamine sector rule. Tetrahedron 37: 1861-1869, 1981.

Hansen, T.J., Angeles, R.M., Keefer, L.K., Day, C.S. and Gaffield, W.: N-Nitroso-thialdine. Synthesis, x-ray crystallography, and N-N rotational barrier. Tetrahedron 37: 4143-4149, 1981.

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. 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 (in press).

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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 (in press).

Lunn, G., Sansone, E.B. and Keefer, L.K.: Reductive destruction of N-nitrosodimethylamine as an approach to hazard control in the carcinogenesis laboratory. Fd Cosmet. Toxicol. 19: 493-494, 1981.

Roller, P.P., Keefer, L.K., Bradford, W.W. and Reist, E.J.: Synthesis, analysis, and stability studies of ¹⁴C-methyl(acetoxymethyl)nitrosamine. J. Labelled Comds. Radiopharm. 18: 1261-1272, 1981.

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 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05259-01 LCM																				
PERIOD COVERED October 1, 1981 to September 30, 1982																						
TITLE OF PROJECT (80 characters or less) Chemical Structure Studies in Carcinogenesis Research																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table border="0" style="width: 100%;"> <tr> <td style="width: 40%;">PI: Peter P. Roller</td> <td style="width: 30%;">Chemist</td> <td style="width: 15%;">LCM</td> <td style="width: 15%;">NCI</td> </tr> <tr> <td>OTHERS: Snorri S. Thorgeirsson</td> <td>Acting Chief</td> <td>LCM</td> <td>NCI</td> </tr> <tr> <td>James L. Cone</td> <td>Chemist</td> <td>LCM</td> <td>NCI</td> </tr> <tr> <td>Narayan Sundaram</td> <td>Visiting Fellow</td> <td>LCM</td> <td>NCI</td> </tr> <tr> <td>C.-H. Robert Lee</td> <td>Senior Staff Fellow</td> <td>LCM</td> <td>NCI</td> </tr> </table>			PI: Peter P. Roller	Chemist	LCM	NCI	OTHERS: Snorri S. Thorgeirsson	Acting Chief	LCM	NCI	James L. Cone	Chemist	LCM	NCI	Narayan Sundaram	Visiting Fellow	LCM	NCI	C.-H. Robert Lee	Senior Staff Fellow	LCM	NCI
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Narayan Sundaram	Visiting Fellow	LCM	NCI																			
C.-H. Robert Lee	Senior Staff Fellow	LCM	NCI																			
COOPERATING UNITS (if any) L.M. De Luca, Differentiation Control, LCCTP, NCI; R. Lehrman, LCP, NCI; R. Pandey, Fermentation Laboratory, FCRF; S.K. Yang, USUHS, Bethesda, MD																						
LAB/BRANCH Laboratory of Carcinogen Metabolism																						
SECTION Analytical Chemistry Section																						
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205																						
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																						
SUMMARY OF WORK (200 words or less - underline keywords) This project involves both independent and collaborative research projects in chemical carcinogenesis where <u>mass spectrometry</u> and <u>nuclear magnetic resonance</u> can be used as analytical tools to determine the structure and to confirm the identity of organic molecules, or to monitor chemical or biochemical reaction processes of interest. Current activities include: (1) Chemical and mass spectrometric studies on various mass labeled analogs of several derivatives of <u>7,8,9,10-tetrahydrobenzo(a)pyrene</u> , which revealed selective chemical reactivities and diagnostic mass spectral fragmentation patterns; (2) synthesis of mass labeled open chain <u>dialkyl nitrosamines</u> and subsequent mass spectral studies that allowed a thorough <u>rationalization</u> of the gas phase cation-radical behavior of this class of carcinogens; attempts are progressing to apply this knowledge to the explanation of their solution chemistry; (3) development of methods for derivatization and analysis of carcinogens and other bioactive materials, including <u>phenylthiohydantoin derivatives</u> of amino acids, <u>retinyl phosphate</u> and analogs, and <u>antitumor antibiotics</u> ; (4) the synthesis of specifically methylated and <u>arylaminated oligodeoxynucleotides</u> is being initiated for <u>NMR conformational</u> and <u>FAB mass spectrometric</u> studies.																						

Project Description

Objectives: (1) To study, in some detail, the mass spectra of carcinogens such as N-nitroso compounds, aromatic hydrocarbons and aromatic amines, as well as some of their possible metabolites, with the aim of applying this knowledge to develop appropriate analytical methods. (2) To apply the mass spectrometry and nuclear magnetic resonance spectroscopy (NMR) methods for the analysis and identification of metabolites in carcinogen activation and metabolism studies. (3) 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 mechanisms. (4) To study the effect of carcinogens on the conformation, structure and spectral behavior of chemically well-defined oligodeoxynucleotides.

Methods Employed: A high resolution double focusing mass spectrometer system was used, interfaced with a gas chromatograph, and run in the electron impact ionization mode. A number of synthetic and metabolic samples were also prepurified using high pressure liquid chromatography. In some cases other suitable chemical and spectral characterizations were necessary. The Section's high resolution superconducting-magnet nuclear magnetic resonance (NMR) spectrometer gives important complementary information to mass spectrometry in structural and other studies. The NMR technique is also being applied to the analysis of materials and reaction mixtures that are thermally unstable or non-volatile, i.e., too polar to be vaporized for mass spectral measurements with the presently available instrument.

Major Findings: The analytical instrumentation program operates at three levels of effort: first, we engage in independent projects on our own initiative; second, the facility supports the chemical studies related to nitrosamines and metal complexes within the Analytical Chemistry Section; third, the group engages in collaborative studies on projects of mutual interest with investigators within the Laboratory of Carcinogen Metabolism and elsewhere within the Institute.

1. Independent studies are continuing to examine the chemical reactivity and the spectral behavior of several classes of carcinogens. One such area is the polycyclic aromatic hydrocarbons. The 7,8-trans-dihydroxy-9,10-epoxy derivatives of 7,8,9,10-tetrahydrobenzo(a)pyrene (4H-BaP) are metabolically activated carcinogenic forms of benzo(a)pyrene, binding to DNA at the C-10 position. Our earlier chemical studies demonstrated the unusual chemical reactivity at C-10 of several 4H-BaP derivatives using ^{18}O labeling and mass spectral analysis. Thus, the hydroxyl group at C-10 of certain isomers of 7,8,9,10-tetrahydroxy-4H-BaP is susceptible to solvolytic exchange in acid. Also, the 9,10-trans diol analog exchanges with ^{18}O labeled solvent, isomerizing to the 9,10-cis compound. Such is not the case for the 7,8 analog. Derivatization to the acetanides makes these compounds amenable to mass spectral analysis. Experiments show that the 7,8-cis diol forms an acetamide derivative with ^{18}O -acetone:CuSO₄, with complete loss of the ^{18}O label, as expected, on the basis of the established mechanism, but that the 7,8-trans diol does not form a derivative. Surprisingly, the 9,10-trans diol also forms an acetamide with complete retention of ^{18}O label from ^{18}O -acetone and apparent inversion of configuration at C-10. In a parallel experiment, the 9,10-cis diol formed an acetamide with 33% retention of ^{18}O from the acetone reagent through a probable carbonium ion at C-10 of the molecule. The mass

spectra of the 3 isomeric pairs (7,8 vs 9,10) of dihydrodiols, tetrahydrodiols and of their acetonides show distinct fragmentation behavior that we delineated now through detailed studies.

2. Most nitrosamines, being volatile and thermally stable, are well suited for trace analysis by gas chromatography/mass spectrometry, and this is indeed the accepted method of choice. The detailed fundamental study of nitrosamine mass spectrometry has been the subject of our research in the past several years and publications at the end of this report deal with the results in detail. It is thus well understood that the loss of hydroxyl radical from the molecular ion of open chain dialkyl nitrosamines is accompanied by further rearrangements. One of the consistently observed fragments is the M-60 ion in the case of methyl alkyl analogs. Recently we synthesized the ^{15}N labeled analogs of N-nitrosomethylhexylamine and of N-nitrosomethyloctylamine and the results showed that the M-60 peak is a rearrangement ion corresponding to the loss of $\text{C}_2\text{H}_5\text{N}$, consisting of C-1, C-1' and of the amino nitrogen. In future experiments a photochemical analogy to the proton abstraction from the β carbon of the side will be attempted.

3. Several collaborative projects are concerned with investigations of bioactive materials that may play a role in cancer prevention mechanisms. In the field of peptide analysis, the present activity is confined to the mass spectral confirmation of the phenylthiohydantoin derivatives of amino acids which were obtained by Edman degradation of transforming growth factors by Dr. Sporn's group. In addition to confirming the expected amino acids, which were initially identified by chromatographic retention times, the method can uncover unexpected amino acids. A number of standards have been run to date. Collaborative work with Dr. De Luca's group involves structural studies of a component of hepatoma cells, and also of rat mammary carcinoma cells, which is absent in normal cells. This compound may be a phospholipid. At present, a permethylation procedure is being tried on retinyl phosphate, as a model compound, to protect the polar functional groups before mass spectral analysis. In collaboration with the Fermentation Laboratory, FCRF, high resolution mass spectra and NMR measurements were instrumental in the structure determination of the antitumor antibiotic Fredericamycin A, which was isolated from *Streptomyces griseus* (FCRC-48). This compound has an unusual polyphenolic spiro ring structure, with unusual physicochemical properties.

4. There is strong evidence suggesting that alkylation of DNA by carcinogens, especially at the O-6 position of guanine by nitrosamines, and arylation of C-8 position by AAF, for example, plays a role in the cancer causation process. Our laboratory is initiating the synthesis of several structurally well-defined oligodeoxynucleotides that will be used for NMR conformation studies and for mass spectrometric analysis to determine if the latter method is capable of sequencing short chains and to determine the location of alkylation site.

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 and polycyclic aromatic compounds 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.

Publications:

Bhat, P.V., Roller, P.P. and De Luca, L.M.: Chemical and biological studies on 5,6-epoxyretinol, retinol, and their phosphoryl esters. J. Lipid Res. 22: 1069-1078, 1981.

McCourt, D.W., Roller, P.P. and Gelboin, H.V.: Tetrabutylammonium hydroxide: A reagent for the base-catalyzed dehydration of vicinal dihydro diols of aromatic hydrocarbons. Implications to ion-pair chromatography. J. Org. Chem. 46: 4157-4161, 1981.

Roller, P.P., Slavin, B.W., Angeles, R.M. and Miller, J.R.: Mass spectral rearrangements of acyclic dialkyl nitrosamines: 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, (in press).

Wei, T.T., Chan, J.A., Roller, P.P., Weiss, U., Stroshane, R.M., White, R.J. and Byrne, K. M.: Detection of gilvocarcin antitumor complex by a biochemical induction assay (BIA). J. Antibiotics 35: 529-532, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05260-01 LCM
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Gene Expression and Differentiation in Neoplasia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to examine the regulation of gene expression and differentiation in neoplasia. 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) rat hepatoma cell lines at different stages of differentiation; (2) human promyelocytic leukemia cell line (HL60); (3) a variety of undifferentiated human B-cell lymphoma lines of both American and African origin; and (4) virally induced murine erythroleukemia cell lines. The results obtained so far include: (1) construction of cDNA libraries for several hepatoma cell lines, (2) partial cloning of the genes for gamma-glutamyltranspeptidase and epoxide hydrolase form rat liver, (3) characterization of changes in transcriptional levels of alpha-fetoprotein and albumin genes after in vitro treatment of the hepatoma lines with sodium butyrate and dexamethasone, (4) quantitation and characterization of changes in protein synthesis in HL60 accompanying in vitro differentiation induced by 12-0-tetradecanoyl phorbol-13-acetate (macrophage) or by dimethylformamide (granulocyte), and (5) examination of the expression of selected oncogenes in the human B-cell lymphomas.

Project Description

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) rat hepatoma cell lines at different stages of differentiation; (2) human promyelocytic leukemia cell line (HL60); (3) a variety of undifferentiated human B-cell lymphoma lines of both American and African origin; and (4) virally induced murine erythroleukemia cell line.

Methods Employed: Methods used in these studies include: tissue culture techniques, radioimmunoassays, differential centrifugation, radioisotope measurements using tritium, carbon-14, and iodine-125; phase contrast microscopy, photomicroscopy; enzyme assays involving radiometric, spectrophotometric, and spectrofluorometric determinations; two-dimensional gel electrophoresis; chemical synthesis, and recombinant DNA techniques.

Major Findings: A. Rat hepatomas. We have constructed cDNA libraries for 7777, 8994 and Reuber cell lines. Messenger RNAs (mRNAs) were prepared by a modification of the 8 M guanidine HCl extraction technique and chromatographed over oligo (dT) cellulose to collect the poly(A)-containing fraction. Double-stranded cDNA was synthesized and approximately 15 residues of dCTP were added at the 3' ends of cDNA molecules using terminal transferase. The plasmid pBR322 cleared at the Pst I restriction site was similarly extended with poly(dG) to approximately 15 nucleotide residues. Homopolymer tailed cDNAs and pBR322 were annealed under appropriate temperatures and competent E. coli HB101 were transformed in the presence of CaCl₂. HB101 containing recombinant DNA molecules were identified by their ability to grow in the presence of 12.5 µg/ml of tetracycline. At least 2000 recombinant containing bacteria were obtained for each library. We are presently constructing libraries to trans-stilbene oxide-induced rat liver in a similar manner.

B. Selection of genes coding for differentiation-specific proteins. 1. γ-Glutamyl transpeptidase (γGT) catalyzes the transfer of the γ-glutamyl moiety of glutathione to a number of acceptors. In the liver, hepatocytes show high γGT activity in the fetus but low activity, primarily associated with the plasma membrane of biliary tract cells, in the adult. During chemical carcinogenesis, and particularly malignant hepatocarcinoma, the activity is markedly increased (up to 50-fold). Since the level of γGT in putative premalignant liver cells is elevated, the value of this differentiation-specific protein as a histochemical marker for malignancy has been suggested.

We have assayed the levels of γGT in the three rat hepatoma cell lines and have shown a 20-30 fold increase in γGT enzyme activity in the 7777 (undifferentiated) cell line as compared to the most differentiated Reuber cell line. We are presently purifying this enzyme from the 7777 cell line for antibody production.

We are also using a differential gene expression method to isolate the γGT specific recombinant clone(s) from our 7777 cDNA library. Briefly, this method includes replica plating of the approximately 2000 recombinant clones onto

Whatman 541 filter paper. The bacteria are allowed to grow, lysed and the DNA made single-stranded. Using reverse transcriptase, total ^{32}P -cDNA will be made from 7777 mRNAs and Reuber mRNAs and separately hybridized to replica filters containing the recombinant colonies. Following autoradiography, the intensity of the hybridization reaction for each colony will be compared and those colonies possessing a higher degree of hybridization using ^{32}P -cDNA (7777) as compared to ^{32}P -cDNA (Reuber) will be isolated as potential γGT clones. The DNAs from each of these colonies will be immobilized on nitrocellulose filters and used for hybrid selection and translation. Using antisera made to the purified γGT , recombinant clones containing γGTcDNA will be identified.

2. Epoxide hydrolase. Epoxide hydrolase is an important enzyme involved in the metabolism of polycyclic aromatic hydrocarbons and this enzyme is induced in preneoplastic liver foci. A differentiation-related protein, the enzyme is undetectable at birth and reaches a plateau level within 6 weeks after birth in the mouse. We are presently isolating the gene coding for this protein using a cDNA library made from a rat liver induced by trans-stilbene oxide. Since the mRNA coding for this enzyme is present in low amounts (0.02% of membrane bound polysomal mRNA) and is not appreciably increased in any of the hepatoma cell lines (unpublished observation) trans-stilbene oxide-induced rat liver mRNAs (10-fold increase), were used. To obtain a further selective advantage we will fractionate total mRNA from trans-stilbene oxide-induced rat livers on a methyl mercury agarose gel and selectively elute the regional corresponding to + 1650 bases known to be the approximate size of the epoxide hydratase mRNA. This mRNA will be assayed in an in vitro protein translation system (microsomal nuclease-treated rabbit reticulocyte lysate) to verify the enrichment procedure prior to obtaining ^{32}P -cDNA and hybridization to the trans-stilbene oxide cDNA library. Verification of highly positive cDNA clones will be done by hybrid selection, translation and immunoprecipitation.

3. Isolation of additional differentiation-specific proteins. Similar methods will be used to identify other genes coding for proteins involved in differentiation or chemically induced modulations. The limitations are presently the identification of these proteins and the availability of pure antibodies. The first limitation has been partially circumvented by the quantitative two-dimensional protein analysis technique now available in our laboratory. Using this strong technique, differentiation-specific proteins and proteins induced or repressed during chemical carcinogenesis can be identified. The recent availability of a microtechnique for amino acid sequencing of nanogram quantities of proteins and the construction of synthetic peptides corresponding to these proteins for antibody production is being considered as a long-term means of circumventing the second problem.

C. Characterization of changes in transcriptional levels of genes coding for differentiation-specific mRNAs. cDNA clones to rat albumin and α -fetoprotein (AFP) were obtained from Dr. Thomas Sargent. In vitro ^{32}P nick translated cDNA probes were synthesized and used to hybridize to Northern dot blotted mRNAs obtained from 7777, 8994, and Reuber hepatoma cell lines. Albumin-specific RNA was shown to be increased 20-fold in Reuber cell lines, and 10-fold in 8994 cell lines when compared to 7777 cell lines. Conversely, AFP-specific RNA was transcribed at much higher efficiencies in the more poorly differentiated 7777 cell line when compared to 8994 (80-fold higher) or Reuber (160-fold higher). These

values closely parallel the values observed for AFP or albumin protein synthesis in these cell lines. We are presently comparing the levels of albumin and AFP RNA synthesis in sodium butyrate (BA) and dexamethasone (DEX) treated 7777 and 8994 cell lines as well as the levels of these RNAs when the cells are transplanted in vivo. Radioimmunoassay on the levels of AFP and albumin has shown that BA or BA + DEX (but not DEX) can induce the synthesis of albumin in the 7777 but not the 8994 cell line. Conversely, DEX or DEX + BA (but not BA) represses the synthesis of AFP in the 8994 cell line but has no effect on the synthesis of AFP in the undifferentiated 7777 cell line, again supporting the previous evidence that regulation of synthesis of differentiating proteins by chemicals is dependent on the stage of differentiation of the target cells.

D. Effects of hexamethylene bisacetamide on α -fetoprotein, albumin, and transferrin production by two rat hepatoma cell lines. α -Fetoprotein (AFP), albumin, and transferrin production by two rat hepatoma cell lines, 7777 and 8994, was determined after treatment with hexamethylene bisacetamide (HMBA, 2 to 6 mM). Radioimmunoassays were used to determine the levels of both secreted and intracellular AFP, albumin, and transferrin. Line 7777 normally produces large quantities of AFP and small quantities of albumin, thus resembling the less differentiated fetal liver with respect to the synthesis of these two proteins. Line 8994 normally produces small quantities of AFP and relatively larger amounts of albumin, thus resembling hepatic functions characteristic of a more differentiated state.

After treatment with HMBA for a period of 28 to 96 hours a three-fold increase in AFP secretion by 7777 and a dose-related increase in AFP, albumin, and transferrin secretion by 8994 were observed. In contrast, the secretion of albumin and transferrin in 7777 was inhibited by 60 and 40%, respectively, following treatment with HMBA.

The intracellular concentration of AFP in 7777 and AFP, albumin, and transferrin in 8994 was increased by pretreatment with HMBA indicating that HMBA is able to stimulate the synthesis of these proteins. The intracellular concentration of AFP, albumin, and transferrin in 7777, when expressed as a percentage of the extracellular concentration of these proteins, did not change significantly during HMBA treatment, indicating that the observed decrease in secreted albumin and transferrin by 7777 is due to decreased synthesis. Similarly, in line 8994, when the intracellular concentration of the three proteins was expressed as a percentage of the extracellular concentration, the only significant change observed was an increase in AFP after 72 hours of HMBA (5 mM) treatment. The observed changes in the synthesis of AFP, albumin, and transferrin in both 7777 and 8994 after HMBA treatment were reversible, as judged by the return to control values upon removal of HMBA from the culture medium. Thus, HMBA stimulates synthesis of the oncofetal protein AFP, a result that appears to be independent of the stage of differentiation of the cell. However, its effect on the synthesis of albumin and transferrin are opposite in the two cell lines, suggesting that the regulation of the synthesis of these two proteins is controlled by factors or conditions that are dependent upon the stage of differentiation of the hepatoma cell lines.

E. Results from studies on HL60. HL60 is a human promyelocytic leukemia cell which can be induced to differentiate by a variety of chemical agents along either the granulocyte or macrophage pathways. We are using computer analysis of two-

dimensional gel electrophoretograms to quantitate changes in protein synthesis accompanying differentiation induced by 12-O-tetradecanoyl phorbol-13-acetate (macrophage) or by dimethylformamide (granulocyte). We are similarly analyzing two-dimensional gels of *in vitro* translates of polyadenylated mRNA extracted from HL60 cells after pretreatment with these agents for appropriate intervals. We have seen both increases and decreases in multiple translation products associated with differentiation in this system suggesting that modification of several different populations of mRNA has occurred. We hope to identify patterns of modulated proteins and mRNAs associated with differentiation induced by these and other agents in the HL60 system. Attempts will then be made to isolate and identify those proteins consistently involved in the differentiation process, followed by studies to clarify their regulation at the molecular level.

F. Results from studies on human B-cell lymphomas. 1. Expression of oncogenes in human B-cell lymphomas. We have obtained a genomic clone of the chicken c-myc DNA and have used this DNA to assay for the expression of human c-myc RNA in our human B-cell lymphoma cell lines. Poly(A) RNA was isolated and fractionated in a denaturing formaldehyde agarose gel as previously described. The RNA was blotted onto nitrocellulose paper and hybridized to *in vitro* nick translated c-myc DNA. We have found increased (approximately 5-fold) levels of a human c-myc-specific RNA (2.6kb) in several of our tumor cell lines as compared to EBV transformed cord blood lymphocytes and to a fully differentiated human tissue (liver).

The expression of other oncogenes will be examined as they become available to our laboratory. We have obtained a genomic clone of the human c-mos DNA and using this DNA to assay for the expression of human c-mos RNA in our human B-cell lymphomas have found no expression in any of the cell lines, although this gene has been localized to chromosome 8 and has been reported to translocate to chromosome 14 in Burkitts lymphomas (personal communication). We have recently obtained c-fes and will repeat similar experiments using ³²P c-fes DNAs.

2. Genetic organization of oncogenes in human B-cell lymphomas. To determine if specific rearrangements or gene amplification of the c-myc oncogene could account for increased expression in the tumor cell lines, the DNA from each of these cell lines was assayed. 20 µg of DNA was digested to completion with the restriction enzymes Eco RI or HindIII. The DNAs were fractionated on agarose gels, blotted onto nitrocellulose paper and hybridized with *in vitro* ³²P-c-myc DNA. The banding patterns of all the tumors lines were similar to one another and to the cord blood lymphocytes. The infectious mononucleosis cell lines, however, all showed a rearrangement of fragment which was complementary to the 5' end of the c-myc DNA. Additionally, gene amplification of this 5' end was apparent in the infectious mononucleosis lines. This phenomenon is being investigated further.

Although c-mos has been reported to translocate to chromosome 14 in Burkitts lymphoma and could possibly be involved in gene regulation and not gene expression, no rearrangement of human c-mos was detectable in any of the cell lines as assayed by restriction endonuclease digestion and hybridization.

3. Transfection experiments in NIH 3T3 cells. We are presently collaborating with Dr. Eric Westin to determine if we can get transformation of NIH 3T3 cells using DNAs from these human B-cell lymphomas.

4. Expression of oncogenes in other tumors. We have also assayed the rat hepatoma cell lines for increased expression of c-myc and c-mos and have shown no increase in gene expression when compared to normal rat liver RNA. The expression of other oncogenes will be examined as they become available to our laboratory.

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:

Heilman, C.A., Engel, L., Lowy, D.R. and Howley, P.M.: Virus specific transcription in bovine papillomavirus-transformed mouse cells. Virology 119: 22-34, 1982.

Hughes, E.H., Schut, H.A.J. and Thorgeirsson, S.S.: Effects of hexamethylene bisacetamide on alpha-fetoprotein, albumin and transferrin production by two rat hepatoma cell lines. In Vitro 18: 157-164, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05261-01 LCM
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Metabolism and Mutagenicity of Chemical Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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6.6	4.6	2.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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.

Project Description

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 system; 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.

1. In the *Salmonella* mutagenesis system, N-hydroxy-phenacetin (N-OH-PA) is activated into a mutagen in the presence of microsomal and/or cytosolic fractions from rat liver (Mol. Pharmacol. 18: 117, 1980). In order to evaluate the relative role of metabolic activation vs detoxification in determining the mutagenicity of N-OH-PA, we have investigated the mutagenic activation of N-OH-PA in the *Salmonella*/hepatocyte system (Biochem. Biophys. Res. Commun. 94: 837, 1980). Incubation of N-OH-PA (0.5-10.0 μM for 30-60 minutes in the presence of hepatocytes ($10^6/\text{ml}$) and TA100 resulted in a mutation frequency not significantly different than that of DMSO control alone (20 $\text{His}^+/\text{10}^7$ bacteria). Pretreatment with nontoxic concentrations of diethyl maleate (DEM) for 10 minutes resulted in a dose-dependent depletion of intracellular hepatocyte glutathione (GSH) levels with a greater than 99% depletion following 10 mM DEM. The mutagenicity of N-OH-PA in the presence of GSH-depleted hepatocytes, however, was not significantly different than that with untreated cells. At higher concentrations (10 μM) N-OH-PA was slightly toxic (30% killing) to the bacteria. Pretreatment of the hepatocytes with 0.15 mM salicylamide, an inhibitor of sulfation and glucuronidation, followed by incubation with N-OH-PA in sulfate-free medium, resulted in dose-dependent increase in the mutagenicity of N-OH-PA (61.0 $\text{His}^+/\text{10}^7$ bacteria at 5 μM N-OH-PA). The results of these studies indicate that the lack of mutagenicity of N-OH-PA in the *Salmonella*/hepatocyte system results from a very efficient detoxification of this hydroxamic acid in the intact hepatocyte. GSH is not critically involved in determining the mutagenicity of N-OH-PA in the *Salmonella*/hepatocyte system.

2. Coincubation of isolated and intact rat hepatocytes and tester strains (TA100, TA98, etc.) of *Salmonella typhimurium* (*Salmonella*/hepatocyte system) has been employed to determine both bacterial mutagenicity and DNA damage in the hepatocytes as measured by alkaline elution following treatment with several N-acetyl-arylamines (Biochem. Biophys. Res. Commun. 94: 837, 1980). We have now substituted the primary rat hepatocytes with a highly differentiated rat hepatoma cell line (Reuber H4-II-E), and examined the metabolic processing and genotoxic effects of three carcinogenic arylamines: N-hydroxy-2-acetylaminofluorene (N-OH-AAF), N-acetoxy-2-acetylaminofluorene (N-Ac-AAF) and N-hydroxy-2-aminofluorene (N-OH-AF). Low levels of DNA breakage, measured by alkaline elution technique, were observed after exposure to N-OH-AAF (up to 100 μM) induced dose-dependent increases in DNA breaks in the Reuber cells. Similar data was obtained for the bacterial mutation frequency for the three arylamines. Employing host cells with differing

metabolic capacity such as the Reuber vs primary hepatocytes may allow a determination of the relative importance of the different metabolic pathways in mutagenicity and/or genotoxicity of arylamines.

3. Pyrazole, a potent inhibitor of alcohol dehydrogenase, was found to be a potent inducer of the activity of low K_m dimethylnitrosamine demethylase (DMN-d). One injection of pyrazole (200 mg/kg body weight) to weanling Wistar rats changed the microsomal DMN demethylase activity by 1.7, 1.9 and 2.5 times the control values at 6, 12 and 24 hours after the injection, respectively. Pyrazole administration reduced arylhydrocarbon hydroxylase (AHH) activity. When animals were injected with pyrazole (200 mg/kg body weight) for 1, 2, 3 or 4 consecutive days, the values for DMN-activity were 277, 297, 306 and 319% of the control values. The corresponding values for AHH were 91, 67, 57 and 45% for 1, 2, 3 and 4 injections, respectively. Pyrazole-induced DMN-d activity was NADPH-dependent and was inhibited by CO; n-butanol gave a 50% inhibition at a concentration of 2×10^{-3} M. The corresponding value for metyrapone was 1×10^{-2} M. Cytochrome P-450 was slightly increased by pyrazole and its CO-complex gave an absorption maximum around 451 nm. When the microsomal proteins were separated using sodium dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis, a large increase in a band at about 51,000 daltons was found in the liver microsomes of pyrazole-treated animals.

4. The correlation between the in vivo toxicity and in vitro mutagenicity of dimethylnitrosamine (DMN) and the activity of DMN demethylase I (DMN-dI) after pyrazole treatment of rats was studied. The biological effects of pyrazole were measured either as toxicity to the rats or as mutations to a Salmonella strain TA92. A dose-response relationship was observed between DMN-dI activity and the administered dose of pyrazole. Pyrazole administration increased the toxicity of DMN when measured as LD₅₀ or as histopathological effect on the liver.

Phenobarbital and methylcholanthrene administration did not have any effect on the activity of DMN-dI or on the number of histidine revertant colonies when tested using the liquid suspension method in the presence of DMN and NADPH generating system. When microsomes from the pyrazole-treated animals were used in the mutagenesis assay there was a linear correlation between DMN-dI activity and the number of histidine revertant colonies. It is concluded that pyrazole treatment of animals increases the activity of liver DMN-dI, the toxicity of DMN and the number of mutations.

5. The mutagenicity of N-hydroxy-2-acetylaminofluorene and N-hydroxyphenacetin and their respective deacetylated metabolites, N-hydroxy-2-aminofluorene and 2-nitrosofluorene, and N-hydroxyphenetidine and p-nitrosophenetole was determined in nitroreductase deficient Salmonella tester strains TA98FR and TA100FR. The mutagenicity of N-hydroxy-2-acetylaminofluorene mediated by either rat liver microsomes or rat liver 105,000 g supernatant fractions was no different in either TA98 (nitroreductase proficient) or TA98FR (nitroreductase deficient). Similarly, the mutagenicity of N-hydroxyphenacetin mediated by hamster liver microsomes was not affected by either the presence or absence of nitroreductase activity in TA100. N-Hydroxy-2-aminofluorene and 2-nitrosofluorene were equipotent direct acting mutagens in both TA98 and TA98FR, as were both N-hydroxyphenetidine and p-nitrosophenetole in TA100 and TA100FR. Ascorbate (5 mM) and NADPH (1 mM) had no significant effect on the mutagenicity of either N-hydroxy-2-acetylaminofluorene, N-hydroxy-2-aminofluorene, or 2-nitrosofluorene in TA98 or TA 98FR, whereas

ascorbate and NADPH markedly inhibited the mutagenicity of both N-hydroxyphenetidine and p-nitrosophenetole in both TA 100 and TA 100FR. Ascorbate appears to inhibit the mutagenicity of N-hydroxyphenetidine and p-nitrosophenetole as a result of the nonenzymatic chemical reduction of these compounds to nonmutagenic derivatives.

6. 2-Acetylaminofluorene (AAF) undergoes cytochrome P-450 dependent oxidation via N- and C-hydroxylations. N-Hydroxylation is considered to be the initial and obligatory step in the metabolic activation of 2-AAF to a carcinogen, while C-hydroxylation appears to be detoxification pathways. For an estimate of the significance of these pathways in the metabolism of AAF, a kinetic study was undertaken using rat liver microsomes and substrate concentrations ranging from 0.02-200 μM . N-Hydroxylation accounted for approximately 50% of the metabolites at low substrate concentrations ($< 0.2 \mu\text{M}$) but less than 5% at concentrations of AAF above 10 μM . The K_m and V_{max} for this reaction were 0.04 μM and 6.0 pmoles/min/mg, respectively. While N-hydroxylation was best described by a single enzyme system (single catalytic site), two enzyme kinetics (two catalytic sites) were observed for 1-, 3-, 5- and 7-hydroxylations. For example, Eadie-Scatchard plots revealed a high affinity ($K_m = 0.07 \mu\text{M}$) low capacity (4.87 pmoles/min/mg) enzyme and a low affinity ($K_m = 99 \mu\text{M}$) high capacity (1289 pmoles/min/mg) enzyme for the 7-hydroxylation of AAF. These data indicate that more than one form of cytochrome P-450 is involved in each of the C-hydroxylations, whereas only one form catalyzes the N-hydroxylation.

7. AAF metabolism has also been used as a probe to study the human cytochrome P-450 system. Human liver samples were kindly supplied by Dr. D.S. Davies and Dr. A.R. Boobis, Hammersmith Hospital, Royal Postgraduate Medical School, University of London, UK. Similar to the rat, two catalytic sites in the cytochrome P-450 system were involved in 7- and 9-hydroxylations. For example, the kinetic parameters for 7-hydroxylation were: $K_m = 0.36 + 0.05 \mu\text{M}$; $V_{max} = 39.8 \pm 4.3$ pmoles/mg $^{-1}$ /min $^{-1}$; and $K_m = 97 + 20 \mu\text{M}$, $V_{max} = 655 + 74$ pmoles/mg $^{-1}$ /min $^{-1}$. The 1-, 3- and N-hydroxylations were best described by one enzyme system. These data add to the increasing evidence for a similar multiplicity of cytochrome P-450 in man as in the rat.

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:

Boobis, A.R., Brodie, M.J., McManus, M.E., Staiano, N., Thorgeirsson, S.S., and Davies, D.S.: Metabolism and mutagenic activation of 2-acetylaminofluorene by human liver and lung. Adv. Exp. Med. Biol. 136: 1193-1203, 1982.

Dybing, E., Saxholm, H.J.K., Aune, T., Wirth, P.J. and Thorgeirsson, S.S.: Studies on mutagenic and carcinogenic N-substituted aryl compounds -- cosmetics and drugs. Natl. Cancer Inst. Monogr. 58: 21-27, 1981

Evarts, R.P., Haliday, E., Negishi, M. and Hjelmeland, L.M.: The induction of microsomal dimethylnitrosamine demethylase by pyrazole. Biochem. Pharmacol. 31: 1245-1250, 1982.

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Thorgeirsson, S.S.: Chemical mutagenesis. In Foster, H.L. and Small, P.D. (Eds.): The Mouse in Biomedical Research. Vol. IV, New York, Academic Press, 1982, pp. 329-339.

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Thorgeirsson, S.S., McManus, M.E. and Glowinski, I.B.: Metabolic processing of aromatic amides. In Horning, M.G. (Ed.): Drug Metabolism and Drug Toxicity (in press).

Thorgeirsson, S.S., Schut, H.A.J., Staiano, N., Wirth, P.J. and Everson, R.B.: Mutagenicity of N-substituted aryl compounds in microbial systems. Natl. Cancer Inst. Monogr. 58: 229-237, 1981.

Thorgeirsson, S.S., Wirth, P.J., Staiano, N. and Smith, C.L.: Metabolic activation of 2-acetylaminofluorene. Adv. Exp. Med. Biol. 136: 897-919, 1982.

Wirth, P.J., Alewood, P., Calder, I. and Thorgeirsson, S.S.: Mutagenicity of N-hydroxy-2-acetylaminofluorene and N-hydroxyphenacetin and their respective deacetylated metabolites in nitroreductase deficient Salmonella TA 98FR and TA 100FR. Carcinogenesis 3: 167-170, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05262-01 LCM
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanism of Chemically Induced Murine Hepatomas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Michael E. McManus	Visiting Fellow	LCM	NCI
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COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:

3.8

PROFESSIONAL:

2.3

OTHER:

1.5

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(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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) the time course of chemically induced hepatoma formation, and the genetic control of the changing enzyme patterns during this process; (2) isolation and characterization of preneoplastic cell populations; (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) diverting the blood from the splanchnic area directly into inferior vena cava, by performing porta-caval shunt operations, resulted in changes in the liver, such as expression of γ-glutamyltranspeptidase activity and lack of glucose-6-phosphatase activity, similar to those produced by hepatocarcinogens; and (2) transplantation of normal rat hepatocytes into the spleen of isogenic animals resulted in growth of the cells. Similar experiments using preneoplastic hepatocytes obtained by treating newborn rats with a single dose of diethylnitrosamine followed by administration of phenobarbital, are in progress.

Project Description

Objectives: The main 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. The research is currently focused on the following areas: (1) time course of chemically induced hepatoma formation and the genetic control of the changing enzyme patterns during this process; (2) isolation and characterization of preneoplastic cell populations; (3) modulating effects of blood flow and oxygen tension on hepatoma formation and development; and (4) the role of genetic predisposition in hepatoma development.

Methods Employed: The principal methods employed are (1) recording spectrophotometry of turbid solutions, (2) enzyme assays involving radiometric, spectrophotometric, and spectrophotofluorometric determinations of product formation or substrate disappearance, (3) radioisotopic measurements using tritium and carbon-14, (4) differential centrifugation, (5) polyacrylamide gel electrophoresis, (6) column and thin-layer chromatography, (7) high pressure liquid chromatography, (8) tissue culture technique, (9) microsurgical procedures, (10) cell separation techniques, and (11) histochemical staining techniques.

Major Findings: 1. Microsurgical procedure to perform porta-caval shunt operations (PCS) in rats has been established. PCS operations produce changes in the liver which are similar, namely, expression of γ -glutamyltranspeptidase activity and lack of glucose-6-phosphatase activity to those produced by hepatocarcinogens.

2. A procedure for transplantation of normal rat hepatocytes into the spleen of isogenic animals has been established, and growth of these cells in the spleen has been obtained. Similar experiments using preneoplastic cells, obtained by giving a single injection of diethylnitrosamine to newborn rats followed by administration of phenobarbital, are in progress.

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. and Brown, C.A.: 2,4-Diaminoanisole-induced thyroid pigmentation in rats inhibited by m-phenylenediamine. Toxicol. Lett. 8: 257-264, 1981.

Evarts, R.P., Brown, C.A. and Mostafa, M.H.: Production of kidney tumors in rats with low dose of dimethylnitrosamine after partial hepatectomy. J. Natl. Cancer Inst. 68: 293-298, 1982.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Computer Analysis of Carcinogenesis by Two-Dimensional Gel Electrophoresis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Snorri S. Thorgeirsson	Acting Chief	LCM	NCI
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COOPERATING UNITS (if any)

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TOTAL MANYEARS:

2.8

PROFESSIONAL:

1.8

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project was initiated to study the mechanism of chemical carcinogenesis by employing the technique of quantitative two-dimensional electrophoresis of total cellular proteins. The technique of two-dimensional gel electrophoresis allows the simultaneous separation of hundreds of polypeptides in a single polyacrylamide gel. We have acquired, and are further developing, 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. Other semi-automatic programs match together the spot patterns found in different gels and can flag those spots which are missing or vary significantly in one electrophoretogram or the other. Still other programs link together a series of gels which may constitute an experiment and allow the investigator to quantitatively follow the synthesis of almost any protein through that experiment or through a series of related experiments.

Project Description

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 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, (4) radioisotope measurements, and (5) high pressure liquid chromatography.

Major Findings: The computer programs developed here for the quantitation of the two-dimensional gels include:

1. The Comtal driver. This is responsible for arbitrating the transfer of data and commands between the Comtal and the VAX. It has been significantly modified from a driver given us by the Department of Electrical and Computer Engineering at the University of New Mexico.
2. The Comtal Command I/O library. This library package acts as the main interface between user level programs and the Comtal driver. It is written in such a way that the programmer may treat the various image and graphics planes (or memory arrays) in the Comtal in the same way as he would a regular file. No special I/O calls or procedures are necessary. Thus, a command, "open(/dev/image.1/);" will open Comtal image one for reading in the same way as if this image plane had been located on one of our disk drives.
3. ctmatch -- This sets up landmark matches to be used by the automatic match program and displays the segmented images of two films on the Comtal. The first film is colored blue and is fixed. The second film is in monotone and is displayed as a "small area image" over the first film. The operator can slide image 2 over image 1 so that some of the corresponding spots overlap. 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 match, which automatically matches up the remaining spots in the two gel patterns.
4. ctedit -- This edits gel patterns matched by the computer and displays the segmented images of two films after the films have been automatically matched with the match program. The first film is displayed in color with the matched contours in green and the unmatched in magenta. The second film is displayed as a "small area image" over the first image and is in monotone. Unmatched contours are outlined in black in the second image. This image may be roamed over the color image as in the ctmatch program outlined above. Auto-matched contours may be added or deleted interactively. The colors and outlining will be changed as the contours are matched or unmatched.

5. ctmosaic -- This displays a given spot as it appears through several analyzed films. The targeted spots are matched and defined in a summary data base file called mul.rb. This file is created by the assist program and summarizes the synthesis of every polypeptide as it is affected through an experiment. Each spot and a region surrounding it will be displayed in a grid arrangement on the Comtal screen. If there is an empty or unmatched column in the row, a region surrounding the position where the spot should be will be displayed. Regions may be displayed and roamed as "small area images" in much the manner of ctmatch and ctedit above.

6. process -- This automatically processes a set of films. This is a C-shell script designed to aid a user in analyzing multiple scans of two-dimensional gel electrophoretograms. The program is interactive and designed to be self-explanatory. The program knows about film names, directories, backups, etc., and tries to keep track of where the experimenter is in the analysis.

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 beginning to focus on studies which will analyze, catalog and compare the capacity 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., Vo, K.-P., Neilsen, C., Geiduschek, E.P. and Xuong, N.H.: Computer analysis of two-dimensional gels: Semi-automated matching. Clin. Chem. 28: 867-875, 1982.

Miller, M.J., Xuong, N.-H. and Geiduschek, E.P.: A quantitative analysis of the heat shock response in S. cerevisiae. J. Bacteriol. (in press).

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PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05264-01 LCM

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Conformation Changes in DNA and RNA Induced by Chemical Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C.-H. Robert Lee	Senior Staff Fellow	LCM	NCI
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	Snorri S. Thorgeirsson	Acting Chief	LCM	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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TOTAL MANYEARS:

1.6

PROFESSIONAL:

1.6

OTHER:

0

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(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 (ELD), electrophoresis of circular DNA and potential energy calculation. The results obtained so far include: (1) observation of A- and B-form DNA conformation in solution in electric field, and that DNA conformation in solution, in the absence of electric field, possesses a coiled coil-like tertiary structure; (2) electrophoresis of circular DNA reveal that organic solvents and alkylated ammonium ions unwind, whereas metallic and ammonium ions as well as polyamines wind the DNA helix; and (3) potential energy calculations indicate that there are four stable conformations for dinucleoside phosphates in solution.

Project Description

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. Using electric linear dichroism, we observed the A- and B-form DNA conformation in electric field in solution. It is also concluded that the DNA conformation in the absence of electric field possesses a coiled coil-like tertiary structure in solution.

2. Electrophoresis of circular DNA shows that organic solvents and alkylated ammonium ions unwind DNA helix, whereas metallic and ammonium ions as well as polyamines wind DNA helix.

3. Potential energy calculations reveal that there are four stable conformations for dinucleoside phosphates in solution.

4. Studies of complex formation between tryptophan pyrolysates and DNA (including oligonucleotides) by NMR, CD, ELD methods and electrophoresis of circular DNA are in progress. Our NMR results indicate that the pyrolysates prefer to intercalate with GC base pair rather than AT base pair.

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 should provide means towards the goal of both defining cancer cause and devising effective programs for cancer prevention.

Proposed Course: Continue the course outlined under Objectives.

Publications:

Lee, C.-H. and Charney, E.: B-Form and A-form DNA in an electric field. Int. J. Biol. Macromol. 4: 121-122, 1982.

Mizusawa, H., Lee, C.-H., Kakefuds, T., McKenney, K., Shimatake, H. and Rosenberg, M.: Base insertion and deletion mutation induced in an Escherichia coli plasmid by benzo[a]pyrene-7,8-diol-9,10-epoxide. Proc. Natl. Acad. Sci. USA 78: 6817-6820, 1981.

October 1, 1981 through September 30, 1982

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 $\text{Na}^+\text{-K}^+$ ATPase pump as it is inhibited by ouabain. Differentiation is also inhibited by the

Ca⁺⁺ ionophore A23187 indicating that increases in intracellular Ca⁺⁺ are not sufficient for differentiation to occur. Studies of intracellular ion concentrations have shown that both Na⁺ and K⁺ are elevated during Ca⁺⁺-induced differentiation. Ouabain and A23187 block the K⁺ increase but not the Na⁺ increase. These results have pointed to an increase in intracellular K⁺ as a mediator of terminal differentiation.

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. We have found that after carcinogen exposure in primary cultures of mouse keratinocytes, some cells resist the Ca⁺⁺ signal to differentiate and continue to proliferate under high Ca⁺⁺ conditions, producing countable foci which stain red with rhodamine B. Cells obtained from mouse skin initiated in vivo show the same characteristics. Cell lines derived from these foci are not tumorigenic when first isolated but upon prolonged culture with no further treatment become highly malignant. Recent studies in collaboration with Dr. Edward Scolnick have shown that infection of epidermal cells with certain transforming retroviruses imparts resistance to Ca⁺⁺-induced differentiation and this effect is dependent on an intact transforming gene.

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, was undertaken. A library of cDNA clones was prepared to total poly(A)RNA isolated from newborn epidermis. Clones corresponding to the 55, 59 and 67 kilodalton keratins have been isolated, characterized and partially or completely sequenced. Specific mRNA has been identified for each probe in newborn, adult and embryonic skin but not in normal epidermal cells in culture. The repression of expression of these keratin genes in cultured cells does not appear to be due to a developmental switch but is more likely related to factors in the culture system. We have been able to detect transcripts of all 3 keratins in a malignantly transformed mouse epidermal cell line suggesting that regulation of keratin gene expression in these cells differs from normal cultured cells.

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 of animal strains through selective breeding 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. Susceptibility is a property of the skin itself and 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, 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 constitutively initiated cells which resist normal differentiation signals. These may be embryonic cells which normally disappear near parturition but persist into adulthood in SENCAR mice.

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-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-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-N²-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 highly sensitive quantitative immunoassays for monitoring carcinogen binding and for morphological localization of binding sites.

Currently these assays are able to detect one adduct per 10⁷ 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 cell culture, immunofluorescence studies with anti-BP-dG antibodies have shown that bound benzopyrene 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. The BP-dG antibody has also been used in immunoassays to screen DNA obtained from lung cancer patients and controls. Several positive samples have been obtained in lung tissue from cancer patients and in one case from DNA obtained from circulating lymphocytes. Exposure histories are currently being assessed for the patients studied. 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 cis-DDP-DNA immunoassay has been used to measure platinum bound to DNA of ascites tumor cells recovered from mice receiving cis-DDP therapy. This capability forms the background to apply immunoassays to monitor platinum binding to DNA of cancer patients receiving 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 heterogeneous 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. Putative initiated cells, isolated in our laboratory, cannot be stimulated to differentiate by phorbol esters thus providing a mechanism for their clonal expansion if they were a subpopulation of a large normal cell mass. Retinoids prevent the terminal differentiation induced by phorbol esters. This could play a role in the anti-promoting activity of retinoids. Studies on the progression of benign to malignant tumors *in vivo* suggest 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 epithelial differentiation in most epithelial tissues. In recent years 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 play a role as specific carriers of the sugar, mannose. In the past year we have been able to show that the amount of retinylphosphate available for mannosylation in microsomal membranes is directly proportional to the degree of morphological differentiation of the cell from which the microsomes were derived and inversely proportional to growth rate.

In particular an investigation was conducted of nine hepatocellular carcinomas of the Morris series varying in the degree of differentiation and growth rate. Microsomes from these tumors and their host liver were studied for their ability to synthesize retinyl phosphate mannose (Ret-P-Man) and dolichylphosphate mannose (DoI-P-Man) from their endogenous acceptors Ret-P and DoI-P. In normal rat liver microsomes the molar ratio Ret-P/DoI-P as derived from measurement of radioactivity in the highly sensitive mannosylation reaction is about 0.3 in rat, hamster and guinea pig liver. In hepatocellular carcinoma tissue the ratio varies from 0.1 to 0 from the most differentiated and slowest growing to the least differentiated and fastest growing tumors. It is of interest that such changed ratio does not result from a change in enzyme activity but it reflects

an actual specific decrease in the amount of Ret-P available in the membrane. This finding may explain changes in cell surface properties and glycosylation patterns in neoplastically transformed liver tissue.

A study of the pool sizes of retinylpalmitate, the most abundant storage form of vitamin A and the cellular retinol binding protein, has revealed a depletion in both these molecules in the hepatocellular carcinoma tissue. Thus, this tissue appears depleted of vitamin A.

A second area of interest focuses on the biochemical mechanism of action of tumor promoters and counteraction by retinoids. We had previously found that the availability of cell surface macromolecules, such as the epidermal growth factor (EGF) receptor, was greatly enhanced in fibroblasts cultured in the presence of retinoic acid, while phorbol esters decreased the number of available receptors. Antagonism between retinoids and phorbol esters was also found at the level of the biosynthesis of procollagen α_1 and α_2 ; again for this molecule retinoids enhanced its synthesis and specifically the incorporation of mannose, whereas tumor promoters inhibited such incorporation. A third biochemical target of the antagonistic action of retinoids and tumor promoters appears to be the cell surface glycoprotein, fibronectin. It appears that retinoids induce retention of this molecule at the cell surface while tumor promoters cause its shedding into the medium.

The emerging suggestion is that the retinoids are necessary to maintain the functional expression of the cell surface in a variety of cells and that phorbol esters directly interfere with this function.

It is our immediate aim to attempt to clarify whether a central and unique mechanism in the biosynthesis of cell surface molecules requiring vitamin A is being interfered with by tumor promoters.

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 receptors; (2) elucidates the functional importance of receptors in promoter action, identifies endogenous ligands with specific affinity for receptors of exogenous promoters, studies the role of endogenous ligands in normal growth control; and (3) identifies and studies cellular mediators which are critical for promoter action.

The efforts of this Section are directed at understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. 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 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.

Current work is focused on characterization of phorbol ester receptors. Pharmacological evidence in vivo implies the presence of subclasses of receptors (e.g., those for first stage promotion, second stage promotion, complete promotion). Over the past year, we have been able to demonstrate that $[20\text{-}^3\text{H}]$ 12-deoxyphorbol 13-isobutyrate ($[^3\text{H}]\text{DPB}$) binds to mouse skin homogenates in a manner consistent

with the presence of a major and a minor binding site. Comparison with [³H]-phorbol 12,13-dibutyrate ([³H]DPBu) binding indicates that PDBu binds to a third major site in addition to the two recognized by DPB. Structure-activity analysis of the two major binding sites indicates distinct structure-activity relations, with the PDBu selective site showing relatively greater preference for lipophilic derivatives such as PMA than does the major DPB site. In other studies, we demonstrated that the first stage promoter 4-O-methylPMA, at concentrations (50 nM) consistent with its potency as a first stage promoter, was able to inhibit binding to the minor DPB site.

Genetics provides an alternate approach to demonstrate the role of receptors and the possible relationship between receptor subclasses. Analysis has been pursued at two levels. Mouse strains differing in their susceptibility to tumor promoters have been compared for their relative abundance of receptor subclasses. In vitro, cultured cell variants and mutant nematodes (Caenorhabditis elegans) altered in phorbol ester responsiveness have been screened for changes in binding activity. No changes were observed in the cultured cells. Analysis of mouse strains and the nematodes is continuing.

As part of our effort to biochemically characterize the phorbol ester receptors, we prepared the photoaffinity label phorbol 12-p-azidobenzoate 13-benzoate (PpABB). Upon UV irradiation, PpABB bound specifically and irreversibly to brain membranes. Identification of the resulting adducts indicated that specific binding was to phosphatidyl serine and phosphatidyl ethanolamine. Since specific lipid labeling was blocked by pre-treatment with protease, we conclude that the identified lipids are specifically associated with a protein receptor. Efforts to develop other photoaffinity labels selective for the protein moiety of the receptor are in progress.

The high evolutionary conservation of the phorbol ester receptor argues for the presence of an endogenous compound which normally interacts at the binding site. Since brain contains the highest level of receptors, we therefore screened brain for potential inhibitors of binding. We found a major, heat-stable, low molecular weight inhibitor. It was identified as ascorbic acid and shown to act irreversibly through a free radical mechanism to cause lipid peroxidation. Its action can be blocked by free radical inhibitors, and efforts to identify other inhibitors are in progress.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04484-05 CCTP
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Modulation of Cell Surface and Growth Parameters by Retinoids and Tumor Promoters

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Laboratory of Cellular Carcinogenesis and Tumor Promotion

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2

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1.85

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0.15

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Upon differentiation of embryonal carcinoma cells induced by retinoids, the "apparent" membrane microviscosity increases dramatically. Only biologically active retinoids induced differentiation and caused an enhancement in microviscosity. Several embryonal carcinoma cell lines have a relatively lower "apparent" microviscosity than their differentiated derivatives suggesting that, in general, differentiation is accompanied by an increase in microviscosity. At higher concentrations retinoids cause a reduction in "apparent" membrane-microviscosity of various cells, whether the analogue is biologically active or not. The cell surface appears to be a target for the action of retinoids and tumor promoting agents. These groups of compounds were found to exert opposing actions on the availability of epidermal growth factor receptors and on the biosynthesis and secretion of procollagen in a variety of cell lines. Generally, retinoids had an enhancing effect on these parameters, whereas tumor promoters had an inhibitory effect.

Project Description

Objectives:

To study the molecular mechanisms by which retinoids regulate and tumor promoters disrupt functional characteristics of the cell surface, such as growth factor reception, fibronectin and procollagen synthesis and association with the cell surface.

Methods Employed:

1. Cell culture. Embryonal carcinoma cells and their differentiated derivatives were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) and 100 U/ml each of penicillin and kanamycin and 100 µg/ml of streptomycin. Mouse fibroblast cells were cultured in DMEM containing 10% calf serum. Binding of ^{125}I -labeled mouse EGF (Collaborative Research, Waltham, MA) to cultured cells and binding of [^3H]retinoic acid and [^3H]retinol to their binding proteins in the cytosol were determined.

2. Binding of epidermal growth factor. Cells were grown in complete medium in 35 mm cluster dishes. At the time of the EGF binding assay, medium was removed and cells washed twice with 2 ml binding buffer (DMEM containing 1 mg/ml of BSA and 50 mM Bes pH 6.0). Then 1 ml of binding buffer containing 0.825 ng of ^{125}I labeled mouse EGF (Collaborative Research, 1.4×10^5 dpm) was added to each well. After 60 min at 37°C, unbound ^{125}I -EGF was removed, cells were washed four times with 2 ml buffer and solubilized in 0.75 ml lysing buffer (0.1 M Tris-HCl, pH 7.4 containing 0.5% SDS and 1 mM EDTA) and the wells washed twice with 0.5ml lysing buffer. Radioactivity was counted in a Packard β-counter. Nonspecific binding was determined in the presence of 10 µg unlabeled EGF.

Internalized radioactivity was determined by washing the cells three times with ice-cold binding medium after incubation with ^{125}I -EGF, followed by treatment with 0.1 ml solution of acetic acid (0.2 M pH 2.5) containing 0.5 M NaCl for 6 min at 4°C. Cells were then rinsed two times with 0.5 ml of the same solution and then solubilized in lysing buffer. Cell-surface associated ^{125}I -EGF was calculated from the difference between total cell-associated and internalized radioactivity.

3. Labeling of secreted proteins. Cells were grown in complete medium in the presence or absence of 10^{-6} M retinoic acid for 72 hrs. In some instances TPA (100 ng/ml) was added at 48 hrs. and incubation continued for another 24 hrs. At that time, incorporation of ^{35}S -methionine into secreted products was determined. Cells were washed twice with medium without serum and methionine and then incubated in the same medium containing 20 µCi/ml of ^{35}S -methionine (990 Ci/mmol). After a 2 hr incubation period, medium was removed and cells were centrifuged at 1500 x g for 10 min. Then a 100% TCA solution was added to the supernatant until a final concentration of 10% was reached. After the addition of 50 µg of BSA as a carrier, medium was incubated in ice for 30 min and then centrifuged for 30 min at 20,000 x g. Precipitate was washed with ether and solubilized in 10 mM Tris-HCl buffer (pH 7.4) containing 0.5% SDS.

In the case of $2\text{-}^3\text{H}$ -mannose and ^{14}C -proline incorporation, the procedure was the same as for ^{35}S -methionine, except that medium containing low glucose or medium without proline was used, respectively.

4. Determination of "apparent" microviscosity. Membrane microviscosity was determined by fluorescence polarization analysis with the aid of the fluorescent probe 1,6-diphenyl 1,3,5-hexatriene (DPH). DPH was dissolved in tetrahydrofuran and added to cells at a final concentration of 10^{-6} M. Membrane microviscosity was measured 1 hr. later in an Aminco SPF 500 spectrophotofluorometer equipped with motorized Glan-Thompson polarizers.

5. Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed according to the method of Laemmli. Polyacrylamide gradient electrophoresis was performed using 6-22% acrylamide. Gels were processed and prepared for fluorography as described by Bonner and Laskey.

Major Findings:

1. Effect of retinoids on membrane microviscosity and EGF binding of embryonal carcinoma cells. Retinoids can induce differentiation of a variety of embryonal carcinoma cell lines. Upon differentiation a large number of cell surface characteristics are altered. Induction of differentiation of embryonal carcinoma cells, OC 15 S1, occurs after one day of exposure to retinoic acid and the process of differentiation can be observed by a change in cell morphology: the cells undergo a change from a typical embryonal carcinoma cell towards an epithelial cell with an endoderm-like morphology. This differentiation process occurs with simultaneous changes in "apparent" membrane microviscosity. After 24 hrs. of treatment retinoic acid causes a rapid increase in the lipid microviscosity, concomitantly with the changes in cell morphology. The lipid microviscosity reaches a maximum after 3 days of exposure to retinoic acid and remains at that level even after the removal of retinoic acid. In untreated embryonal carcinoma cells microviscosity remains largely unchanged over this incubation period. Another cell surface characteristic that is altered during differentiation of embryonal carcinoma OC 15 S1 cells is the binding of epidermal growth factor (EGF). Embryonal carcinoma cells, OC 15 S1, have low EGF binding, whereas their differentiated derivatives, OC 15 D1, have a much higher EGF-binding capacity. The increase in "apparent" microviscosity occurs faster than the enhancement in EGF binding. The half-times of the maximum increase in microviscosity is at 39 hrs. after retinoic acid treatment, whereas that of EGF binding is at 60 hrs.

In order to determine if the low "apparent" microviscosity is a more general property of embryonal carcinoma cells, we determined the membrane microviscosity in different embryonal carcinoma cell lines and their differentiated derivatives. All three embryonal carcinoma cell lines tested show a low membrane viscosity, whereas all their differentiated derivatives have a relatively higher "apparent" microviscosity independent of whether they are of a fibroblastic (like PCC 4 D1) or an epithelial (like F9 D1 and OC 15 D1) cell type.

Retinoic acid, its 13 cis-analog, its TMMP analog and retinol are all enhancing the "apparent" microviscosity; these analogs have been shown to be biologically active analogs in that they are able to induce differentiation of embryonal

carcinoma cells. The pyrimidyl and the phenyl analogs of retinoic acid do not enhance microviscosity and are poor inducers of differentiation of these cells. The specificity with which retinoids enhance "apparent" microviscosity also correlates well with their ability to compete for the binding sites of either the retinol or retinoic acid-binding proteins present in the cytosol of OC 15 S1 cells.

In contrast to these specific effects which occur at low concentrations of retinoids, retinoic acid decreases "apparent" microviscosity of the membrane at least in certain cells at high, non-physiological concentrations. This effect may be due to direct insertion of large amounts of retinoids in the membrane.

2. Effect of retinoids and tumor promoters on adhesion and EGF-receptor activity of fibroblasts. In contrast to TPA treatment, RA treatment of mouse fibroblasts caused an increase in adhesiveness and EGF binding and a reduction in saturation density. Furthermore, in 3T3 A31-1-BP-2 cells RA inhibited, whereas TPA enhanced, focus and colony formation. It can be concluded from these results that in this culture model RA can inhibit the expression of the transformed phenotype by preventing progression from the preneoplastic to the neoplastic state of the cell, whereas TPA promotes this process. The increase in adhesiveness produced by retinoids may be associated with the restoration of contact inhibition and reduction in saturation density, resulting in inhibition of focus formation and reduction of colony formation in soft agar.

3. Effect of RA and TPA on procollagen synthesis. Tumor promoting, but not non-promoting, phorbol esters produced decreased levels of 180,000 and 150,000 M.W. glycoproteins in mouse JB-6 cells which are promotable to tumor cell phenotype by phorbol esters. These glycoproteins isolated from JB-6 cells were shown to be procollagen α_1 and α_2 with appropriate hydrolytic techniques. Antipromoting concentrations of retinoic acid blocked the action of phorbol esters. Similar results were obtained in 3T6 mouse fibroblasts.

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 in attempts 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 spontaneously transformed mouse fibroblasts, BALB/c 3T12-3 cells, to resemble the "normal phenotype." At the molecular level, it appears that retinoids modify adhesive properties of transformed cells by their action on the biosynthesis of specific cell surface glycoproteins. Retinoids also seem to affect, profoundly, the availability of receptors for 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 of the biochemical targets for the opposing actions of retinoids and tumor promoters.

Proposed Course:

The following seem to be logical developments of this research: characterization of cell surface glycoproteins responsible for altered growth and adhesion of neoplastically transformed cells; modulation of the synthesis of these glycoproteins by retinoids and antagonism by tumor promoters such as phorbol esters. Study of the mechanism responsible for the increase in EGF receptor sites caused by retinoids.

Publications:

- Dion, D., Bear, J., Bateman, J., De Luca, L.M., and Colburn, N. Tumor promoting phorbol esters inhibit procollagen synthesis in promotable JB-6 mouse epidermal cells. J.Natl. Cancer Inst., 1982, in press.
- Dion, L.D., De Luca, L.M., and Colburn, N.H.: Phorbol ester-induced anchorage independence and its antagonism by retinoic acid correlates with altered expression of specific glycoproteins. Carcinogenesis 2: 951-958, 1981.
- Jetten, A.M. and De Luca, L.M.: Studies on the antagonistic actions of TPA and retinoic acid. In Hecker, E. (Ed.): Carcinogenesis 7. Raven Press, New York, 1982, pp. 513-518.
- Jetten, A.M., Meeks, R.G., and De Luca, L.M.: Enhancement in apparent membrane microviscosity during differentiation of embryonal carcinoma cells induced by retinoids. Exp. Cell Res. 138: 494-498, 1982.
- Jetten, A.M.: Effects of retinoic acid on the binding and mitogenic activity of epidermal growth factor. J. Cell. Physiol. 110: 235-240, 1982.

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Model Systems for the Study of Chemical Carcinogenesis at the Cellular Level

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
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8.0

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3.5

OTHER:

4.5

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 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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^{++} . Carcinogen exposure to mouse skin in vivo or mouse epidermal cells in culture yields cellular foci which resist Ca^{++} -induced differentiation but are not tumorigenic. Exposure to some transforming retroviruses also imparts resistance to differentiation. Transformed keratinocytes express an altered profile of keratins, the major skin differentiation proteins. To explore altered regulation of specific differentiation proteins during carcinogenesis, keratin genes have been cloned and sequenced and the amino acid sequence of the proteins deduced. Tumor promoters induce terminal differentiation in some basal cells while stimulating proliferation in others. Carcinogen-altered preneoplastic cells are resistant to the differentiation-inducing effects of tumor promoters, a property providing a selective advantage to these cells during promotion. In vivo studies indicate that genotoxic carcinogens, but not tumor promoters, can accelerate malignant conversion of benign lesions.

Project Description

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 a mouse epidermal cell culture as a major model to approach the stated objectives. Previous studies have shown that this model functions biologically in a highly analogous fashion 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 into 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 flame photometry. Cellular functions, including the production of specific differentiation products, are monitored by enzyme assays, gel electrophoresis, amino acid analysis and radioimmunoassay. The progression to the malignant phenotype is monitored by growth rates, soft agar assay, karyotypic abnormalities, enzymatic changes 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 filter hybridization, translation arrest 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) improved assays for quantitation and selection of carcinogen altered cells; 3) biochemical and molecular genetic characterization of specific marker molecules and assessment of their regulation in normal and transformed cells; 4) enhanced 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. Essentially 100% of the attached cells are in the proliferating cell pool.

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. A variety of morphological, biochemical and immunological techniques have indicated that low calcium culture selects for basal cells, while higher calcium levels induce differentiation in a manner closely resembling the in vivo process.

The mechanism by which calcium induces epidermal differentiation has been studied in some detail. We have determined that the Ca^{++} effect cannot be reproduced by many other cations nor can it be blocked by inhibitors of macromolecular synthesis, microtubule disrupters or several calcium antagonists. The Ca^{++} effect is independent of cyclic nucleotides, markedly accelerated by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and inhibited by the Ca^{++} ionophore, A23187, and the inhibitor of Na-K ATPase, ouabain.

Ca^{++} -induced differentiation is associated with a substantial increase in epidermal transglutaminase activity, the formation of $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ dipeptide bonds and a marked accumulation of cornified cell envelopes (40-fold increase above 0.02 mM Ca^{++} cells). Both ouabain and A23187 block the production of cornified cell envelopes in the presence of 1.2 mM Ca^{++} . Likewise both agents block the formation of desmosomes and the marginal band (the structure forming a cornified cell envelope) when studied by electron microscopy, while control cells in 1.2 mM Ca^{++} form these structures in abundance. The block in differentiation caused by A23187 and ouabain has focused attention on the role of Na^+ and K^+ ions in epidermal differentiation. Several drugs which act as K^+ ionophores or specific blockers of Na^+ or Ca^{++} transport were without effect on differentiation. Cells plated in 1.2 mM Ca^{++} medium showed a 5-fold increase in intracellular Na^+ and a 2-fold increase in intracellular K^+ when compared to proliferating cells in 0.09 mM Ca^{++} medium. When cells grown under low Ca^{++} conditions are switched to high Ca^{++} , intracellular Na^+ rises 1.5- to 2.5-fold after a 12-hour lag. Intracellular K^+ rises by 50% within 6 hours of a switch from low to high Ca^{++} . When ouabain is used in high Ca^{++} medium to inhibit differentiation, intracellular Na^+ rises rapidly to 3-6-fold above control values. However, the expected rise in K^+ is completely blocked and intracellular potassium decreases significantly. When A23187 is used in 1.2 mM Ca^{++} medium to block differentiation, both Na^+ and K^+ increases are blocked and intracellular K^+ actually decreases significantly after 6 hours. These findings have suggested that the increase in intracellular K^+ observed during Ca^{++} -induced differentiation may be important for the completion of the differentiation process.

Further studies on the modulation of differentiation have been conducted with retinoic acid. This agent is known to alter epidermal differentiation and to inhibit skin tumor promotion, but its 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 other epidermal cells with high transglutaminase activity show increases in both of these products. Analysis of the transglutaminase induced by retinoic

acid suggests it is the same enzyme induced by tumor promoters and high Ca^{++} medium. This paradoxical action of retinoic acid provides a novel mechanism by which this agent could modify differentiation and carcinogenesis.

Additional studies, performed in collaboration with John Stanley of the Uniformed Services University of the Health Sciences, have demonstrated specific protein changes associated with epidermal differentiation. These experiments show that the basement membrane zone antigen, bullous pemphigoid, is a 220 Kd protein synthesized exclusively by the proliferating basal cells. Synthesis of this protein ceases upon induction of terminal differentiation. In contrast, pemphigus antigen is a 130 Kd protein synthesized exclusively by differentiating keratinocytes but not by basal cells. These two important antigens play a role in epidermal cell adhesion to the basement membrane or to neighboring cells and thus could be important factors in regulating the process of differentiation.

The emphasis we have placed on understanding the regulation of normal 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 colonies while normal cells slough from the culture dish. During the past year technical improvements in our ability to select for altered cell foci have developed. By changing the selection procedures to a gradual increase in Ca^{++} concentration, colonies of altered cells can be selected within two weeks of carcinogen exposure to newborn epidermal cells. This earlier selection procedure reduces the number of spontaneous colonies seen in the newborn assay. In collaboration with John Lechner of the Laboratory of Human Carcinogenesis we have utilized new medium to grow adult mouse keratinocytes with little or no serum. This improved medium has allowed us to reduce selection time to several weeks in this assay as well. Further studies, performed with Dr. Ed Scolnick of the Laboratory of Tumor Virus Genetics, have indicated that infection of newborn epidermal cells by certain oncogenic retroviruses (Harvey and Kirsten) results in cultures resistant to Ca^{++} -induced differentiation. This effect is dependent on a functioning p21 transforming gene and results in cells which proliferate at a high rate in any Ca^{++} concentration. This provides a specific molecular probe for use in studies to define an alteration in the regulation of terminal differentiation.

Cell lines have been developed from carcinogen altered foci and their properties studied in detail. All have a stable epithelial morphology. They have no or low tumorigenic potential when first isolated but upon prolonged culture may become highly tumorigenic. Tumors are either carcinomas or mixed carcinomas and sarcomas. Analysis of DNA content by flow cytometry suggests a clonal origin for each line. Tumorigenicity does not correlate with growth in agar, gamma-glutamyl transpeptidase activity, transglutaminase activity or the synthesis of keratin proteins. The ability to grow in agar shows a negative correlation to the synthesis of keratin proteins. Additional analysis of these lines, particularly stable nontumorigenic lines, is proceeding.

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, cloning of genes for keratin peptides, the major differentiation proteins of

epidermis, was undertaken. A library of cDNA clones was prepared to total poly (A)RNA isolated from newborn mouse epidermis. From this cDNA library we have isolated clones corresponding to the 55, 59 and 67 kilodalton keratins, which are the major keratins synthesized in this tissue. The presence of keratin cDNA sequences within these cDNA clones has been confirmed by DNA sequence analysis in collaboration with Peter Steinert. Most of the amino acid sequence of the 59 and 67 Kd keratins has been deduced from the sequence of their cloned cDNAs. These sequence data support a model for subunit structure in which long stretches of amino acids in an α -helical conformation are interspersed by stretches that are clearly non- α -helical. There are also long stretches of glycine residues at both ends of these polypeptides. The cloned cDNAs have been used as specific probes for keratin gene transcripts. By RNA blot analysis we have detected specific mRNA species which are abundant in newborn mouse epidermis and code for the 55, 59 and 67 Kd keratins. Those RNAs are approximately 1600, 2000 and 2400 nucleotides in length, respectively. Primary cultures of mouse epidermal cells do not contain the mRNAs coding for the 55, 59 and 67 Kd keratins synthesized in newborn mouse epidermis, although that is the tissue from which the primary cultures were derived. These results suggest that keratin gene expression changes dramatically in epidermal cells when they are grown in culture. The repression of expression of these keratin genes in culture cells does not appear to be due to a developmental switch which may have been induced by placing the cells in culture since we have been able to detect transcripts of these genes in embryonic and adult skin. It is more likely that factors within the culture system repress the expression of these genes. We have been able to detect transcripts of the 55, 59 and 67 Kd keratin genes in a malignantly transformed mouse cell line, PAM 212. The keratins synthesized by these cells are very similar to those synthesized by newborn epidermis. This cell line may be useful in elucidating factors which influence keratin gene expression in epidermal cells.

In vivo and in vitro studies have indicated that phorbol ester tumor promoters markedly effect epidermal differentiation. Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Our studies over the past year provide 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 activity within 12 hours of exposure. This activity increase 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 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. When non-tumorigenic carcinogen-altered epidermal cells (putative initiated cells) are studied by these techniques, TPA does not induce transglutaminase activity or terminal differentiation. However, preliminary studies indicate TPA can stimulate DNA synthesis and cell proliferation in these cell lines.

Our in vitro studies have suggested a mechanism for multistage carcinogenesis involving 3 steps. A genetic change in the program of terminal differentiation characterizes the first alteration produced by initiators of carcinogenesis. This is a preneoplastic change. Tumor promotion involves cell selection and clonal expansion of initiated cells but does not alter the preneoplastic character of these cells. A third step is required to make this conversion. In vivo studies conducted under NCI Contract CPI-5744 at Microbiological Associates have indicated that genotoxic agents can accelerate the conversion of benign lesions to malignant tumors. Animals were initiated once and promoted for 10 weeks until benign tumors developed. The conversion rate during 40 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 40 weeks, the conversion rates were much accelerated and enhanced. Animals not receiving prior initiation and promotion did not develop 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 and steroids. The routine isolation of cells resistant to selection for 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 induce progression can also be studied. Antigenic markers have become useful tools to detect normal and abnormal states and to provide a rapid assay for differentiation. Greater understanding of mechanisms of tumor promotion and the stages of tumor progression 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 is simultaneously developing the analogous human tissue model in vitro. 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 explored further 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 further define the role of that enzyme in differentiation. The results obtained with normal keratinocytes will be compared to those with preneoplastic and neoplastic keratinocytes.

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-05177-02-CCTP) will be used to monitor binding. 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. Parallel development of mutagenesis assays will continue and frequency comparisons for initiation and mutation under the same treatment conditions will be performed. The mechanism by which p21 transforming protein can alter differentiation will be explored at the molecular level in collaboration with Ed Scolnick. As the murine epidermal system becomes routinely used for mechanistic questions, a parallel assay will be developed using human keratinocytes.

The isolation and characterization of specific expressed epidermal genes, in combination with the sensitive immunological probes for carcinogen binding, could allow the study of the functional and structural consequences of exposure to initiators and promoters. These probes will be used to monitor changes in the number of gene copies, gene location and regulation of expression. Epidermal messenger RNA changes will also be monitored during transformation and after single exposures to initiators and promoters. The molecular basis for altered expression of keratin genes in transformed keratinocytes will be studied by isolating genomic clones and performing restriction analysis and sequencing.

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. This will be explored in great detail in the next year. Subpopulations will be isolated after promoter treatment and studied individually for specific markers. The number and character of TPA receptors will be analyzed on each population and their response to various pharmacologic agents will be explored. Antibodies to each cell type will be prepared in rabbits, and after absorption of anti-mouse activity, localization of each cell type in the epidermis will be studied by indirect immunofluorescence. The proteins induced by TPA will be studied in greater detail to see if any are directly involved in producing the biological responses seen. This will be studied in whole basal cell populations and in selected subpopulations. Reconstruction experiments with normal and initiated 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. New in vitro findings will be tested in vivo on mouse skin to assure validity of the data.

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CONTRACTS IN SUPPORT OF THIS PROJECT

MICROBIOLOGICAL ASSOCIATES INC. (N01-CPI-5744)Title: Rodent and Rabbit Facility as a Resource to LCCTPContractor's Project Director: Dr. Martin WenkProject Officer (NCI): Dr. Henry HenningsObjectives:

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 Z01 CM-05177-01 and the genetic susceptibility studies of project Z01 CM 0517-01.

Major Findings:

Project Z01 CP 04504-10 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 40 weeks did not alter the carcinoma incidence from that of control animals in which promotion was terminated at 10 weeks. However, administration of either urethane (systemically), MNNG (topically) or 4 NQO (topically) markedly accelerated and enhanced (6-fold) the conversion of papillomas to carcinomas.

Retinoic acid is a potent inhibitor of tumor promotion when given in conjunction with TPA. However, retinoic acid alone, administered 3x per week in initiated animals, can act as a tumor promoter. Of interest in this study was the finding that only some animals in the experimental group were susceptible to the promoting influences of retinoic acid.

Formaldehyde is a controversial hazardous agent to which humans are exposed. Current studies in mice are testing formaldehyde as an initiator or promoter of skin carcinogenesis.

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.

Project Z01 CM-05177-01 LCCTP: Rabbits have been immunized with several new DNA adduct antigens with excellent immunological responses. One new antiserum currently under study contains cis-platinum-DNA antibodies which can recognize

cis-platinum-DNA adducts in an in vivo model for cancer chemotherapy. Another new antibody is specific for the deacetylated adduct of the acetyl aminofluorene-guanine product (AF-dG) and recognizes femtomolar quantities of this adduct in DNA.

Project Z01-CM-05178-01 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. Current studies are underway to assess the tumor incidence in other tissues of SENCAR mice after systemic administration of DMBA or urethane. Some animals are also receiving topical administration of TPA. Complete autopsies will be performed in August 1982. To date no premature deaths have occurred in any group.

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. In vivo studies have also provided unique immunological reagents to probe the interaction of carcinogens with DNA and to pursue molecular epidemiological studies with human DNA from individuals exposed to environmental carcinogens or cancer chemotherapeutic drugs. The work contributed by this animal support contract 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.

Date Contract Initiated: December 30, 1980

Current Annual Level: 320,000

Man Years: 3.0

MICROBIOLOGICAL ASSOCIATES INC. (N01-CPO-5637)

Title: Biochemistry and Cell Culture Resource

Contractor's Project Director: Dr. Roger Curran

Project Officer (NCI): David L. Morgan

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:

Human epidermal cells in culture will be used to set up a differentiation assay for early carcinogen-induced changes. Likewise studies with tumor promoters will be continued in this model. Retinylphosphate will be supplied to LCCTP and other scientists as needed.

Date Contract Initiated: June 30, 1980

Current Annual Level: 100,000

Man Years: 1.0

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04798-12 CCTP

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Metabolism and Mode of Action of Vitamin A

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	Luigi M. De Luca	Research Chemist	LCCTP NCI
Other:	Carol S. Silverman-Jones	Microbiologist	LCCTP NCI
	Michele R. Brugh	Biochemist	LCCTP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

Differentiation Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.5

PROFESSIONAL:

0.65

OTHER:

1.85

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Vitamin A is necessary for maintenance of normal differentiation. As retinyl phosphate (Ret-P) this vitamin participates in mannosyl transfer reactions in a manner distinct from that of dolichylphosphate (Dol-P). Since amounts of Ret-P and Dol-P available for mannosylation can be assayed in the same incubation containing rat liver microsomes, the effect of different stages of differentiation and tumor growth rates on such ratio was determined, utilizing the Morris series as well as chemically induced hepatocellular carcinomas. The transplanted tumors had less than 5% the amount of A found in the host tissue. Moreover, a decrease in the Ret-P/Dol-P ratio from 0.3 in normal tissue to 0.1 in the moderately differentiated and 0.025 in the poorly differentiated and undifferentiated hepatomas was found. Such remarkable change correlated with the absence of the cellular retinoid binding protein in these tumors. The Ret-P/Dol-P ratio in the tumor was found similar to that of microsomes from vitamin A-depleted liver tissue. The consequences of this deficiency in Ret-P on cell surface glycosylation are being investigated in vitamin A deficiency and in tumor tissue.

Project DescriptionObjectives:

To study the microsomal membranes of normal and neoplastically transformed hepatic tissue regarding their ability to synthesize and utilize the lipid intermediates Ret-P-Man and Dol-P-Man and to correlate the concentration and activity of these intermediates to the degree of differentiation and growth characteristics of the transformed tissue.

Methods Employed:

In addition to procedures already described in the literature, the following methods have been developed.

1. Preparation, incubation and extraction of liver or tumor microsomal vesicles:

Normal male rats, Osborne-Mendel strain (weighing 150-200 g), starved overnight, were killed by bleeding under light diethyl ether anaesthesia. The livers were homogenized with 2 volumes of saline (0.9% NaCl) by a glass/teflon homogenizer. After centrifugation at 2,500 g for 20 min, the resulting supernatant was centrifuged at 105,000 g for 60 minutes. The pellet was resuspended in a small volume of saline and stored in liquid nitrogen until used. The series of Morris hepatomas were grown in ACI and Buffalo rats subcutaneously. Host liver and tumor tissue was processed as above to obtain microsomes. The incubation mixture contained 0.4 μCi of GDP- ^3H -mannose (or 0.1 to 0.2 μCi of GDP- ^{14}C mannose), 4 mg of bovine serum albumin/ml, GDP-D-mannose (24 μM final concentration), 30 mM Tris/HCl buffer (pH 8), 5 mM MnCl_2 or as stated, 8 mM-NaF, 2 mM-ATP, 5 mM AMP and about 1 mg of the microsomal protein in a final volume of 200 μl . After incubation for appropriate time intervals, the lipids were extracted with the following procedure which yields two phases. Five volumes (1 ml) of chloroform/methanol (2:1, v/v) were added, the tube was stirred and two phases were allowed to separate by low speed centrifugation. Under these extraction conditions approximately 98% of the Dol-P-Man is recovered in the lower (organic) phase, while Ret-P-Man partitions about 40-50% in the lower and 60-50% in the upper (aqueous) phase. Alternatively, fifteen volumes (3 ml) of chloroform/methanol (2:1, v/v) were added to yield a monophasic extract.

Extracts were dried under a stream of nitrogen and immediately dissolved in suitable volumes of chloroform/methanol (2:1) containing 10 μg of synthetic Ret-P ready for application on thin layers of silica gel. Chromatography was usually performed in chloroform/methanol/water (45:35:6, by volume), (Solvent A) on two identical plates. One plate was used for determination of radioactivity, the other one for fluorography. Fluorography was carried out on a Kodak made film XR-5 after spraying with Enhance (New England Nuclear), after a procedure developed by Bonner and Laskey, 1974.

2. Preparation of vitamin A-depleted Syrian golden hamsters. Vitamin A-depleted male Syrian golden hamsters were prepared essentially as previously described by placing the mothers on a vitamin A-free diet at birth of the experimental animals. These were weaned on a vitamin A-deficient diet at about 21 days. At

40 days hamsters prepared in this way began to show signs of vitamin A deficiency, such as eye lesions and reduced growth rate. At about this stage of deficiency hamsters were anaesthetized by ethyl ether and killed by bleeding from the neck. Livers were removed and microsomes were prepared as for rat liver microsomes. Microsomes from normal male Syrian golden hamsters were prepared in the same way.

Major Findings:

1. Ratio Ret-P/Dol-P in normal liver. Utilizing the newly developed bovine serum albumin-based assay for the synthesis of lipid intermediates, it was possible to measure the amount of endogenous Ret-P and Dol-P from the amount of radioactive mannose incorporated into Ret-P-Man and Dol-P-Man in 15' at 37°C. The molar ratio of Ret-P/Dol-P was in the range of 0.25 to 0.35 with absolute values for Ret-P of approximately 3.3 pMol and for Dol-P 10 pMol per mg of microsomal protein. A similar Ret-P/Dol-P ratio (0.3) was found for different strains of rats as well as for guinea pig and hamster liver under identical conditions of incubation. Distinctive characteristics between Ret-P-Man and Dol-P-Man synthesis were found. Thus a divalent cation (Mn^{++} , Co^{++} or Mg^{++}) is essential for Ret-P-Man but not for Dol-P-Man synthesis, in the BSA assay. The optimal concentration in Ret-P-Man synthesis from endogenous Ret-P is higher for $MgCl_2$ (10 mM) than for Co^{++} and Mn^{++} (5 to 10 mM). Triton X-100 (0.5%) completely inhibits Ret-P-Man synthesis; it also causes partial inhibition of endogenous Dol-P-Man synthesis as assayed in the BSA system.
2. Ratio Ret-P/Dol-P in hepatoma tissue. The ratio Ret-P/Dol-P was investigated for hepatoma tissue as well as host liver tissue for nine Morris hepatomas, a guinea pig hepatic carcinoma cell line (line 10) and four acetylaminofluorene-induced hepatocellular carcinomas. The hepatoma microsomes in general showed a remarkable reduction in the Ret-P/Dol-P ratio from 0.3 found in the host liver to 0.1 or less. The least differentiated and fastest growing tumors showed the smallest ratio (0.025 to 0) and in general, except for a well-differentiated Morris hepatoma for which the ratio was close to 0.1, the ratio was less than 0.05. This low ratio was similar to that (0.05) found in microsomal membranes from livers of vitamin A-deficient animals, thus suggesting a specific deficiency in Ret-P resulting from a deficiency of vitamin A in the tumor. In fact, retinyl palmitate concentrations in the hepatoma tissue from the Morris series was near zero, as found for the microsomes from vitamin A-depleted hamster liver. Moreover, a measurement of cellular receptors for retinol (cRBP) in the fastest growing and least differentiated hepatomas showed a deficiency of cRBP as well.
3. Subcellular distribution of Ret-P and Dol-P in normal rat liver membranes. The subcellular distribution studies showed that the endoplasmic reticulum of rat liver contains both the mannosyl transferases as well as the endogenous acceptors, Ret-P and Dol-P, for Ret-P-Man and Dol-P-Man synthesis. On the contrary Golgi apparatus membranes, though containing the mannosyltransferase to synthesize Ret-P-Man from guanosine-diphospho-mannose and exogenous Ret-P, do not contain significant amounts of the endogenous acceptor, Ret-P and Dol-P, thus suggesting a specific function for Ret-P in glycosylation reactions of the endoplasmic reticulum. As found with total microsomal vesicles, the endoplasmic

reticulum utilized Ret-P and Dol-P in mannosylation of endogenous acceptors. However, while Dol-P was active in the transfer of mannose to oligosaccharide-lipid molecules, Ret-P functioned in a direct transfer to protein.

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 newly found involvement of the phosphorylated vitamin in glycoprotein biosynthesis may explain its effect on mucus secretion, adhesion and the maintenance of normal phenotypic expression.

The reported findings establish a direct 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 may, in the end, explain altered functional cell surface characteristics of the tumor cells if one considers that a variety of cell surface molecules, including fibronectin, procollagen and growth factor receptors are glycoprotein in nature.

Proposed Course:

Having established that a specific deficiency in Ret-P-Man synthesis from endogenous Ret-P exists in hepatocellular carcinoma microsomes, the possibility will be probed that specific glycoproteins or glycoprotein domains containing mannose derived from Ret-P-Man are absent or defective in the tumor tissue.

Publications:

Bhat, P.V., Roller, P.P. and De Luca, L.M.: Chemical and biological studies on 5,6-epoxyretinol, retinol and their phosphoryl esters. J. Lipid Res. 22: 1069-1078, 1981.

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Sundaresan, P.R. and Bhat, P.V.: Ion pair high pressure liquid chromatography of cis-trans isomers of retinoic acid in tissues of vitamin A-sufficient rats. J. Lipid Res. 23: 448-455, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP-05177-02 CCTP
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Use of Immunological Techniques to Study the Interaction of Carcinogens with DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	S.H. Yuspa	Chief	LCCTP	NCI
	J. Nakayama	Visiting Fellow	LCCTP	NCI

COOPERATING UNITS (if any)

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Dr. John Stanley - Uniformed Services U. of Health Sciences	Dr. I.B. Weinstein - Columbia University
	Dr. Brian Laishes - McArdle Laboratories
	Dr. David Kaufman - University of NC

LAB/BRANCH
Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION
In Vitro Pathogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	4.25	PROFESSIONAL:	2.25	OTHER:	2.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies have employed antibodies to carcinogen-DNA adducts to investigate the nature and extent of binding of 2-acetylaminofluorene (AAF), benzo[a]pyrene (BP) and Cis-dichlorodiammineplatinum (II) (Cis-DDP) to DNA in vivo and in vitro. Using quantitative immunoassays AAF, BP and Cis-DDP adducts have been determined in biological samples by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) with lower limits of detectability in the range of 0.1-0.2 femtomoles/μg DNA or one adduct in ten million nucleotides. Anti-G-8-AAF is currently being utilized to monitor adducts in cultured cells during transformation and in rat liver during hepatocarcinogenesis. Anti-BPdG-DNA is being utilized with electron microscopy and immunofluorescence to morphologically localize BpdG adducts in native DNA and the nuclei of cultured cells. Quantitative ELISAs for BpdG-DNA and Cis-DDP DNA are in use for monitoring adducts in cultured cells and whole animals, and their potential for detection of adducts in human tissue is currently under investigation.

Project DescriptionObjectives:

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 patients environmentally exposed to carcinogens 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 radioimmunoassay (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-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-acetylaminofluorene (AAF). The antisera obtained have been employed to detect picomoles of each C-8 adduct in a competition radioimmunoassay utilizing ^3H -G-8-AAF or ^3H -G-8-AF with lower limits of sensitivity in the range of one adduct per 3.5×10^{-5} nucleotides. A linear regression equation has been established which allows precise quantitation of each C-8 adduct in an unknown mixture and proportions can also be determined by comparison with standard mixtures. In the past year the limits of detectability for C-8 adducts have been lowered further with the development of the enzyme-linked immunosorbent assay (ELISA) for use with this antibody. The standard curve 50% inhibition for ELISA is at 50 femtomoles, reducing the lower limit of sensitivity to about 0.5 femtomole of C-8 adduct/ μg DNA or one adduct in 10^7 nucleotides.

A variety of cultured cells from different species have been exposed to N-acetoxy-acetylaminofluorene (N-Ac-AAF) and the deacetylated C-8 adduct was found to predominate in all cells except primary rat hepatocytes. The validity of the RIA approach in performing such studies was confirmed by concomitant HPLC analysis of DNA-binding profiles. The data indicated that specific patterns of AAF-DNA binding are determined at the cellular level and that experimental techniques could be developed to modify the quantity and type of adduct formed. Studies have been initiated to assess the effect of such modifications on cell transformation in the BALB/c primary epidermal system. With the low levels of

carcinogen exposure used for adequate cell viability in the transformation experiments, the increased sensitivity of the ELISA assay is a requirement for adduct determination.

Immunological techniques have also been applied to study binding of AAF to liver and kidney DNA during liver carcinogenesis protocols. In collaboration with Dr. Brian Laishes at the McArdle Laboratory for Cancer Research, RIA has been used to detect C-8 adducts in liver and kidney DNA of male Wistar-Furth rats fed 0.02% or 0.04% 2-AAF either continuously up to 16 weeks or for a specific time followed by an interval for repair. The formation of C-8 adducts in liver DNA increased from 80 fmoles/ μ g DNA at 24 hr to a plateau of approximately 230 fmoles/ μ g DNA at 30 days and thereafter. During the first week of continuous feeding, about 80% of the total C-8 adducts in the liver DNA were deacetylated (dG-8-AF) but the proportion of dG-8-AF increased to 100% by 30 days on the AAF-containing diet. When rats were fed 2-AAF for 3 or 7 days followed by control diet for one month, the adduct levels in the liver were reduced by 90% indicating that repair processes were functioning efficiently. When dietary administration of 2-AAF was for 28 days, the adduct levels were higher (than after 3 or 7 days), the rate of removal or repair remained constant and therefore the quantity of adducts remaining on the DNA after one month on control diet were substantial. At this time dG-8-AF was the persistent C-8 adduct and there was no dG-8-AAF detectable in liver DNA. Adduct profiles in kidney DNAs from the same animals indicated formation and removal similar to that occurring in liver but the binding levels were several-fold lower than those observed in liver.

Antisera have also been 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-N²-deoxyguanosine (BPdG). The antisera have a higher affinity for BP-substituted DNA than for the isolated BPdG adduct (suggesting antibody recognition of the DNA backbone) and do not cross-react with deoxyguanosine, DNA or the carcinogen alone. The ability of this antiserum to recognize BPdG in intact (non-hydrolyzed) DNA makes it particularly useful for morphological studies. During the past year, in collaboration with Dr. David Kaufman of the University of North Carolina, the visualization of BPdG adducts on DNA fibers has been quantitated by ultrastructural techniques. Antiserum was incubated with calf thymus DNA modified in vitro to various extents with BPDE-I, subsequently incubated with ferritin-conjugated goat anti-rabbit IgG and examined by electron microscopy using a modification of the Kleinschmidt basic protein monofilm technique. No specific binding of antibodies was visualized with unmodified DNA or when pre-immune serum was incubated with unmodified or BPDE-I-modified DNA. In early studies DNAs modified higher than 0.005% gave less than quantitative indications of extents of modification due to aggregations of DNA and antibodies. However in the most recent investigation the use of monovalent Fab fragments has yielded quantitative results. This system is being used to examine BPDE-I modification of DNA from nuclei of regenerating rat liver and to study the modification of DNA during BPDE-I-induced transformation of 10T 1/2 cells.

Qualitative studies were also performed at the cellular level by examining cultured mouse epidermal cells exposed to BPDE-I in vitro by indirect immunofluorescence. 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 did not yield fluorescence. Specific fluorescence was abolished by treatment of fixed, permeabilized cells with DNAase. RNAase treatment removed the particularly intense bright staining localized in spots in the nucleus but did not change overall nuclear fluorescence. While immunofluorescence was readily seen one hour after BPDE-I exposure, by 24 hours it could no longer be observed suggesting removal of adduct during that time to levels below detectability by this technique. These studies have been carried out in collaboration with Dr. John Stanley of the Uniformed Services University of the Health Sciences. The quantitative analysis of BpDg formation, previously accomplished by RIA, has been enhanced by the development of a highly sensitive ELISA yielding a standard curve with 50% inhibition at 20 femtomoles and allowing detection of adduct from biological samples in the range of 0.1 fmole/ μ g DNA. The lower limits of sensitivity of this assay have been determined in animal models. Mice have been injected i.p. with increasing amounts (0.1-3 mg) of BP, and BpDg adducts have been monitored in lung DNA. The levels of deoxyguanosine adducts determined by immunoassay (0.4 fmoles/ μ g DNA) agreed favorably with overall binding measured by [3 H] BP (0.8 fmole/ μ g DNA). Using the ELISA assay, samples of human lung and white blood cells from individuals exposed to B(a)P through lifestyle (smoking) or occupation (coke oven and shale oil workers) have been screened for evidence of BP-DNA adducts. In this pilot study lung tissue DNA from four lung cancer patients gave positive results in the ELISA assay but samples from the occupationally exposed groups were not significantly different from the controls. Collection of the clinical material has been coordinated by Dr. I. B. Weinstein of Columbia University.

In an attempt to further expand the use of this sensitive technology to monitor human exposure, rabbit antibodies have been elicited against DNA modified with the chemotherapeutic drug cis-dichlorodiammineplatinum (II) (cis-DDP) in collaboration with Dr. Stephen Lippard of Columbia University. By ELISA the antiserum has been determined to be specific for cis-DDP-DNA and does not cross-react with either the drug, cis-DDP, or unmodified DNA alone. The standard curve falls in the range of 20-60 femtomoles for 50% inhibition depending on the assay conditions and this allows for detectability in the range of 0.2 femtomoles/ μ g DNA. In collaboration with Dr. Leonard Zwelling of NCI this assay has been used to quantitate adducts in DNA from cultured L1210 cells exposed to cis-DDP. DNAs from control cells or cells treated with trans-DDP or phenylalanine-mustard (another cross-linking agent) are not recognized by the anti-Cis-DDP-DNA. DNAs from tumor ascites extracted from mice carrying L1210 tumors and injected with Cis-DDP have levels of adducts measurable in this assay. This constitutes the first evidence that Cis-DDP adducts formed in vitro are similar to those formed in vivo. Currently clinical studies are being developed to assay DNA extracted from circulating nucleated cells in individuals receiving cis-DDP chemotherapy to investigate the possibility that this assay is sensitive enough to detect adducts formed in humans.

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 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 our most recent results indicate that even intramolecular localization is possible. Antibodies may also be useful to probe for adducts in humans exposed to carcinogens, to monitor a biological effect of a specific exposure, to gather data on dose response in prospective epidemiological studies and to assist the clinician in adjusting dosage on individuals exposed to DNA-interacting chemotherapeutic agents.

Proposed Course:

The remarkable sensitivity and specificity of antibodies raised against carcinogen-DNA adducts provides a new approach to bioassay and mechanistic studies. As an applied methodology, this technology may allow for detection of adducts in tissues from human populations exposed to environmental carcinogens such as benzo(a)pyrene, or it may be useful in the clinical monitoring of biological effectiveness of a chemotherapeutic agent such as Cis-DDP. These possibilities are both under investigation. Utilizing immunoassays for C-8 adducts of acetylaminofluorene with DNA, we will continue to investigate adduct formation and removal both during cell transformation in the primary epidermal system and during hepatocarcinogenesis in the rat. Since the kinetic data currently obtained in the rat liver system indicate greater complexity than we had anticipated we will be investigating localized areas in the liver by immunofluorescence and probably by autoradiography as well. Collaborative studies utilizing electron microscopic immunohistochemistry (ferritin labeling) to detect specific localization of BP adducts on DNA will focus on DNA from 10 T 1/2 cells exposed *in vivo* and on a search for selective binding sites in fractionated DNA. In particular, binding to initiation sites in replicons will be examined after isolation of these sites by density gradient centrifugation. We will also use this approach to determine if gaps in nascent DNA after exposure of cells to BPDE-I are localized opposite adducts. In this laboratory immunofluorescence techniques will be applied to study distribution of bound BPDE-I in cultured cells and animal tissues while quantitation of BpDg adducts will be carried out on parallel samples by ELISA. The time course of BpDg removal will be monitored in primary mouse keratinocytes by both immunofluorescence and ELISA. Investigations will be initiated to determine whether or not presence of the antibody on DNA is able to prevent adduct removal. The ELISA for Cis-DDP-DNA will be employed in attempts to monitor the binding of Cis-DDP to DNA after exposure in cancer patients. Other studies are designed to elucidate the mechanism of action of Cis-DDP in biological systems and in investigations designed 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:

Hsu, I.C., Poirier, M.C., Yuspa, S.H., Grunberger, D., Weinstein, I.B., Yolken, R.H., and Harris, C.C.: Measurement of benzo[a]pyrene-DNA adducts by enzyme immunoassays and radioimmunoassay. Cancer Res. 41: 1091-1095, 1981.

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Poirier, M.C., True, B., and Laishes, B.A.: Determination of 2-AAF adducts by immunoassay. Second International Conference on Carcinogenic and Mutagenic N-Substituted Aryl Compounds, Hot Springs, Arkansas, March 30-April 1, 1982. Carcinogenesis, in press.

Poirier, M.C., True, B., and Laishes, B.A.: The formation and removal of guan-8-yl)-DNA-2-acetylaminofluorene DNA adducts in liver and kidney DNA of male rats administered dietary 2-acetylaminofluorene. Cancer Res. 42: 1317-1321, 1982.

Poirier, M.C., Yuspa, S.H., True, B., and Laishes, B.A.: Specific patterns of DNA adduct formation and removal in N-acetoxy-2-acetylaminofluorene-exposed cultured cells and in organs from rats fed 2-acetylaminofluorene. Proceedings of Symposium on Organ and Species Specificity in Chemical Carcinogenesis, Raleigh, N.C., March 2-4, 1982, (In press).

Poirier, M.C., Stanley, J.R., Beckwith, J.B., Weinstein, I.B., and Yuspa, S.H.: Indirect immunofluorescent localization of benzo[a]pyrene adducted to nucleic acids in cultured mouse keratinocytes. Carcinogenesis 3: 345-348, 1982.

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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Section, LCCTP, NCI	Health, FDA

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LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

3.25

PROFESSIONAL:

2.25

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

SENCAR mice are markedly susceptible to two-stage skin carcinogenesis compared to BALB/c mice. Susceptibility is a property of the skin itself and 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, 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 constitutively initiated cells which resist normal differentiation signals. These may be embryonic cells which normally disappear near parturition but persist into adulthood in SENCAR mice.

Project Description

Objectives:

To elucidate the cellular mechanism of genetically derived, enhanced sensitivity to carcinogenesis.

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, SENCAR mice are exposed to carcinogens and tumor promoters in vivo or SENCAR epidermal cells are cultured and studied in vitro. Comparisons are made with BALB/c as a representative resistant strain. 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. Epidermal thymocyte activating factor was measured by stimulation of thymidine incorporation and interleukin 2 production in thymocytes. Effects of various agents on epidermal cell growth kinetics have been determined in culture by cell counts and thymidine incorporation. Poly A RNA was isolated from cells by extraction with guanidine-HCl and chromatography on oligo dT cellulose. This RNA has been translated in vitro and the products examined by SDS-polyacrylamide gel electrophoresis. The various RNA species have been separated by gel electrophoresis and viral-specific sequences detected by hybridization of cloned probes to Northern blots. DNA, extracted with SDS and purified by NaCl precipitation, pronase digestion, and phenol extraction, was treated with restriction endonucleases and the fragments separated by gel electrophoresis. Cloned viral-specific probes were hybridized to Southern blots. Transglutaminase was assayed by incorporation of radioactively labeled putrescine into acid precipitable form using casein as recipient. Cultured epidermal cells are assayed for resistance to terminal differentiation by culture under conditions of reduced or increased extracellular calcium.

Major Findings:

Earlier studies have shown that the increased susceptibility of SENCAR mice to skin carcinogenesis relative to other strains such as BALB/c cannot be accounted for by differences in metabolism of polycyclic aromatic hydrocarbon carcinogens. Furthermore, the sensitivity is a property of the skin itself rather than a systemically mediated phenomenon. Literature reports that athymic nude mice normally resistant to carcinogen-induced skin tumor formation became susceptible after thymus transplants suggested that an immune component in the skin itself may be involved in the susceptibility. Epidermal Langerhans cells (LC) play a major role in cell-mediated immune functions of the skin, including allergic contact sensitivity and antigen and alloantigen presentation. We therefore did a comparative study of LC number and function in BALB/c and

SENCAR epidermis. Similar numbers of LC were detected in epidermis and fresh epidermal cell suspensions of both newborn and adult BALB/c and SENCAR mice by several analytical methods. Moreover, functional studies, including contact sensitization and alloantigen-presenting ability, in adults showed no significant strain differences. However, in primary newborn epidermal cell cultures examined 24 hrs. after plating there were more than 4X as many F_C -positive BALB/c cells as SENCAR. This difference may result from SENCAR LC being less adherent to plastic than BALB/c LC. Alternatively, cultured SENCAR LC may survive less well in vitro or may lose their F_C receptor more rapidly than BALB/c. In any case, we have been unable to demonstrate LC functional differences between strains. In connection with this study we showed that treatment of primary cultures with compounds having tumor promoting activity such as 12-O-tetradecanoylphorbol-13-acetate (TPA), teleocidin, and mezerein, but not the nonpromoting phorbol ester 12-deoxyphorbol isobutyrate, lead to a marked increase in cells with F_C receptors. Since these cells stain for Ia antigen but not for keratin, we believe that tumor promoter treatment results in actual increased numbers of LC rather than induction of F_C receptors in other cell types. Neither retinoic acid nor fluocinolone acetonide, compounds having anti-promoter activity in vivo, was able to block the increase in LC brought about by TPA, although fluocinolone acetonide did strongly suppress the baseline LC level. It was possible to devise a procedure combining the TPA effect on LC with their resistance to trypsin to obtain cultures enriched to approximately 90% LC.

Another immunological area examined was epidermal thymocyte-activating factor (ETAF) which appears to be produced by keratinocytes. Under a variety of conditions, with and without TPA, in high and low calcium levels, SENCAR primary epidermal cultures produced amounts of ETAF similar to those found in BALB/c cultures.

Comparative epidermal growth factor (EGF) binding studies with BALB and SENCAR primary epidermal cell cultures demonstrate that EGF binding capacity increases with cell density. There is an approximate 2-fold increase in binding per cell for a 10-fold increase in cell density. At comparable cell densities, however, the two mouse strains bind similar amounts of EGF per cell. TPA strongly depresses EGF binding ability of primary epidermal cells. BALB/c and SENCAR are similar in this regard. At 100 ng/ml TPA-EGF binding is depressed to 10-30% of control (no TPA) values within less than 4 hr. Similar studies with chemically initiated cell lines derived from newborn BALB/c epidermal cells suggest that initiated cells are resistant to the TPA-mediated reduction in EGF binding capacity. One of these lines has an EGF binding capacity more than 3x that of primary cells. It is possible that in the presence of tumor promoters such an initiated cell, being better able to utilize available growth factors, would have a growth advantage compared to normal cells.

Normal basal epidermal cells are induced to terminally differentiate in vitro by calcium levels greater than 0.1 mM. Malignant cells resist this differentiation signal and nonmalignant, resistant colonies can be isolated from carcinogen-treated cultures in proportion to initiator dose. In such a calcium resistance selection experiment we isolated a line, designated A7, which is very rapidly

proliferating in high calcium media. A7 was isolated from SENCAR primary epidermal cells which were treated with TPA very early in culture. A7 binds EGF roughly similar to primary cells. It is essentially unresponsive to TPA in growth kinetics experiments. Poly A RNA isolated from A7 cells and translated in vitro does not appear to contain keratin message. Scatchard plots of phorbol dibutyrate (PDBU) binding by A7 cells differ from all other cells examined, including newborn mouse dermal fibroblasts, in that A7 cells have only a single class of receptors. Host cell reactivation experiments in which ultraviolet light-irradiated herpes simplex virus was used to "infect" A7 cells to test their DNA repair capacity revealed that these cells were not infected, even with high doses of virus. Similar behavior was seen with newborn mouse dermal fibroblasts. These cells are similar to cells derived from human epidermal cell cultures after TPA treatment. Similar cells were not obtained when dermal fibroblasts were treated by a similar protocol. The origin of these cells and their possible role in carcinogenesis remains to be determined.

Studies of binding of PDBU to BALB/c and SENCAR epidermal cells have not suggested any strain differences with regard to tumor promoter binding.

Although expression of infectious endogenous retrovirus is highly repressed in both SENCAR and BALB/c epidermal cells, we have been able to detect virus-related sequences in poly A RNA preparations from primary epidermal cells as well as lines derived from primaries by carcinogen treatment or by long-term culture in low calcium media. Of particular interest, however, is an RNA species of approximately 30S which contains sequences homologous to the viral long terminal repeat (LTR) region, which contains elements that regulate gene expression. This sequence appears to be absent in primary epidermal cells of both strains but is found in several calcium-resistant lines. Experiments using recombinant DNA technology have demonstrated that LTRs can control expression of endogenous oncogenic sequences.

Transglutaminase (TGA) is an enzyme which catalyzes the protein cross-linking characteristic of differentiating epidermis. The activity of this enzyme is increased by TPA treatment in a way that suggests enzyme induction by the tumor promoter. We have shown that TPA induces TGA in primary newborn epidermal cells of both BALB/c and SENCAR with a similar dose-response and time course. Recent experiments with the inhibitor chymostatin suggest the induction of TGA may occur via the lysosomal protease cathepsin D since the inhibitor reduces TPA-induced TGA activity in a dose-dependent fashion.

There are two kinds of data which imply the existence of endogenously initiated cells in SENCAR epidermis. In vivo studies show that skin tumors are obtained in SENCAR mice treated with promoters alone, i.e., not chemically initiated. Such tumors are quite rare in BALB/c. In vitro experiments to select calcium-resistant colonies after carcinogen treatment produce relatively large numbers of colonies in untreated SENCAR cultures but only rarely in untreated BALB/c dishes. BALB/c colonies are more common in newborn epidermis and quite rare in adults. These cells may be embryonic and disappear shortly after birth in normal animals but persist into adulthood in the SENCAR. If they are indeed

precursors of the tumors in animals treated with promoter alone and can therefore be considered initiated, these cells are of great interest. We have improved procedures for culturing adult epidermis and have recently begun to isolate colonies of calcium-resistant cells from adult SENCAR epidermis.

Growth kinetics studies *in vitro* have compared newborn BALB/c and SENCAR primary epidermal cell cultures with regard to TPA and steroid responses. Again, the two mouse strains were remarkably similar except that noticeable morphological changes were seen in SENCAR but not BALB/c in the presence of hydrocortisone or fluocinolone acetonide while epidermal cell growth was affected only slightly.

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:

Improved procedures for culturing adult epidermal cells will permit initiator dose-response studies with BALB/c and SENCAR epidermal cells and selection for calcium resistant colonies to try to determine whether SENCAR epidermis is especially sensitive to initiators. Identifying whether SENCAR susceptibility to skin carcinogenesis is at the initiation or promotion stage will greatly simplify studies to elucidate the mechanism. *In vivo* studies comparing initiation-promotion protocols with multiple initiator doses will also be helpful in this regard.

Special emphasis will be placed upon development of lines of constitutively initiated SENCAR epidermal cells. These will be characterized in a variety of ways--DNA repair capacity, binding of tumor promoters and growth factors, in

in vitro growth kinetics and response to possible modulating factors, induction of markers of differentiation and proliferation, etc.--and compared with chemically initiated cell lines and primary epidermal cells. If constitutively initiated cells in the SENCAR skin are persistent embryonic cells and are essential components of SENCAR susceptibility, this small subpopulation of epidermal cells might be expected to have special properties that could not be observed in the presence of a large excess of normal cells.

Characterization of the nature of virus-specific mRNA sequences in a variety of cell lines of defined origin and biological and biochemical properties is now underway. The genetic composition of these sequences will be examined and DNA restriction patterns from these cells will be studied for evidence of genetic rearrangements, especially with regard to regulatory sequences.

Exploration of the mechanism of transglutaminase induction will continue with emphasis on the role of this enzyme in normal differentiation. We will determine whether chymostatin which inhibits TPA-mediated increase in TGA will also block enzyme induction or activation by calcium, and, if so, will this modify calcium-induced terminal differential of basal epidermal cells in culture. We hope that by isolating and studying what are probably the specific cells responsible for the sensitivity of SENCAR mice and by determining whether these animals are especially susceptible to the initiation phase of carcinogenesis to simplify the problem of elucidating the mechanism of susceptibility.

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INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Mechanism of Action of Phorbol Ester Tumor Promoters

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

Molecular Mechanisms of Tumor Promotion Section

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TOTAL MANYEARS:

4.0

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 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The efforts of this section are directed at understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. To characterize the phorbol ester receptor biochemically we have prepared photoaffinity labels and affinity columns. The photoaffinity labeling studies in brain suggest that the receptor is a lipoprotein, with phosphatidylserine and phosphatidylethanolamine in proximity to the ligand binding site. The subdivision of promotion into multiple stages implies the existence of subclasses of receptors. In fact, we have been able to demonstrate three subclasses of phorbol ester receptors with distinct structure-activity requirements in mouse skin homogenates. As part of a genetic analysis of receptors, we have screened cultured mouse cells and nematode variants altered in their responsiveness to the phorbol esters. All variants so far are altered at steps distal to ligand binding. During assays for possible endogenous ligands in brain acting at the phorbol esters receptor, ascorbic acid was the major inhibitor detected. Its inhibition, however, was irreversible, being mediated through induction of lipid peroxidation.

Project DescriptionObjectives:

The early events in the interaction of phorbol ester tumor promoters with cells and tissues are being characterized. There are five specific aims: 1) analysis of the postulated subclasses of receptors which mediate distinct biological responses, e.g., stage 1 promotion, stage 2 promotion, complete promotion, and hyperplasia; 2) biochemical identification and purification of receptors; 3) genetic analysis of receptors; 4) identification of modulators of binding activity; and 5) identification of the biochemical response(s) directly coupled to receptor occupancy. Since the phorbol ester receptor appears to mediate promotion in mice and since people possess phorbol ester receptors, the biochemical process directly mediated by this receptor and modulators of its activity may be of importance in human cancer causation.

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 of receptors, are being used for receptor purification and biochemical analysis. Mouse skin is 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:

Substantial progress has been made in two areas. First, the existence of subclasses of phorbol ester receptors has been demonstrated in skin, and their structure-activity relations have been analyzed. Second, specific photoaffinity labeling of phorbol ester receptors has been achieved, identifying specific lipids associated with the receptor. In addition, although no endogenous competitive inhibitor of phorbol ester binding has been found, a major low molecular weight noncompetitive inhibitor in brain was demonstrated. It was identified as ascorbic acid and shown to act through induction of lipid peroxidation. In collaborative studies, variants of cultured cells and mutant cells altered in their responsiveness to phorbol esters were examined for altered phorbol ester binding. No changes were observed, indicating that the alterations were at steps distal to receptor binding.

Mezerein is the best studied example of a second stage promoter. At higher concentrations, however, it is also able to act as a complete promoter. The potency of mezerein for inhibiting [^3H]PDBu binding was consistent with the [^3H]PDBu

binding site being the site mediating the action of complete promoters. We predicted, therefore, that an additional high affinity binding site for mezerein should exist. A problem in analysis, however, had been that we did not have a system in which we could both measure biological responses coupled to the putative high affinity mezerein receptor (stage 2 receptor) and the lower affinity (for mezerein) receptor (the major [^3H]PDBu binding site) as well as measure binding itself under the same incubation times and culture conditions. The possibility therefore existed that the different apparent potencies of mezerein for different responses were a pharmacological artifact of differential stability, serum binding, etc., under the different assay conditions. Using the G-292 osteosarcoma cell line we are now able to exclude this possibility (Jaken, Shupnik, Blumberg, and Tashjian, manuscript submitted). In these cells, mezerein is 25-fold more potent in causing a decrease in binding of epidermal growth factor to the EGF receptor than it is for inducing prostaglandin E_2 production under the same incubation conditions. This differential potency was not seen for phorbol myristate acetate (PMA) and phorbol 12,13-dibutyrate (PDBu). Inhibition of [^3H]PDBu binding by mezerein corresponded to its potency for inducing PGE_2 production. This may be a valuable cell system for screening for mezerein analogs and for developing a binding assay selective for mezerein analogs.

The short-chain substituted 12-deoxyphorbol analogs, like mezerein, were predicted from *in vivo* and *in vitro* biological data to be selective for a subset of receptors. Although inflammatory and toxic, these derivatives possess only very weak promoting activity. We therefore prepared [^{20-3}H]12-deoxyphorbol 13-isobutyrate ([^3H]DPB) and assayed it for binding to mouse skin membrane preparations. Mouse skin was of particular interest for analysis because, using low specific activity PDBu, we had previously observed a curved Scatchard plot for binding consistent with receptor heterogeneity.

[^3H]DPB bound in a specific, saturable, and reversible manner. Analysis of the binding data yielded a curvilinear plot, consistent with two binding sites present at 0.14 (site 1) and 1.55 (site 2) pmol/mg protein and possessing binding affinities of 6.9 nM and 86 nM, respectively. Comparison with [^3H]PDBu binding indicated that PDBu bound to these two sites with affinities of 0.69 and 10.3 nM, respectively. In addition, a third PDBu site was present at 1.87 pmol/mg (site 3) and possessed an affinity of 53 nM. By competition, DPB could be shown to bind to this third site as well. Its affinity, however, was only 5300 nM. Binding affinities measured by competition were consistent with the values obtained by analysis of direct binding. Experimental versus predicted values were as follows: DPB inhibition of [^3H]DPB binding, 86 nM versus 120 nM; PDBu inhibition of [^3H]DPB binding, 9.5 nM versus 12.5 nM; PDBu inhibition of [^3H]DPB binding, 45 nM versus 47 nM.

Extensive structure activity analysis of sites 2 and 3 indicated similar affinities for the more lipophilic phorbol esters such as phorbol 12-myristate 13-acetate and phorbol 12,13-didecanoate (PDD). Although less lipophilic derivatives showed reduced affinity at both sites, the decrease was greater at site 3. Thus, whereas PMA had affinities of 1.6 nM and 0.6 nM at sites 2 and 3, respectively, the corresponding affinities for PDBu were 10 nM and 53 nM. A similar difference has been observed for the dependence on chain length of inflammation and tumor promoting activity. Neither site was selective for mezerein.

4-0-Methylphorbol 12-myristate 13-acetate (MPMA), which initially had been suggested by Hecker to be a negative control for PMA, was subsequently shown by Slaga to be a first stage promoter if applied at a concentration 40-fold higher than that for PMA. Likewise, under conditions in which binding of [3 H]PDBu was predominant to sites 1 and 2, we found that MPMA inhibited binding to site 1 with an affinity of approximately 50 nM. Although further structure-activity analysis needs to be done, for which higher specific activity ligand would be useful, this data suggests site 1 as a potential target for mediating the action of first stage promoters.

Structure-activity analysis provides one approach to demonstrating the role of a specific binding site in biological responses. An alternate approach is genetic, to show that some variants altered in their response to an agent are also altered in their binding activity. Mouse variants would be of greatest utility. Although we had previously shown that of a dozen mouse strains examined, all possessed phorbol ester binding, we are now in the process of re-examining this question by looking at the relative abundance of subclasses of binding sites. Reproducible differences were not seen between CD-1 and C57BL/6, AKR, Balb/c, and CD-1 nu/nu. Possible changes in another strain are being studied further.

Dr. Colburn (NCI) and Dr. Herschman (UCLA) have isolated variants of JB6 mouse epidermal cells and 3T3 cells, respectively, by selecting for reduced mitogenic responsiveness to PMA. In collaborative studies, we screened these variants for altered phorbol ester binding. No changes were seen.

The nematode *Caenorhabditis elegans* provides one of the most manipulable genetic systems in a multi-cellular eukaryote. In collaboration with Dr. K. Lew (Childrens Hospital, Boston), we had previously shown that *C. elegans* possessed a level of phorbol ester receptors similar to that of vertebrate systems and that these receptors showed similar structure-activity requirements. In the intact animal, the phorbol ester had several biological effects suitable for selection, including a reduced size of the adult in animals grown in the presence of PMA (thus permitting a filtration assay for variants). We have therefore collaborated with Dr. Lew on the isolation of nematode variants.

A problem with the system was that the nematodes are relatively impermeable to PMA; whereas the binding affinity for PMA is 0.6 nM, the biological response is seen only at a half-maximally effective concentration (ED₅₀) of 170-460 nM. The strategy was therefore to isolate supersensitive variants. Although the predominant class of variants should show a decreased permeability barrier, multiple selection cycles should exhaust this pathway, and interesting variants may then arise. After 5 cycles of selection, variants showing an ED₅₀ for PMA of 0.2 nM were obtained; no change in binding activity was found.

As part of our effort to biochemically characterize the phorbol ester receptors we prepared a photoaffinity derivative, [20- 3 H]phorbol 12-p-azidobenzoate 13-benzoate (PpABB). The aryl azide side chain, upon UV-irradiation, generates a nitrene. In the dark, PpABB bound reversibly to brain particulate fractions with a dissociation constant (Kd) of 0.9 nM. Specific binding of PpABB, at a

concentration equal to its K_d , represented 85% of the total bound. At saturation, 32 pmol PpABB were bound per mg of brain protein, a level similar to that observed with [3H]PDBu. Under the conditions used (above the K_d for PpABB) irradiation caused 45% of the PpABB binding to become irreversible. Most of this binding (approximately 62%), including virtually all of the specific irreversible binding, was to phospholipid rather than to protein. The specifically labeled phospholipids were identified as phosphatidyl serine, phosphatidyl ethanolamine and phosphatidyl ethanolamine plasmalogen. Although the PpABB was specifically labeled lipid, labeling was blocked by pre-treatment of membranes at 100° for 5 min or by papain digestion. It therefore seems likely that the identified lipids are specifically associated with a protein receptor and are preferentially labeled either because of the location or reactivity of the nitrene generated on the photoaffinity probe.

To purify the protein portion of the receptor, conditions for solubilization are being optimized and an affinity column prepared. With either a free or blocked side chain, the ligand used for coupling to the affinity column has been shown to possess high affinity (10^{-8} M) for the membrane receptor.

The high evolutionary conservation of the phorbol ester receptor argues for the presence of an endogenous compound which normally interacts at the binding site. Since brain contains the highest level of receptors, we therefore screened brain for potential inhibitors of binding. Boiled or acidified preparations from calf cortex showed the presence of a heat-stabile, low-molecular weight factor which inhibited [3H]PDBu binding in a dose-dependent, irreversible, and noncompetitive manner. The inhibitory factor was identified as ascorbic acid. As in other cases in which ascorbic acid alters the activity of membrane proteins, inactivation of phorbol ester binding by ascorbic acid was not stereospecific and appeared to be mediated by a free radical mechanism, correlating with the stimulation of lipid peroxidation. Inactivation by ascorbate could be blocked by inhibitors of lipid peroxidation, permitting the further screening of extracts in the presence of ascorbate. Although ascorbate presumably does not normally inactivate phorbol ester receptors *in vivo*, free radical-mediated receptor inactivation could potentially play a role in systems in which phorbol esters stimulate the generation of active oxygen species or fatty acid hydroperoxides.

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 has 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 necessary 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 both provide a new avenue for the rational development of inhibitors of promotion as well as shed light on the mechanism of current classes of 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 same five specific aims will continue to be pursued over the next year. The multiple receptors in skin homogenates will be compared to the binding in intact keratinocytes using the cultured cell systems developed by the *In Vitro* Pathogenesis Section of LCCTP. Higher specific activity [³H]PDBu will be synthesized to facilitate the structure-activity analysis of site 1 in skin homogenates. Using intact cells of a cultured cell line for which we have observed 2 distinct binding components in the intact cells, we will determine the structure-activity requirements of the two sites and which cellular responses correspond to each site. In addition, radioactive ligands potentially specific for the mezerein site will be synthesized and new methods will be used to identify possible cytoplasmic receptors for the phorbol esters.

To determine the localization of phorbol ester receptors on cells, phorbol ester-ferritin or phorbol ester-peroxidase conjugates will be prepared. Although it is assumed that the membrane receptors are on the outer face of the plasma membrane, their orientation is not, in fact, known. Likewise, their distribution within the plane of the membrane and the effects of phorbol ester binding on this distribution is not known.

To biochemically characterize receptors, additional photoaffinity probes will be synthesized which may have better efficiency for labeling the protein moiety of the receptor. The affinity purification studies will be continued. As purification of the receptor provides information on its nature, assays to identify the biochemical activity directly coupled to receptor occupancy will be performed.

Using different extraction techniques, further screening for endogenous inhibitors will be conducted. Particular emphasis will be placed on detection of labile compounds or membrane components insoluble in aqueous solution. Studies on the mutants will continue.

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ANNUAL REPORT OF THE
LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

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. Major research efforts of the LCMB in these areas are centered within the Molecular Biology, Animal Virology and Field Studies, and Experimental Oncology Sections.

During the past year, significant progress has been made by LCMB scientists within the Molecular Biology Section and the Animal Virology and Field Studies Section 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.

Moloney murine sarcoma virus (MSV) arose by recombination of Moloney murine leukemia virus (MuLV) and cellular sequences present within the normal mouse genome. These latter sequences, designated v-mos, have been shown to be essential for viral transforming activity. The development of molecular cloning and DNA sequencing techniques has made detailed analysis of viral

genome structure possible. During the past year, we reported the first complete sequence analysis of a transforming retrovirus genome. The genome of Moloney-MSV was shown to be comprised of 5,828 bases. Its long terminal repeat (LTR) possessed putative signals for the initiation and termination of transcription and possessed other structural similarities with transposable elements of prokaryotic and eukaryotic organisms. The MSV genome has the coding capacity for the Moloney murine leukemia virus gag gene product and contains large deletions in pol and env genes. A large open reading frame encompassing its cell-derived mos sequences codes for its putative transforming protein. The MSV-transforming region has the coding capacity for a protein of 409 amino acids and a molecular size of approximately 44,000 daltons. From the predicted amino acid sequence of the Moloney-MSV transforming protein, we have obtained synthetic peptides. These should be useful in immunologically identifying the MSV-transforming protein in MSV-transformed cells. Studies to date have already shown that antibodies prepared against synthetic MSV peptides are capable of recognizing the in vitro translational products of MSV RNA.

Abelson murine leukemia virus (A-MuLV) is a mouse-transforming retrovirus which induces lymphomas in vivo and transforms fibroblasts as well as B lymphoid cells in tissue culture. We utilized the integrated Abelson-MuLV genome cloned in bacteriophage λ to localize viral genetic sequences required for transformation. Comparison of the biological activity of cloned A-MuLV genomic and subgenomic fragments revealed that subgenomic clones which lacked the 5' LTR and adjoining sequences (300 bp downstream of LTR) were not biologically active. In contrast, subgenomic clones, which lacked the 3' LTR and as much as 1.3 kbp of the A-MuLV cell-derived abl gene, were as efficient as wild-type virus DNA in transformation. The A-MuLV encoded polyprotein P120 and its associated protein kinase activity were detected in the 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.

Another mouse acute transforming virus, BALB-MSV, was initially isolated from a spontaneously occurring BALB/c sarcoma. The integrated form of the viral genome was cloned from the DNA of a BALB-MSV transformed nonproducer NRK cell line in the Charon 9 strain of bacteriophage λ . In transfection assays, the 19 kilobase pair (kbp) recombinant DNA clone transformed NIH/3T3 mouse cells with an efficiency of 3×10^4 focus-forming units per pmol. Such transformants possessed typical BALB-MSV morphology and released BALB-MSV after helper virus superinfection. A 6.8 kbp DNA segment within the 19 kbp DNA possessed restriction enzyme sites identical to those of the linear BALB-MSV genome. LTRs of approximately 0.6 kbp were localized at either end of the viral genome by the presence of a repeated constellation of restriction sites and by hybridization of segments containing these sites with nick-translated Moloney murine leukemia virus LTR DNA. A continuous segment of at least 0.6 and no more than 0.9 kbp of helper virus-unrelated sequences was localized toward the 3' end of the viral genome in relation to viral RNA. A probe composed of these sequences detected six Eco RI-generated DNA bands in normal mouse cell DNA as well as a smaller number of bands in rat and human DNAs. These studies demonstrate that BALB-MSV, like previously characterized avian and mammalian transforming retroviruses, arose by recombination of a type C helper virus with a well-conserved cellular gene.

The nature of the cell-derived (bas) sequences of BALB-MSV was determined. Molecularly cloned bas sequences demonstrated no detectable homology with the onc genes of other mouse transforming viruses, but exhibited a high degree of sequence homology with the cell-derived sequence (ras) gene of the rat-derived Harvey murine sarcoma virus (Ha-MSV) genome. The Ha-MSV ras shared a colinear 750 bp region of homology with bas. Moreover, BALB-MSV transformation was associated with the expression of high levels of a 21,000 dalton protein, immunologically related to the ras gene product, p21. Thus bas and ras represent retroviral transforming gene homologs that were independently transduced by mouse type C viruses from the genomes of different species.

BALB- and Harvey-MSVs induce sarcomas and erythroleukemias in susceptible animals. An in vitro colony assay that detects transformation of lymphoid cells by Abelson-murine leukemia virus was utilized 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 which 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 Abelson-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.

Simian sarcoma virus is the only known transforming retrovirus isolated from a primate. Our laboratory initially characterized this virus in tissue culture and recently obtained molecular clones of infectious SSV DNA. We sought to identify the species of origin of the cell-derived (sis) sequences of simian sarcoma virus. A molecular clone comprised of sis DNA detected related nucleotide sequences at low copy numbers 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.

The sequence of the transforming region of simian sarcoma virus (SSV) was determined by using molecularly cloned viral DNA. This region encompassed the

1.0 kbp 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 within *v-sis* and helper viral sequences. 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. Efforts are presently in progress to utilize the results of nucleotide sequence analysis of the SSV-transforming gene to obtain antibodies capable of recognizing the translational product of this gene in SSV-transformed cells.

Nucleotide sequence analysis of the long terminal repeat (LTR) of the integrated simian sarcoma virus showed that the simian virus LTR comprised 504 nucleotides with an inverted repeat of seven bases at its 5' and 3' termini. At the site of simian sarcoma virus integration, cellular flanking sequences adjacent to the proviral LTR contained a direct repeat of four bases. A 13-base sequence after the 5' LTR was found to be complementary to prolyl tRNA, suggesting that tRNA^{Pro} may serve as the primer for reverse transcription of simian sarcoma virus RNA. The U₅ and R regions, derived respectively from the 5' end and terminally redundant sequences of the viral RNA, were found to have similar organization and sequence homology close to that of Moloney murine sarcoma virus or Moloney murine leukemia virus. These results indicate that regions within LTRs with known functionally important sequences have been most well-conserved during retrovirus evolution.

LCMB scientists have continued studies aimed at characterization of independent sarcoma virus isolates of the outbred cat. A new strain of feline sarcoma virus (GR-FeSV) was isolated from a spontaneous sarcoma of an 8-year old domestic house cat. The virus induced sarcomas at high incidence after a short latent period in fetal and newborn kittens and transformed cat embryo fibroblasts *in vitro* after 5 days. Compared to Gardner-Arnstein and the Snyder-Theilen strains of FeSV, GR-FeSV induced more pleomorphic sarcomas and larger, more rounded and discrete foci of cell transformation. GR-FeSV was shown to be defective for replicaton, and nonproducer transformed clones from several species were obtained at limiting GR-FeSV dilution. The defective sarcoma virus could be rescued from such transformants by superinfection with replication competent type C viruses. The primary translational product of the GR-FeSV genome is a 70,000 dalton polyprotein that contains the amino-terminal domain of the FeLV gag gene precursor protein and a sarcoma virus-specific polypeptide. These results differentiate GR-FeSV from previously isolated FeSV strains, and establish it as an independent spontaneously occurring sarcoma virus isolate of the domestic cat.

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.

We have applied radioimmunological techniques to demonstrate evolutionary linkage among distantly related oncoviruses by radioimmunological techniques. Antibodies elicited against proteins of a given virus often bind analogous proteins of oncoviruses isolated from species belonging to different orders or families of mammals. The designation "interspecies" has been applied to antigenic determinants recognized by an antibody in this kind of reaction. Broadly reactive interspecies antigenic determinants have been demonstrated in the major structural proteins of all known mammalian type C viruses. These findings have been extended to include other translational products representing more than two-thirds of the type C viral coding capacity. All of these studies have led to the conclusion that mammalian type C viruses arose from a common progenitor.

During the past year, collaborative studies involving scientists within the Molecular Biology Section have shown that the major core protein (p28) of MMC-1, an endogenous type C virus of rhesus monkey (Macaca mulatta), exhibits extensive homology in its NH₂-terminal amino acids to the sequences of the major structural protein (p30) of known mammalian type C viruses. Similarly, interspecies antigenic determinants shared by all the above viral proteins were detected in MMC-1 p28. Competition radioimmunoassays, together with the results of statistical analysis of the primary structure data, provided evidence that MMC-1 p28 is not more closely related to primate type C viruses of the Papio genus than to those isolated from rodents, cats or New World monkeys. MMC-1 p28 was found to be closely related, structurally, to the p30 protein of the avian reticuloendotheliosis virus (REV-A), a horizontally transmitted type C virus of putative mammalian origin. In addition, MMC-1 p28 and REV-A p30 shared a specific subset of antigenic determinants not present in any of the other avian or mammalian type C viruses studied. These findings suggest that MMC-1 and REV may have a common evolutionary origin.

Research within the Molecular Biology and Animal Virology and Field Studies Sections is also aimed at elucidating how leukemia viruses cause malignancies. Mouse leukemia viruses have been reported to induce tumors involving cells within the T lymphocyte lineage. We found striking differences in the target cells for in vivo transformation by two clonal replication-competent type C viruses, Moloney- and Rauscher-MuLV. Moloney-MuLV-induced tumors and lymphoma cell lines exhibited Thy.1 antigen in the absence of detectable Fc or C3 receptors, indicating their T cell origin. However, Rauscher-MuLV primary tumors and lymphoma cell lines of the same mouse strain invariably exhibited Fc receptors in the absence of Thy.1 antigen, suggesting that these tumors were of the B lymphoid cell lineage. The pattern of immunoglobulin synthesis by individual Rauscher-MuLV tumor cell lines was determined both by biosynthetic and radioimmunologic techniques. Rauscher-MuLV lymphoma lines most frequently

expressed immunoglobulin heavy (μ) chain in the absence of detectable light (λ or κ) chains. All of these findings established that the target of neoplastic transformation in response to Rauscher-MuLV is an immature cell within the B lymphoid lineage.

To investigate the basis for their cell specificity, we have molecularly cloned Rauscher- and Moloney-MuLV and analyzed their molecular organization. The physical map obtained for integrated Moloney-MuLV DNA, molecularly cloned in its Hind III permuted form, was consistent with that previously published (Gilboa et al., 1979). We cloned the Rauscher-MuLV genome in its integrated form. Cellular DNA was isolated from a clonal line of NRK cells productively infected with Rauscher-MuLV and digested with Eco RI. This enzyme was previously shown not to cleave the unintegrated linear Rauscher-MuLV genome. Rauscher-MuLV-specific DNA was enriched by RPC-5 column chromatography and sucrose density gradient and used for cloning in lambda phage Charon 4A. The cloned molecule is approximately 14 kbp in length. It was shown to contain an approximate 9 kbp viral DNA, as well as host flanking sequences. The cloned DNA induced virus production in NIH/3T3 cells upon transfection. Restriction enzyme analysis showed a strong correlation between the maps of cloned Rauscher-MuLV DNA and unintegrated linear Rauscher-MuLV DNA. Detailed restriction enzyme mapping of the cloned integrated Rauscher-MuLV DNA was performed. Efforts are now under way to construct recombination viruses between Rauscher- and Moloney-MuLV genomes in order to map the region of the viral genome responsible for target specificity for transformation.

Scientists within the Molecular Biology and Animal Virology and Field Studies Sections have successfully initiated new research thrusts utilizing knowledge gained from our studies in model systems to investigate mechanisms involved in naturally occurring human malignancies. One major effort has emanated from our 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. In addition, onc genes have been utilized to search for expression of onc-related transcripts in human neoplastic and normal cells.

Human DNA was analyzed for the presence of sequences homologous to the transforming gene (v-mos) of Moloney murine sarcoma virus (MSV). A single 2.5 kbp Eco RI-generated fragment of human DNA was identified 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 varying 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 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.

Polyadenylated RNAs of certain human tumor cell lines were shown to contain transcripts related to the cell-derived transforming onc genes of molecularly cloned primate, murine or avian transforming retrovirus genomes. Markedly elevated levels of single transcripts related to either SSV or MC29 onc genes were present in certain human tumor cell lines established from solid tumors. These findings are consistent with transcriptional derepression of these genes in certain tumors. More complicated patterns of transcripts related to BALB-MSV and Abelson-MuLV onc genes were detected in human normal and neoplastic cells. These findings raise additional questions concerning the mode of processing of such transcripts and their source from one or more related genes. Nonetheless, the almost universal presence of these transcripts in human cells argues strongly that these viral onc-related genes possess some important function(s).

Total cellular poly (A)-enriched RNA from a variety of fresh human leukemic blood cells and hematopoietic cell lines was analyzed for homology with molecularly cloned DNA probes containing the onc sequence of A-MuLV, Ha-MSV, SSV, and avian myelocytomatosis virus strain, MC29. Results with the fresh blood cells paralleled those obtained with the cell lines. With A-MuLV and Ha-MSV, multiple RNA bands were visualized in all cell types examined without significant variation in the relative intensities of the bands. When SSV was used as the probe, expression of related onc sequences was absent in all of the hematopoietic cell types examined except for one neoplastic T-cell line (HUT 102), which produces the human T-cell leukemia (lymphoma) retrovirus, HTLV. In this cell line, a single band (4.2 kilobases) was observed. With MC29 as the probe, a single band of 2.7 kilobases was visualized in all cell types examined with only a 1- to 2-fold variation in intensity of hybridization. An exception was the promyelocytic cell line, HL60, which expressed approximately 10-fold more MC29-related onc sequences. With induction of differentiation of HL60 with either dimethyl sulfoxide or retinoic acid, a marked diminution in the amount of the MC29-related, but not the A-MuLV-related, onc message was observed.

Total cellular RNAs from a variety of fresh and culture-derived human hematopoietic neoplastic cell types at various stages of differentiation and human sarcoma, carcinoma, melanoma, and glioblastoma cell lines were enriched for poly (A)-containing sequences, fractionated by gel electrophoresis, and blot hybridized to a cloned DNA probe containing the transforming sequences (v-amv) of avian myeloblastosis virus (AMV), a virus known to cause myeloid leukemias in chickens. Expression of RNA sequences homologous to AMV was detected in all immature myeloid and lymphoid T-cells in addition to the single erythroid cell lines examined, but not in mature T-cells or in B-cells, including lymphoblast cell lines derived from patients with Burkitt's lymphoma. In addition, induction of the cell line, HL60, a promyelocytic leukemia line, to differentiate with dimethyl sulfoxide or retinoic acid resulted in a reduction of the level of expression of the human cellular gene c-amv homologous to v-amv. There was no detectable c-amv mRNA in any of the solid tumor cell lines examined. Thus, expression of the human c-amv gene could be correlated with the stage of differentiation of different hematopoietic cell types determined by morphologic and marker studies. Expression of c-amv could not be correlated with the extent of methylation in HL60 and HL60 induced to differentiate with dimethyl sulfoxide.

The development of DNA-mediated gene transfer techniques has recently made it possible to identify transforming genes in a variety of tumors, including some of human origin. During the past year, 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 xenocompetent mice. Moreover, they contain human DNA sequences and were able to transmit their malignant phenotype in additional cycles of transfection. Southern blot analysis of the T24 bladder tumor-derived transformants showed that a single fragment of human DNA specifically cosegregated with the malignant phenotype, suggesting that it contained the T24 oncogene. Therefore, these human sequences were molecularly cloned with lambda Charon 9A as the cloning vector. The resulting recombinant DNA molecule, designated λ T24-15A, was shown to contain a 15 kbp-pair Eco RI insert of human cellular DNA. This DNA transformed NIH/3T3 fibroblasts with a specific activity of 20,000 focus-forming units per pmol of cloned DNA. These findings demonstrated the successful molecular cloning of a biologically active oncogene present in T24 human bladder carcinoma cells. Studies are presently underway to identify and isolate additional biologically active human oncogenes by using this same strategy.

LCMB scientists within the Molecular Biology Section also demonstrated a major new link between human oncogenes isolated from human tumor cell DNAs and the transduced cellular (onc) sequence acquired by retroviruses from the host cell genome. It has been well established that of the more than two dozen independent acute transforming retrovirus isolates to date, some contain the same or closely related onc genes. These findings have suggested that vertebrates may contain a limited number of cellular genes that can acquire transforming properties when recombined with retroviral sequences. Thus, it was of potential interest and importance to determine whether the transforming gene isolated from T24 human bladder carcinoma cells was related to the onc gene of any known transforming retrovirus. We were able to establish a high degree of relationship between this human oncogene and the onc gene of BALB-MSV. By restriction mapping and heteroduplex analysis, these sequences were localized to a 3.0 kbp region required for transformation by the T24 oncogene. This transforming gene was expressed as a 1.2 kbp polyadenylated transcript, which also contained v-bas-related sequences. Moreover, antiserum known to detect the immunologically related onc gene products of BALB- and Harvey-MSVs recognized elevated levels of a related protein in T24 cells as well as T24 oncogene-induced transformants. Our finding that a molecular clone of the normal human homologue of v-bas was indistinguishable by restriction enzyme analysis from the T24 oncogene implies that rather subtle genetic alterations have led to its activation as a human transforming gene. Presently, studies are under way to determine, at the nucleotide sequence level, the basis for the alteration of the normal human v-bas homologue to a transforming gene in T24 cells. It appears likely that research on the mechanisms of transformation by retroviral onc genes will provide major insights into the mechanisms by which normal human cells become malignant.

The Experimental Oncology Section has been involved in both immunological and molecular biological studies of carcinomas, with emphasis on mammary neoplasia. Monoclonal antibodies reactive with the major envelope and internal structural

proteins of mouse mammary tumor viruses (MTVs) were generated. Different monoclonals were shown to be reactive with type-specific, group-specific, and interspecies determinants of MTVs. The monoclonals to type-specific determinants were used to demonstrate that differences exist between each of six MTVs derived from different strains of laboratory mice (Mus musculus). MTVs grown in murine as well as feline cells were tested in order to rule out the possibility that the differences observed were due to host determinants. Antibodies to MTV interspecies determinants were obtained by immunizing mice with type B retroviruses obtained from Mus cervicolor (MC-MTV) and Mus cookii (MCo-MTV) and testing subsequent monoclonals for reactivity with MTVs of Mus musculus. The monoclonals generated have been used to demonstrate the diversity of expression of individual antigenic determinants in primary mammary tumors of various Mus strains and species. The phenomenon of diversity of expression of a defined antigen, (a) among mammary tumors and (b) within a given tumor mass, has striking parallels to that observed in human breast cancers using monoclonal antibodies to human tumor-associated antigens.

A number of monoclonal antibodies reactive with human mammary tumor cells have been generated and characterized. Mice were immunized with membrane enriched fractions of metastatic human mammary carcinoma lesions; splenic lymphocytes were fused with non-Ig secretor myeloma cells to generate subsequent hybridoma cultures synthesizing 11 monoclonal antibodies reactive with human mammary carcinoma cells. The monoclonals could be placed into five major groups based on reactivity in solid phase RIAs and fluorescent-activated cell sorter analyses. Some of the monoclonals demonstrated a "pancarcinoma" activity, reacting with the surface of some non-breast carcinomas, but none of the 11 monoclonals reacted with the cell surface of melanomas, sarcomas, various hematopoietic malignancies, and numerous apparently normal cell lines. Two additional monoclonals were also generated and were shown to be reactive with purified carcino-embryonic antigen. The immunoperoxidase technique was used on fixed or frozen tissue sections to determine the extent of reactivity of the different monoclonals with various types of primary mammary tumors and with metastatic lesions in lymph nodes and at distal sites. One of the 13 monoclonals described, B72.3, showed the most restricted range of reactivity for tumor versus any normal tissues, and has been studied extensively. The monoclonal antibodies developed to date react with approximately 85 percent of human mammary tumors; studies are in progress to generate additional monoclonal antibodies to the thus far unreactive mammary carcinomas.

Antigenic variation was observed in the expression of specific tumor-associated antigens within individual mammary tumor masses using monoclonal antibodies. This variation was demonstrated by both the pattern and cellular localization of reactivity with a given antibody. This diversity was also observed in human mammary tumor cell lines grown *in vivo* and *in vitro*. Analyses of DNA content and cell surface binding of monoclonal antibodies during logarithmic growth phase, and at density-dependent arrest, demonstrated that the expression of some tumor-associated antigens is related to S-phase of the cell cycle. Membrane expression of the reactive antigens appeared to be stable despite prolonged exposure to antibody. Antigenic drift was observed with continued passage of mammary tumor cell lines; consistent with this finding, "the same" mammary tumor cell line obtained from different sources exhibited distinct phenotypes. Preliminary results indicate that exposure of mammary tumor cells to certain compounds may enhance the cell surface expression of some tumor-associated antigens.

Monoclonal antibodies to human mammary tumor metastases were tested for reactivity to novel and known tumor-associated antigens. The monoclonals were used to immunoprecipitate antigens from a radiolabeled breast tumor metastasis extract. Monoclonal antibody B72.3 immunoprecipitated a high molecular weight polypeptide complex of approximately 220,000 daltons. B6.2 and four other antibodies immunoprecipitated a 90,000 dalton polypeptide. The four other antibodies cross-react in a radioimmunoassay (RIA) for monoclonal B6.2 but differ in their ability to compete with the binding of B6.2. Two antibodies, B1.1 and F5.5, were shown to differentially react with carcino-embryonic antigen. The high molecular weight complex identified by monoclonal B72.3 has been preparatively purified using molecular sieving and antibody affinity chromatography, without loss of immunoreactivity. Studies are in progress to develop RIAs with several of the monoclonals described.

Studies to localize human mammary tumors using radioactively labeled monoclonal antibodies were also performed. Purified IgG, F(ab¹)₂ fragments, and Fab¹ fragments of monoclonal B6.2 have been generated. The IgG and its fragments were radiolabeled with ¹²⁵I without loss of immunoreactivity and were injected into athymic mice bearing human mammary tumor transplants. The radiolabeled antibody localized in the tumor within 24 hours with the tumor to tissue ratio rising over a 96 hour period. The F(ab¹)₂ was better than the IgG and gave tumor to liver and spleen ratios of 15 to 20:1, and tumor to muscle and brain ratios of 50 to 110:1. No localization was observed in mice bearing human melanomas, or with radiolabeled normal murine IgG in human mammary tumor-bearing mice. The ability of the radiolabeled antibody to localize in mammary tumors was sufficient to give high quality gamma scans of tumor-bearing mice. Several monoclonal antibodies are now being labeled with other radioactive isotopes that may be more appropriate for clinical studies.

Finally, human and nonhuman primate (nhp) monoclonal antibodies reactive with human mammary tumor cells were generated. Lymphocytes from lymph nodes obtained at mastectomy from breast cancer patients have been fused with murine non-immunoglobulin (Ig) secretor myeloma cells to obtain human-mouse hybridoma cultures that synthesize human monoclonal antibodies. Ig production was stable in several of the cloned cultures through the 60- to over 300-day observation period. Levels of Ig synthesis were comparable to those of standard mouse-mouse hybridomas. Several assays were used to demonstrate that the Ig's produced by the interspecies hybridomas were indeed human and not murine. The immunologic reactivities of the human Ig's were assayed with the immunoperoxidase method using tissue sections of the primary tumor from the patient whose lymphocytes were used for fusion, as well as using tissue sections from other primary tumors. One human IgM monoclonal was used to discriminate between mammary carcinoma cells (from lymphocytes of the same breast). This same antibody reacted with selected non-breast carcinomas and metastatic mammary carcinoma cells in lymph nodes and at distal sites. Experiments are in progress to develop nonhuman primate monoclonal antibodies using lymphocytes from old world monkeys and chimpanzees immunized with membrane enriched extracts of human mammary tumors. Human myeloma cells are being compared with murine non-Ig secretor myeloma cells for use in fusions with both lymphocytes from human lymph nodes of mastectomy patients and with the lymphocytes from hyperimmunized nonhuman primates.

Another research program within the Experimental Oncology Section concerns the identification of new retroviral genes in feral species of the genus Mus. Several pedigreed breeding colonies of feral species of Mus have been established. One of these colonies, Mus cervicolor popaeus, contains three maternal lineage groups which exhibit a high incidence of mammary tumors. Histologic examination of these tumors revealed a significant number of adenocarcinomas rarely seen in inbred strains of Mus musculus. Type B retroviral particles were observed in some tumors and in the milk of Mus cervicolor popaeus mice. Purification of these viral particles has led to the development of monoclonal antibodies to the associated viral proteins and interspecies RIAs for the MMTV p28, gp36, and gp52 proteins. High stringency blot hybridization analysis of restricted cellular DNA from the different species of mice, using cloned MMTV proviral DNA as a probe, has led to the identification of Mus musculus, Mus caroli, and Mus pahari breeding colonies which lack the genetically transmitted MMTV genome (designated MMTV-negative mice). One other Mus musculus colony was found to contain only the MMTV long terminal repeat region of the viral genome in its germline. By lowering the stringency of blot hybridization we have identified a new class of highly diverged MMTV-related sequences (designated MMTV-B) in the cellular DNA of all species of the genus Mus, including MMTV-negative mice.

Investigations within this section have also continued to analyze the genetic organization, evolution and role that novel endogenous retroviruses play in neoplasias of mice. The unintegrated proviral genomes of representatives from two novel classes of murine endogenous retroviruses (designated M432 and type C-I) isolated from Asian species of mice were molecularly cloned. The major internal protein of the Mus cervicolor M432 retrovirus was found to be immunologically related to the core protein of the noninfectious Mus musculus intracisternal A-particles (IAP) and unrelated to the proteins of type B and type C retroviruses. A detailed comparison of recombinant DNA containing the M432 and IAP proviral genomes has revealed two discrete regions of nucleic acid sequence homology. Thus, it is possible that the M432 class of retrovirus results from recombinational event(s) between the IAP genome and either other rapidly evolving cellular DNA sequences or another as yet unclassified retroviral DNA sequence. The IAP genome is reiterated 1000 times in the Mus musculus cellular genome, but to a lesser extent on other Mus species. The Mus cookii type C-I retroviral genome was found to share significant sequence homology to the gag-pol region of the woolly monkey infectious primate type C viral genome. This is consistent with previous immunological results suggesting that the woolly monkey-gibbon ape group infectious primate type C viruses in this endogenous retroviral class are highly conserved in all species of Mus. In Mus musculus, but not other murine species, at least 100 copies of type C-I-related sequences were found on the Y chromosome.

LCMB efforts to elucidate mechanisms of carcinogenesis are complemented by the In Vitro Carcinogenesis Section. Research of this section is aimed at determining mechanisms of spontaneous and carcinogen-induced malignant transformation of cultured cells of rodent and human origins. Special emphasis is being directed to the development of culture systems utilizing human epithelial cells.

Over the past year, studies by this section have emphasized the importance of DNA damage and deficient repair as crucial elements in the stepwise process of carcinogenic transformation in human cells. Cytogenetic analysis of

chromosomal damage induced by irradiation (visible light or x-irradiation) has proved useful in elucidating deficiencies in DNA repair. Since a chromatid (half-chromosome) at metaphase is thought to contain a single continuous DNA double helix, a chromatid break observed at the first metaphase following irradiation would represent an unrepaired DNA double-strand break. Gaps in chromatid regions are generally interpreted as representing single-strand breaks. In a collaborative study, a line of "normal" human skin fibroblasts (i.e., diploid and nontumorigenic), designated KD, differed from its malignant neoplastic derivative, HUT-14, in response to low-intensity x-irradiation during the G₂ phase of the cell cycle. The malignant cells had significantly more chromatid breaks and gaps after exposure to 25, 50, or 100 rad than their normal counterparts. Results from alkaline elution of cellular DNA after irradiation showed that normal and malignant cells were equally sensitive to DNA single-strand breakage by x-irradiation. Caffeine or cytosine arabinoside (ara-C), inhibitors of DNA repair, significantly increased the incidence of radiation-induced chromatid damage in the normal cells but had little influence on the malignant cells.

Lines of human skin fibroblasts derived from ataxia telangiectasia, Gardner's syndrome, Fanconi's anemia, Bloom's syndrome, and xeroderma pigmentosum (XP-A, C, E, variant) donors, all genetically predisposed to a high risk of cancer, were found to differ (except XP-A) from six lines of skin fibroblasts from normal donors. The former showed a significant increase in chromatid breaks following exposure to low-intensity fluorescent light during late S-G₂ phase of the cell cycle. However, cells from patients predisposed to cancer did not differ from normal cells if light treatment was given during the G₁ or S phase. This damage could be prevented by mannitol, a scavenger of $\cdot\text{OH}$ generated during light exposure. The failure of late S-G₂ light exposure to produce chromatid breaks in XP-A cells, which are known to be deficient in endonuclease activity, suggests that endonuclease incision of DNA base damage is a necessary step in chromatid breakage induced by light exposure during late S-G₂. A possible explanation of these observations is that cells from genetic variants with a high risk of cancer are deficient in a mechanism of excision repair operative at the late S-G₂ stage of the cell cycle.

A number of environmental agents, including ultraviolet light, chemical carcinogens, visible light, as well as x-rays, are known to produce DNA-protein crosslinks in mammalian cells. Such crosslinks may play a role in epigenetic events leading to malignant transformation. The reagent, transdiaminodichloro-platinum, an inducer of DNA-protein crosslinks, was used to study the induction, consequences and repair of crosslinks in L1210 mouse cells and normal human fibroblasts. Two pathways of repair were revealed. One pathway is coupled to DNA replication. In rapidly growing populations of L1210 cells, the rate of repair is coupled to and limited by the rate of DNA synthesis. However, when the doubling time was increased from 12 hours (20% serum supplement) to 30 hours (1% serum), the repair of crosslinks, though slower, was twice the rate of DNA synthesis. Furthermore, in the presence of aphidicolin, an effective inhibitor of S phase DNA synthesis, complete repair of crosslinks occurred slowly in both L1210 and normal human fibroblasts. This repair can be inhibited by cycloheximide, an inhibitor of protein synthesis and by α amanitin, an inhibitor of RNA polymerase. These results implicate a second pathway, the induction of a required repair component(s), not dependent on DNA synthesis.

Another aspect of the section program concerns the nutritional requirements for proliferation and function of epithelial cells in culture. This is an area of increasing importance in carcinogenesis studies since most tumors are the result of malignant alteration of epithelial cells. Studies have continued on the role of dissolved oxygen in modulating epithelial cell proliferation and function. Based on current quantitative data, the rate of oxygen utilization by epithelial cells is significantly higher than that of fibroblasts. In addition, a gas phase (approximately 40 mm Hg, O₂) which is optimal for clonal growth of fibroblasts, retards proliferation of human epidermal cells or a line of rhesus monkey kidney cells grown in serum-free medium. The frequency of hemicyst formation in cultures of monkey kidney cells was found to be dramatically reduced at lowered dissolved oxygen concentration. The influence of calcium on proliferation of human epidermal cells was further evaluated under optimal culture conditions; it was possible to obtain reproducible high yields of proliferating human epidermal cells taken from foreskin. Efforts are now underway to develop appropriate human epithelial transformation systems using chemical carcinogens or transforming retroviruses.

The Viral Immunology Section has as its major goals the development of valid and reliable assays for identification of individuals at high risk for developing early cancer as well as approaches aimed at immunoprevention of cancer. A fibroblast assay was calibrated in skin fibroblasts derived from a group of 40 members of a Gardner's syndrome family with complete defined cancer profiles provided (in coded form) by Dr. Eldon Gardner, the geneticist who described the syndrome. Triplicate specimens from each of the lines were sent to three collaborating laboratories (Sloan-Kettering Institute for Cancer Research, the University of Southern California, and the LCMB), and to date have been run completely in one (USC) and partially in the LCMB. Cell lines were thawed and passaged 3-7 times in each laboratory and tested for focus formation using the Ki-MSV (LCMB) or the Ki-MSV (BaEV) (USC) viruses. In both laboratories, the fibroblasts derived from the high risk individuals were found markedly more sensitive to viral transformation than were those derived from family members not carrying the Gardner's trait; the latter transformed only at the highest virus concentrations or not at all. The correlation of high risk with positives in the assay was 75-80%. However, 7-9% of the "normal" risk cell lines responded positively as well. In collaboration with Dr. J. Frankel, State of Florida Department of Health, Tampa, similar studies were undertaken with skin fibroblasts derived from heavy smokers with and without lung or other cancers. Results were again significant, but the same percentage of normals was positive in the assay. In addition, two known Gardner's syndrome patients, post-surgery, tested consistently negative in repeated tests. Although the degree of validity in cancer patients and individuals known to be high genetic risk for cancer is significant, the basis for such sensitivity remains to be determined. Moreover, the unexplained positives and negatives raise important questions which would have to be resolved prior to considering the assay for diagnostic purposes.

The objectives of the Experimental Ontogeny Section include the investigation of exogenous growth factors on the proliferation and differentiation of fetal tissues. Growth stimulatory as well as anti-proliferative and anti-viral factors have been solubilized from mouse placental tissues taken after the tenth day of gestation. High salt extracts of placental tissue contained an acid-sensitive anti-viral activity which was specific for mouse cells and

demonstrated serologic cross-reactivity with mouse fibroblast interferon. Two peaks of activity were resolved by gel filtration. One possessed a molecular weight greater than 65,000, and the other was approximately 30,000. Both peaks of activity were sensitive to temperature and to acid treatment (pH 2.0 at 24 hours). Both peaks of anti-viral activity were also found to display anti-proliferative activity for mouse cells.

Placental-associated growth factor (P1-GF) induced mitogenic activity in mouse cells as well as a variety of heterozygous cell lines. The mitogenic activity was trypsin-sensitive, unstable in one molar acidic acid (pH 3.0), but moderately stable in 0.5 molar acidic acid (pH 3.5). Findings that the activity was not disrupted by disulfide bond disruption helped to distinguish P1-GF from EGF. By rate zonal sedimentation at neutral pH, P1-GF appeared to have a molecular weight of around 25,000 - 30,000. Studies are presently underway to further characterize this biologically active molecule.

A number of administrative changes within the LCMB have taken place this year or will be effected by the beginning of the next year. The Experimental Ontogeny Section, which is located at FCRF, has recently been transferred to the Comparative Carcinogenesis Laboratory at FCRF. Dr. Jeffrey Schlom, Chief of the Experimental Oncology Section, has been given the opportunity to develop his own laboratory. The focus of the new laboratory will include present programs within that section. A number of projects currently underway with other scientists within the LCMB will continue to be actively pursued collaboratively. Finally, Dr. Robert Huebner, who has headed the Viral Immunology Section for more than 15 years, will retire at the end of the present year. The research of this Section will be refocused to pursue our recent advances in the investigation of transforming genes of human malignancies.

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 multi-disciplinary 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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04805-12 LCMB

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cocarcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A. Hellman Assistant Chief LCMB NCI
OTHERS: A.K. Fowler Chief, Experimental Ontogeny Section LCMB NCI

COOPERATING UNITS (if any)

Food and Drug Administration
Bureau of Radiological Health
Rockville, MD 20857

Litton Bionetics, Inc.
Frederick Cancer Research Facility
Frederick, MD 21710

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is designed to gain further insight into the control of genomic expression, particularly as it may lead to deregulatory processes. Systems consisting primarily of primary rodent and human cultures are being used to evaluate modification of genetic expression based on exposure to tumor promoters (such as chemicals), x-ray and ultraviolet light.

Project DescriptionObjectives:

Tumor promoters are a group of compounds which by themselves do not induce cancers, but markedly enhance the production of malignancies if administered after exposure to low doses of carcinogens. The mechanisms of initiation and promotion are not well understood. We are attempting to elucidate mechanisms of tumor promotion utilizing immunological and enzymatic techniques. We are also attempting to define the influences of promoters on the translational process.

Methods Employed:

Cell exposure to tumor promoters, ultraviolet light, and x-irradiation and evaluation of subsequent responses are being studied in vitro. The influence of promoters on DNA transfection is also being explored.

Major Findings:

Tetradecanoyl-phorbol-13-acetate (TPA) produces biological effects in vivo and in vitro. It has been demonstrated that TPA induces proteases in cell culture systems. Proteases are thought to play a role in cellular regulation by derepressing genes via the proteolytic cleavage of repressor proteins. We have previously shown that protease inhibitors suppress induction of endogenous type C virus by agents such as photosensitizers 5-iodo-2-deoxyuridine (IUDR) and ultraviolet-irradiated herpes simplex virus. If TPA could induce type C virus, and if such induction could be suppressed by protease inhibitors, it would strengthen the concept that proteases may be involved in mammalian gene derepression. Having now demonstrated that protease inhibitors modify mammalian gene expression via suppression of retrovirus replication, the concept that protein repressors may be regarded as a means of permitting virus expression cannot be excluded.

Significance to Biomedical Research and the Program of the Institute:

The demonstration that TPA, a protease inhibitor, also induced type C virus (which is a process partially initiated by protease inhibitors) is consistent with protease inhibition of type C virus induction by other agents, and provides additional presumptive evidence for the involvement of proteases in TPA action. Identification of individual events involved in virus induction and repression should eventually provide definitive information on the processes that control mammalian genome expression.

Proposed Course:

In view of the rapid strides made in developing means for detecting, isolating and defining transforming genes in mammalian malignant tissues, efforts to elucidate the mechanisms that modify such transfections and transformation

are of high priority. Attempts are currently underway to define the physiological cofactors associated with the transfectional process in systems presumed to be analogous to those observed with promoters.

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., in press.

Weislow, O.S., Fisher, O.V., Jr., Fowler, A.K., Twardzik, D.R., and Hellman, A.: Depression of mitogen-induced lymphocyte blastogenesis by baboon endogenous retrovirus-associated components. Proc. Soc. Exp. Biol. Med. 166: 522-527, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Comparative Analysis of Fetal and Neoplastic Growth Regulating Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: A.K. Fowler Chief, Experimental Ontogeny Section LCMB NCI
OTHER: P.T. Allen Microbiologist LCMB NCI

COOPERATING UNITS (if any) Litton Bionetics, Inc., Frederick Cancer Research
Facility, Frederick, Maryland 21701

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Experimental Ontogeny Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

1.3

PROFESSIONAL:

0.8

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The expression and control of bioregulatory macromolecules in embryo-fetal development and the effect of their interactions on cellular proliferation, differentiation and immunocompetence are being studied. Mouse placentas contain interferon-like factors (PI-IFN), mitogenic stimulating factors (MSF) and transforming growth factors (TGF). The PI-IFN exhibits species specific antiviral activity and shows serological cross reactivity to mouse fibroblast interferon, but not to mouse immune interferon. The antiviral activity is acid and trypsin sensitive and is blocked by actinomycin D. MSF and TGF, in contrast, do not exhibit species specificity and are moderately stable to acid treatment. Chromatography of high salt placental extracts by gel filtration resolves two major peaks of antiviral activity, one with an estimated molecular weight greater than 65 K and the other approximately 30 K, as well as a more diffuse peak (15 K - 30 K) exhibiting the major growth promoting activities (MSF and TGF). The two peaks of antiviral activity, which vary quantitatively during gestation, also display antiproliferative activity specific for mouse cells. Additionally, treatment of mouse cells with fibroblast interferon abrogates the MSF activity of placental extracts.

Project Description

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 regulation processes. To study the physiological induction and regulation of endogenous retrovirus expression in vivo and their potential influence on normal and pathological growth control mechanisms of the host.

Methods Employed:

Interferons (IFN) are assayed by their antiviral activity on L929 mouse cells using plaque reduction procedures (PR50), inhibition of cytopathogenic effect (CPE), virus yield reduction and inhibition of viral specific RNA synthesis. Stimulation of cellular mitogenesis is measured by the incorporation of radiolabeled thymidine into cellular DNA of quiescent mouse, rat, hamster or human cells. Colony stimulating activity is quantitated by the induction of BALB/3T3 and NRK cell growth in soft agar. Oncornavirus expression is determined by molecular hybridization, viral receptor-ligand interaction and tissue culture techniques.

Major Findings:

1. Identification and partial purification of interferon in mouse placentas. Previous studies have demonstrated the appearance of a pregnancy-dependent antiviral component in the placentas of mice from several different strains. The activity is efficiently extracted and relatively stable in high salt buffers at neutral pH, supplemented with protease inhibitors. Titers as high as 5,000 units/ml have been detected in term placentas of mice from high inducer strains. Partial purification of this activity has been obtained by gel filtration (P-100). Using this procedure, two major molecular species have been identified in term placental extracts, one with an estimated molecular weight greater than 65K (peak 1) and one of approximately 30 K (peak 2). In comparison, the smaller species is predominant in midterm placentas. The antiviral activity in peaks 1 and 2 exhibits species specificity (i.e., protects mouse cells against virus infection but not human, bovine, hamster, rat or dog cells) and is trypsin sensitive. The increment addition of either peak 1 or 2 to mouse cells results in a progressive reduction of EGF induced proliferation, but fails to exert detectable antiproliferative effects on hamster cells even at elevated (5-fold) concentrations. Both peaks of activity are sensitive to temperature, exhibiting a loss of approximately 90% of the antiviral activity by treatment at 56°C for 15 minutes. A significant portion of the antiviral activity is sensitive to acid treatment (pH 2.0 for 24 hrs.). The activity in peak 1 is completely inactivated by acid, while the activity in peak 2 is reduced by 50-70%. The stability of the crude placental extract is intermediate (nearly 25%). Incubation with antisera to L-cell interferon, but not to immune interferon, partially neutralizes the placental antiviral activity. Treatment with beta mercaptoethanol has no effect on the activity. These data suggest that placental interferon may be representative of a new class of interferons.

2. Antiviral activity of placental interferon in vivo. The antiviral activity of placental interferon has been demonstrated by in vitro protection of mouse cells (L929 and JLS V9/NRA) to encephalomyocarditis virus (MM) infection. In vivo, however, placental interferon fails to check virus replication. In fact, the sensitivity of the pregnant mouse to MM virus infection increases with gestational age and elevated placental interferon titer. In particular, the placenta exhibits a higher rate of virus replication than other tissues (brain, spleen, plasma, thymus) of the pregnant mouse, especially at or near parturition when placental interferon titers are highest. These data can be explained, in part, by the apparent insensitivity of cytotrophoblast to interferon mediated suppression of virus replication and to the elevation of estrogen during pregnancy, a hormone reported to modify interferon synthesis. From these data it appears unlikely that a primary biofunction of placental interferons is their viral prophylactic activity.

3. Detection of growth stimulating factors in mouse placentas. Mitogenic and transforming growth factors have been detected in midterm and term mouse placental extracts. These factors are relatively stable to mild acid conditions (pH 3.5) and do not exhibit species specificity. Under acid conditions, molecular sizing by gel filtration distinguishes a 10-15 K component(s) in the crude extract that exhibits the principle growth promoting activity, whereas under high salt conditions, molecular weight estimates are approximately doubled (20-30 K). Exogenous mouse fibroblast interferon abrogates the placental mitogenic activity on mouse (BALB/3T3) cells.

Significance to Biomedical Research and the Program of the Institute:

The role of interferons as a potent bioregulator is becoming increasingly apparent. Besides their antiviral effects, this class of low molecular weight glycoproteins is known to influence a variety of other physiological processes ranging from cell surface and growth control to immunoregulation. Indeed, interferons are regarded as one of nature's most potent and best characterized negative growth regulators. The demonstration of interferons, as well as transforming growth factors, in placentas--an allograft possessing numerous morphological, immunological, endocrinological and biochemical similarities to a variety of cancers--provides a unique model system to investigate and define the mechanisms of growth regulation and the possible role of interferons in normal cellular recognition and differentiation.

Proposed Course:

Efforts to identify and purify interferons and transforming growth factors in embryo-fetal tissues in adequate quantities to permit further characterization and study will be continued. Model systems will be developed to assess and compare growth factors and their interactions on cellular proliferation, differentiation and immunocompetence during normal and neoplastic development.

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, in press.

Saviolakis, G.A., Strickland, J.E., Paisley, T., Hellman, A., and Fowler, A.K.: Expression of oncornaviral RNA in mouse uterus during pregnancy. Biol. Reprod. 26: 806-812, 1982.

Twardzik, D.R., Ranchalis, J.E., and Fowler, A.K.: Interaction of murine thymocyte histocompatibility antigens with envelope glycoproteins of Rauscher murine leukemia virus. In Lapin, (Ed.): Comparative Leukemia Research, in press.

Weislow, O.S., Fisher, O.V., Twardzik, D.R., Hellman, A., and Fowler, A.K.: Depression of mitogenic-induced lymphocyte blastogenesis by baboon endogenous retrovirus associated components. Proc. Soc. Exp. Biol. Med. 166: 522-527, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Genetic Organization and Role of Murine Endogenous Retroviruses in Neoplasias

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert Callahan	Microbiologist	LCMB	NCI
OTHERS:	Edward Kuff	Chief, Biosynthesis Section	LB	NCI
	Kira Lueders	Microbiologist	LB	NCI

COOPERATING UNITS (if any) E. Birkenmeier, Jackson Laboratories, Bar Harbor, Maine;
R. Jaenish, Heinrich-Pette Institut Fur Experimentelle Virologie and
Immunologie, Hamburg, West Germany

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have molecularly cloned M432 retroviral and intracisternal A particle (IAP) genes from M. cervicolor cellular DNA. The 4 kbp major region of homology between the M. musculus and M. cervicolor IAP genes does not include the long terminal repeat regions of the genomes. The molecularly cloned M. cookii type C-I proviral genome was found to share significant sequence homology to the gag-pol region of the woolly monkey infectious primate type C viral genome. This is consistent with previous immunological results, suggesting that the origin of the woolly monkey-gibbon ape group of infectious primate type C viruses is from this class of endogenous murine type C viruses. Sequences related to the type C-I class of endogenous retroviral genome are highly conserved in all species of Mus. We have found that in M. musculus, but not in other murine species, at least 100 copies of type C-I-related sequences are located on the Y chromosome.

Project Description

Objective:

To characterize at a molecular and biological level novel classes of endogenous retroviruses and assess their role in neoplasia.

Methods Employed:

Retroviral-related sequences were detected in restriction endonuclease digested recombinant and cellular DNAs by the Southern transfer-blot hybridization technique. Standard techniques were used to obtain recombinant clones of cellular and unintegrated retroviral DNA.

Major Findings:

The M432 retroviral and intracisternal A particle (IAP) genes. We have molecularly cloned restriction fragments of M. cervicolor cellular DNA which contain either IAP or M432 retroviral genes. Several of these clones have been analyzed by restriction enzyme mapping. Some of the M. cervicolor and M. musculus IAP genes contain a major 4 kbp region of homology. The 5' and 3' ends of the two genomes, including the LTR regions, are unrelated by blot hybridization. In addition, several of the M. cervicolor IAP genes contain either internal insertions or deletions. The IAP genes appear to be inserted at several heterologous sites within the cellular genome. Clones containing M432 retroviral cellular genes were obtained at one-fifth the frequency of IAP genes. Several of the M432 cellular genes contain deletions corresponding to the portion of the genome coding for envelope proteins.

Endogenous type C viruses. Of the type C viruses isolated from the different Asian species, two subclasses (C-I and C-II) could be distinguished by the following criteria: host range, immunological and nucleic acid sequence homology. The type C-I class is related to the woolly monkey (SSAV)-gibbon ape infectious primate type C retroviruses and the type C II is related to laboratory strains of MuLV. We have molecularly cloned M. cookii type C-I 8.5 kbp unintegrated proviral genome from acutely infected tissue culture cells. We are currently defining the regions of homology shared between the M. cookii type C-I proviral genome, cloned type C-II proviral genomes from M. musculus (AKR and Moloney MuLV) and the infectious, woolly monkey (SSAV) type C proviral genome. Our preliminary results show that a type C-I DNA fragment corresponding to the p30, p10 and the 5' half of the pol region, does hybridize to SSAV proviral DNA but not to AKR ecotropic or Moloney MuLV proviral DNAs. This is consistent with our previous immunological characterization of the viral associated proteins corresponding to this region of the genome. In addition, the type C-I fragment corresponding to the gp70 region of the genome did hybridize with Moloney MuLV proviral DNA, but not with ecotropic AKR MuLV proviral DNA or with SSAV proviral DNA. This result may reflect the high passage history of Moloney MuLV in mice and suggests that their presence is a result of a recombinational event between this viral genome and a M. musculus type C-I retroviral gene.

We have begun to analyze the organization of endogenous type C-I sequences in different species of the genus Mus. In restricted cellular DNA from M. musculus

and M. poschiavinus, a clone of the entire M. cookii type C-I proviral genome hybridized to at least four fragments (14.5, 12.5, 8.4 and 4.5 kbp) in male DNAs that were not present in female DNAs. Because the sum of the sizes of the male-specific bands is larger than a viral genome (8.5 kbp), we conclude that these four bands are derived from more than one type C-I endogenous viral genome. The relative intensity of hybridization of these fragments to type C-I proviral DNA suggests that each is present in multiple copies. From these and other results, we conclude that there are approximately 100 copies of type C-I-related sequences in males from M. musculus inbred strains and M. poschiavinus.

Although male-specific type C-I-related fragments were detected in M. spretus DNA, they do not react as intensely with the probe as in M. musculus male DNA. Whether this reflects a lower copy number or less homologous sequences is unresolved. Restricted cellular DNA from M. caroli also hybridized with type C-I proviral DNA. While M. caroli DNA does not appear to contain male specific sequences unique to the type C-I proviral genome, it does contain a 2.0 kbp internal EcoRI fragment characteristic of the proviral genome. We conclude that the type C-I related sequences in the M. caroli cellular DNA are structurally more closely related to the M. cookii type C-I proviral DNA than to related bands in M. musculus and M. poschiavinus DNAs. These results are consistent with the previously noted conserved nature of the type C-I endogenous retroviral related sequences in cellular DNA from all species of Mus.

Significance to Biomedical Research and the Program of the Institute:

Our observations have led to a model in which the M432 retrovirus is a prototype of a class of retroviridae formed by recombination between an endogenous IAP genome and some as yet unidentified viral or cellular DNA sequence. In addition, our results have raised the possibility that IAP, although extracellularly non-infectious, may be capable of intracellular "infection" with reinfection of its genome at new sites in the cellular DNA or germline. The expression of IAP genes in early embryos prior to the development of the germline also has implications for the genetic events involved in speciation. Thus, the unique amplification of IAP genes in M. musculus or the origin of the transposed pseudo-alpha globin gene in this species may represent specific examples of such a phenomenon. Similarly, the striking observation that type C-I-related sequences are present in many copies on the Y chromosome of some Mus species suggests at least two models: (1) many independent viral integration events occurred within or near a specific sequence present many times on the Y chromosome, or (2) specific amplification of a Y chromosomal element containing virus-related sequences. The conserved pattern of type C-I-related dimorphic restriction fragments in M. musculus and M. poschiavinus suggests the integration or amplification event(s) responsible for multiple copies occurred recently in evolutionary terms. Taken together, our results raise the possibility that amplification of specific endogenous retroviral genes is associated with the phenomenon of speciation.

The role these novel retroviral genes play in neoplasia remains to be determined. However, it is perhaps pertinent that large numbers of IAP are expressed in all pristane-induced plasma cell tumors and certain mammary tumors of BALB/c mice. The presence of type C-I-related envelope sequences in Moloney MuLV, but not the AKR ecotropic type C virus, suggests that these were acquired by recombination between the MoMuLV and endogenous type C-I genomes. This raises the possibility

that some recombinant MCF type C retroviruses may also be chimeras of the ecotropic type C-II and type C-I retroviral genes. Thus type C-I retroviruses may play a role in the etiology of viral induced T-cell leukemias in mice.

Proposed Course:

The M432/IAP group of retroviral genes. Our current and future efforts will focus on the following areas: (1) To gain an insight into the mechanism by which IAP genes have been uniquely amplified in M. musculus, it will be determined whether certain families of M. musculus IAP restriction fragments are located on one or a few chromosomes or are dispersed throughout the cellular genome. In addition, the chromosome(s) on which the M. cervicolor endogenous M432 and IAP genes are located will be determined using mouse/hamster somatic cell hybrids (segregating mouse chromosomes). (2) The biological activity of M. cervicolor popaeus IAP genes will be determined in transfected NIH/3T3 tissue culture cells. This study is made possible by the lack of sequence homology between the M. cervicolor and M. musculus LTR region of the respective IAP genes. Thus it will be possible to distinguish between the endogenous expression of IAP genes in the NIH/3T3 tissue culture cells and that of the exogenous M. cervicolor IAP DNA. Moreover, it will be possible to determine whether biologically active M. cervicolor IAP genes are capable of intracellular "infection" of the host cellular DNA. In collaboration with Dr. Rudolf Jaenisch, we will attempt to insert recombinant DNA containing the M432 proviral genome and the M. cervicolor popaeus IAP gene into the cellular genome of preimplantation BALB/c mice. This approach may afford us the opportunity to look at the regulation of expression of these genes in early embryos as well as adult normal and neoplastic tissue.

The type C-I class of endogenous retroviruses. During the next few months, we will complete the characterization of the recombinant clones of the M. cookii type C-I proviral genome. In collaboration with Dr. S. Tronick (NCI), we plan to compare the type C-I proviral genome with those of various xenotropic and MCF type C viruses of AKR mice and the woolly monkey infectious primate type C virus by heteroduplex analysis. In other studies to be done in collaboration with Dr. E. Birkenmeier, we plan to: (1) identify the region(s) of the type C-I genome which are found only in male M. musculus and determine their organization on the Y chromosome, and (2) determine whether the high level of expression of type C viral gp70 protein in male genital organs correlates with the expression of type C-I male specific sequences using in situ nucleic acid hybridization.

Publications:

Callahan, R., Kuff, E.L., Lueders, K.K., and Birkenmeier, E.N.: Genetic relationship between the Mus cervicolor M432 retrovirus and the Mus musculus intracisternal type A particle. J. Virol. 90: 901-911, 1981.

Phillips, S.J., Birkenmeier, E.H., Callahan, R., and Eicher, E.M.: Male and female mouse DNAs can be discriminated using retroviral probes. Nature 297: 241-243, 1982.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on Reticulum Cell Neoplasm Type B in BALB/c Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R.M. Merwin Research Biologist LCMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of data obtained on over 50 primary murine lymphocytic neoplasms, most of which were composed of B lymphocytes, and derived lines transplanted in mice, was continued. Some of these tumors arose in the spleen and, when transplanted, homed only to the spleen. Cells of these tumors were unstable, changing their homing patterns either during growth in the primary host or during transplantation. Because such homing changes in the primary host are almost always associated with enlargement of the gut-draining mesenteric node, it seems likely substances from the gut play a role in inducing the change. Prior to the change, cells have the characteristics of early B lymphocytes, whereas after the change demonstrate homing patterns like tumors of more mature B lymphocytes with surface immunoglobulins of the A or G isotypes.

Project DescriptionObjectives:

To determine the relationships between the sIg isotypes and other tumor properties by analysis of data already obtained on transplant lines of over 60 lymphocytic neoplasms.

Methods Employed:

Immunofluorescence methods were employed in identifying the isotope of the surface immunoglobulin on tumor cells.

Major Findings:

Evidence was found from analysis of data on over 50 lymphocytic neoplasms of B lymphocytes that cells of tumors that arise in the spleen and that home when transplanted only to the spleen are induced by substances from the gut to become more mature and give rise to secondary tumors in the mesenteric node. Findings supporting these conclusions are as follows. Some tumors that arise in the spleen and grow when transplanted only in the spleen change after a few generations to grow at three sites, including the site of injection, the nodes draining this site and the spleen. This change indicates an instability of the spleen growing cells. Two findings indicate the same instability may lead to a change during development of tumors in primary hosts. First, tumor lines derived from primary tumors in early stages of development yield more spleen growing lines than do primary tumors in late stages of development. Second, some primary tumors growing in both the spleen and node have two types of cells present because transplants yield lines that grow only in the spleen and lines that grow at three sites. That the change is induced by substances from the gut is indicated by the fact the gut-draining mesenteric node is involved. That the change is like a step in normal maturation of B lymphocytes is suggested by the finding that tumors of more mature cells with surface immunoglobulins of the A and G isotypes grow at three sites when transplanted, just as do the transplanted cells after the change.

Significance to Biomedical Research and the Program of the Institute:

With each stage in maturation of B lymphocytes there are changes in surface phenotype, in migration rates and in homing patterns relative to function. Tumor lines provide large numbers of cells arrested at a certain stage of differentiation and as such serve as experimental models of human B lymphocytic neoplasms.

Proposed Course:

To prepare the results of these studies for publication. This project will terminate at end of FY 1982.

Publications:

None

SMI P	SONIAN SCIENCE INFORMATION EXCHANGE CT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04922-16 LCMB
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Retroviral Immunotherapy Trials; Transformation Assay for Cancer Risk

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Huebner	Chief, Viral Immunology Section	LCMB	NCI
OTHERS:	J.S. Rhim	Research Microbiologist	LCMB	NCI
	R. Trimmer	Biological Laboratory Technician	LCMB	NCI

COOPERATING UNITS (if any) Dr. Eldon Gardner, Utah State U., Logan, Utah; Dr. M. Lynch, Creighton U., Omaha, Nebraska; Drs. Murray Gardner and S. Rasheed, USC, Los Angeles, California; A. Krush, Johns Hopkins U., Baltimore, Maryland; and Dr. J. Frankel, State of Florida Dept. of Health, Tampa, Florida

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Viral Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
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CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Retroviral immunotherapy of cancers in dogs, although providing long-term remissions in dogs with early malignancies, did not prevent eventual relapses. Concentrated retroviruses tested in healthy primates proved innocuous at the time of administration and throughout more than a year of observation. No evidence of viral shedding was observed in the sera, urine or feces of treated dogs.

A skin transformation assay for identifying members of families carrying autosomal dominant genes for high cancer risk was 80-100% successful in the initial tests. A follow-up study of 40 specimens derived from cancer syndrome families and provided blindly by Dr. Eldon Gardner was hampered by difficulty in resusciating his cultures after long-term storage. Duplicate cultures provided to and tested by a collaborative group (Drs. M. Gardner and S. Rasheed) correlated significantly (75-80%) with cancer incidence.

Project DescriptionObjectives:

1. Trials of retroviral immunotherapy in dogs with spontaneously occurring cancers of all types.
2. Development of skin transformation assays for identification of individuals at high risk of cancer in families carrying autosomal dominant genes specific for neoplastic disease.

Methods Employed:

1. Preparations of nontransforming retroviruses (RD114, C57L, M55, 1504A), concentrated 1000X, were inoculated intravenously alone or in combination with unconcentrated virus inoculated intraperitoneally. Virus titers ranged from 10^4 - 10^6 . From 2-10 ml of concentrated virus was used, depending on the weight of the dog. The unconcentrated preparations were administered at an approximate ratio of 1 ml/pound.
2. Skin biopsies were cultured to 3-5 passages and then infected with serial log doses of KiMSV. Degree of risk was determined by transformation efficiency at a virus titer of 10^{-2} , at which level it was found that cultures derived from individuals at high risk transformed; those derived from low or normal risk individuals generally did not.

Major Findings:

1. Remissions (up to one year) of primary and metastatic cancers were achieved in eight dogs bearing spontaneous tumors. Tumor types included two adenocarcinomas, 1 hemangiopericytoma, 1 hemangiosarcoma, 2 testicular carcinomas, 1 lung tumor, and 1 squamous cell carcinoma. Treatments were repeated bimonthly. Animals under treatment were not restrained or anesthetized and appeared to suffer no ill effects or discomfort during the period of treatment or afterward. Care was exercised to avoid precipitating anaphylactic shock by slow administration of virus and close monitoring of heartbeat and respiration of dogs during and for several hours after treatment.

In some animals, there was a reduction of tumor size, and in at least one case elimination of any visible signs of tumor. In two cases, residual tumors were removed surgically. However, tumors eventually recurred and proved recalcitrant to treatment after relapse. Each of the animals eventually succumbed to their original tumors.

Although this experimental therapy appears less toxic than other conventional chemotherapies and augments rather than depletes the natural immune defenses of the host, it does not at this time offer any therapeutic advantage over conventional treatments now in use in terms of long-term prognosis, and no additional trials are planned.

Safety tests of retroviruses. The retroviruses utilized in the foregoing studies were safety-tested in two adult chimpanzees, as follows: 5 ml of each of the following retroviruses were inoculated intravenously in consecutive order, M7 (baboon), RD114, C57L, M55 and 1504A. The chimpanzees, which had been lightly tranquilized, demonstrated no stress or untoward reactions during or after treatment, nor have they evidenced any illnesses or symptoms (18 months after treatment).

Tests for retrovirus "shedding" in treated dogs. Saliva, blood, urine and feces were collected from each of three dogs pretreatment and 3 days after treatment with M7 (1 dog) and M55 (2 dogs). Specimens were tested for focus formation in the S+L- mink system. Readings were taken through day 14. All of the mink cells were passed on day 14, including positive controls, and grown for 14 days. The fluid harvest was checked for reverse transcriptase, and cell packs were prepared for complement fixation tests. Results were negative in all tests.

2. Skin transformation assay for cancer susceptibility. Earlier results, in which the Ki-MSV focus-forming assay successfully identified 80% of individuals at high risk for cancer in one test, and 100% in another, were confirmed in a subsequent trial at the University of Southern California (Drs. M. Gardner and S. Rasheed) of 40 specimens from a Gardner's syndrome family provided (blindly) by Dr. Eldon Gardner* to three laboratories. However, studies in our laboratory were hampered by the failure of the initial cultures provided to grow. Duplicate cultures were provided and are now on test. In addition, tests of fibroblasts derived from heavy smokers with and without lung cancers (acquired and tested by another collaborator, Dr. J. Frankel), demonstrated an 80% accuracy in identifying lung cancer patients. Duplicate specimens are now being run in our laboratory as well. However, in all tests, 7-9% of smokers without lung cancer and normal controls were positive as well. Another puzzling result was the finding that two people with Gardner's syndrome who had undergone total colectomies were consistently negative in the skin transformation test, whereas other members of the same family known to be positive for the Gardner's syndrome trait but as yet cancer-free were strongly positive.

These unexplained negatives and positives raise important questions which would have to be resolved prior to considering the skin transformation assay for diagnostic purposes. Although the degree of validity in cancer patients and individuals known to be at high genetic risk for cancer is significant, the basis of such sensitivity remains to be determined. Enhanced transformation of cells derived from susceptible populations may reflect an alteration of receptor or physiological states not necessarily associated but often coincident with cancer. There is also a possibility that the test measures for an antigen present during the neoplastic process, but which is lost with tumor removal. If the latter proved true, the assay could provide a useful tool for monitoring the clinical status of cancer patients post-treatment for early signs of recurrence. Efforts will be made to acquire skin biopsies from "positive" Gardner's syndrome patients prior to evidence of disease and subsequent to surgical removal of their cancers.

*Dr. E. Gardner is the geneticist who first described the Gardner's syndrome as an autosomal dominant trait predisposing to high cancer incidence.

Significance to Biomedical Research and the Program of the Institute:

1. Retroviral immunotherapy of cancer in the dog did not result in indefinite remissions or cures. An investigation of the feasibility of retroviral immunotherapy combined with surgery, conventional chemotherapy, and perhaps immunological augmentation (i.e., BCG) may involve significant numbers of variables and, thus, the use of a small animal model would greatly augment the ability to perform systematic studies required.

2. If demonstrated to be a valid indicator of cancer risk or incipient cancer, the skin transformation assay could provide an important diagnostic and/or prognostic clinical tool for identifying individuals at genetic risk for cancer and for detection of early cancer. It is designed for use in cancer syndrome families, and people heavily exposed to carcinogens (i.e., industrial workers, smokers), and eventually may identify those populations who would be likely candidates for new vaccines or other cancer prevention and/or control measures.

Proposed Course:

Both projects will be terminated in the LCMB by the end of 1982. The skin transformation assay will continue to be evaluated in smokers and industrial workers by Dr. J. W. Frankel, State of Florida Department of Health, Tampa.

Publications:

Rhim, J.S., Arnstein, P., and Huebner, R.J.: Chemical transformation of cultured skin fibroblasts from humans genetically predisposed to cancer. Cancer Detect. Prev. 4: 239-247, 1981.

Rhim, J.S., and Huebner, R.J.: The chimpanzee as an experimental model in cancer research. In Heberling, R.L., and Kalter, S.S. (Eds.): The Use of Nonhuman Primates in Exotic Viral and Immunologic Diseases. Austin, U. Texas Press, in press.

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 Detect. Prev., in press.

Rhim, J.S., Trimmer, R., Arnstein, P., Koh, K.S., and Huebner, R.J.: Susceptibility of chimpanzee skin fibroblasts to viral transformation. In Yohn, D.S., and Blakeslee, J.R. (Eds.): Comparative Research in Leukemia and Related Diseases. New York, Elsevier/North Holland, 1981, pp. 401-402.

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., in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biology of Natural and Induced Neoplasias

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

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1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) In vivo studies using cloned genetically defined mouse leukemia retroviruses administered to newborn mice of known backgrounds revealed significant differences in behavior of these agents, depending on the immunocompetence and the particular breed of the host. These biological differences include virus replication in the recipient, tumorigenicity, and tumor type induced. The specified virus-host interaction is consistent for each agent, whether it is an intact leukovirus prototype or a laboratory recombinant constructed in LCMB laboratories. Many tumor types induced by the various retroviruses have been deep-frozen for future viral, antigenic and genomic studies. 2) 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. 3) Human and animal xenografts on athymic nude mice have been used to study tumor progression and malignant transformation.

Project Description

Objectives:

1. Determine *in vivo* biological activity (including induction of malignancy) by genetically fully mapped mouse leukemia viruses in their natural hosts (newborn mice). These viruses, genetically defined in Dr. Aaronson's laboratory, will pinpoint genomic loci for specific neoplasias.
2. Study *in vitro* transformation of primate cells using selected oncoviruses and promoters. In particular, seek evidence of malignant behavior (cancer cell-like) in previously benign samples after their exposure to viral and chemical cocarcinogens.
3. Study transplanted human and selected animal xenografts for malignant behavior, tumor-specific antigen production, malignancy-related genes or possible new viruses.

Methods Employed:

1. 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 non-pathogenic 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. Normal primate tissue cultures are characterized by karyology and morphologic characteristics, then exposed to oncoviruses with or without chemical promoters. Subsequently, exposed cultures are passaged in parallel with controls and continuously monitored for preneoplastic or neoplastic transformation by frequent visual checks, periodic specific tests for altered behavior (anchorage-independent growth, loss of contact inhibition), as well as *in vivo* tumorigenicity checks in athymic nude mice.
3. Candidate cultures for xenografting are received from collaborating NCI investigators, from other cooperating units, as well as from studies in (2) above. All cultures are maintained until sufficient cell numbers are accumulated for a meaningful malignancy test, usually employing 4 athymic nude mice, each injected with 10^6 - 10^7 test cells.

Major Findings:

1. Murine non-defective oncogenic viruses produce predictable biologic effects *in vivo*, depending on the genetic makeup of the virus as well as the host.
 - a. Rauscher leukemia virus (RLV) produces lymphomas in about 3/4 of euthymic (normal thymus and T-cell function) mice. The majority of the lymphomas are of the B-cell type in all strains tested (129J, NFR inbred, NIH

Swiss outbred). The non-lymphomatous leukemias, comprising about 1/4 of the RLV malignancies, are either erythroleukemia, granulocytic leukemia, or occasionally undifferentiated primitive leukemia. In the athymic nude (rudimentary thymus, absence of mature T-lymphocytes), the proportion of erythroleukemias is increased to about 1/2 of the RLV tumors induced; the other half are lymphomas consisting of immature B cells.

b. Moloney leukemia virus (MoLV) almost always produces lymphomas. In the euthymic mouse the tumor cell type is almost always a malignant T-lymphocyte; in the athymic mouse the histopathology is the same, but 3/4 of the tumors are of the "null" cell type and 1/4 are immature B cells.

c. Most recombinants between cloned Rauscher leukemia and endogenous xenotropic type C virus are less oncogenic than "straight" Rauscher, and the tumorigenicity seems dependent on specific portions of the Rauscher genome in the hybrid. Many recombinants are completely non-tumorigenic in euthymic mice, but leukemogenic in athymic nude siblings within the same litter, suggesting that cell-mediated immunity may significantly contribute to defense against murine leukemia. It is also suggested that the xenotropic gene products may be more antigenic for T-cell response than are the classical Rauscher MuLV products.

d. Grafting of original tumors to syngeneic new hosts has proven a practical method of increasing the amount of tumor tissue available for characterization and the time during which it can be kept viable. (The leukemia cells induced by the MuLVs are very difficult to establish in culture.) Many important tumor types representing specific viral and host genomes have been thus amplified, preserved in DMSO and deep-frozen for future detailed analysis.

2. Transformation studies using normal human and chimpanzee cultures were continued, using DNA oncovirus (Ad12/SV40), as well as transforming RNA sarcoma (baboon-Kirsten) as carcinogens. As in the previous year, we successfully induced permanent morphologic changes in the primate cell cultures, best described as pre-neoplastic. None of these experiments yielded fully malignant transformation, even after use of chemical promoters such as TPA and MNG. This is further evidence of the relatively high resistance of human and chimpanzee to viral and chemical cocarcinogenesis.

3. Xenotransplantation to athymic nude mice continues to be employed as a gauge of malignant status in selected human and animal cultures and tissue specimens. Some of the test materials are from LCMB laboratories, others are tested as a service to colleagues in other laboratories conducting NCI research.

Significance to Biomedical Research and the Program of the Institute:

1. 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. These experiments may later help find loci of susceptibility in human tissues; candidate human cancer viruses, if and when identified, will also be more efficiently tested through data collected in the mouse experiments.

2. 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 with special emphasis on part 1, the in vivo studies of genetically characterized retroviruses.

Publications:

None

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Viruses in Experimental Oncogenesis and Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER

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4.0

PROFESSIONAL:

1.0

OTHER:

3.0

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(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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) the biology of endogenous retroviruses; (2) the molecular biology of retrovirus replication and transformation; and (3) the application of knowledge gained from these studies to the search for the causes and mechanisms involved in human neoplastic transformation.

Project DescriptionObjectives:

1. To study the mechanisms of action of RNA tumor viruses.
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 biochemistry.

Major Findings:

1. The complete nucleotide sequence of a mammalian transforming retrovirus, Moloney murine sarcoma virus, was determined. MSV, a recombinant virus derived of helper viral and cellular sequences, possesses termini resembling prokaryotic transposable elements. The viral genome has the coding capacity for the Moloney murine leukemia virus gag gene product and contains large deletions in pol and env genes. A large open reading frame encompassing its cell-derived sequences codes for its putative transforming protein. The nature of some of the important domains in the viral genome, including those within the long terminal repeat (LTR) and helper viral gag gene, were established.
2. The integrated Abelson-murine leukemia virus (A-MuLV) genome cloned in bacteriophage λ gtWES- λ B was used to localize viral genetic sequences required for transformation. Comparison of the biological activity of cloned A-MuLV genomic and subgenomic fragments revealed that subgenomic clones which lacked the 5' LTR and adjoining sequences (300 bp downstream of LTR) were not biologically active. In contrast, subgenomic clones, which lacked the 3' LTR and as much as 1.3 kbp of the A-MuLV cell-derived abl gene, were as efficient as wild-type virus DNA in transformation. The A-MuLV encoded polyprotein P120 and its associated protein kinase activity were detected in the 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.
3. BALB/c mouse sarcoma virus (BALB-MSV) is a spontaneously occurring transforming retrovirus of mouse origin. The integrated form of the viral genome was cloned from the DNA of a BALB-MSV-transformed nonproducer NRK cell line in the Charon 9 strain of bacteriophage λ . In transfection assays, the 19 kilobase pair (kbp) recombinant DNA clone transformed NIH/3T3 mouse cells with an efficiency of 3×10^4 focus-forming units

per pmol. Such transformants possessed typical BALB-MSV morphology and released BALB-MSV after helper virus superinfection. A 6.8 kbp DNA segment within the 19 kbp DNA possessed restriction enzyme sites identical to those of the linear BALB-MSV genome. Long terminal repeats of approximately 0.6 kbp were localized at either end of the viral genome by the presence of a repeated constellation of restriction sites and by hybridization of segments containing these sites with nick-translated Moloney murine leukemia virus long terminal repeat DNA. A continuous segment of at least 0.6 and no more than 0.9 kbp of helper virus-unrelated sequences was localized toward the 3' end of the viral genome in relation to viral RNA. A probe composed of these sequences detected six Eco RI-generated DNA bands in normal mouse cell DNA as well as a smaller number of bands in rat and human DNAs. These studies demonstrate that BALB-MSV, like previously characterized avian and mammalian transforming retroviruses, arose by recombination of a type C helper virus with a well-conserved cellular gene.

4. The nature of the cell-derived (*bas*) sequences of BALB-MuSV, a spontaneous mouse sarcoma virus isolate, was determined. Molecularly cloned *bas* sequences demonstrated no detectable homology with the *onc* genes of other mouse transforming viruses, but exhibited a high degree of sequence homology with the *ras* gene of the rat-derived Harvey murine sarcoma virus (Ha-MuSV) genome. The Ha-MuSV cell-derived sequence (*ras*) shared a colinear 750 bp region of homology with *bas*. Moreover, BALB-MuSV transformation was associated with the expression of high levels of a 21,000 dalton protein, immunologically related to the *ras* gene product, p21. Thus *bas* and *ras* represent retroviral transforming gene homologs that were independently transduced by mouse type C viruses from the genomes of different species.
5. BALB and Harvey murine sarcoma viruses (MSVs) induce sarcomas and erythro-leukemias in susceptible animals. An *in vitro* colony assay that detects transformation of lymphoid cells by Abelson-murine leukemia virus was utilized 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 which 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, 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 Abelson-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.

6. We sought to identify the species of origin of the cell-derived (sis) sequences of simian sarcoma virus. A molecular clone comprised of sis DNA selected related nucleotide sequences at low copy numbers 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.
7. The sequence of the transforming region of simian sarcoma virus (SSV) was determined by using molecularly cloned viral DNA. This region encompassed the 1.0 kbp 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 polyadenylational sites were identified downstream within v-sis and helper viral sequences. The recombinational event that led to the generation of SSV occurred in the middle of two functional codons, indicating that SSAV provided their regulatory elements for transcription as well as the initiation codon for translation of SSV cell-derived transforming sequences.
8. Nucleotide sequence analysis of the long terminal repeat (LTR) of the integrated simian sarcoma virus showed that the simian sarcoma virus LTR comprised 504 nucleotides with an inverted repeat of seven bases at its 5' and 3' termini. At the site of simian sarcoma virus integration, cellular flanking sequences adjacent to the proviral LTR contained a direct repeat of four bases. A 13-base sequence after the 5' LTR was found to be complementary to prolyl tRNA, suggesting that tRNA^{Pro} may serve as the primer for reverse transcription of simian sarcoma virus RNA. The U₅ and R regions, derived respectively from the 5' end and terminally redundant sequences of the viral RNA, were found to have similar organization and sequence homology close to that of Moloney murine sarcoma virus or Moloney murine leukemia virus. These results indicate that regions within LTRs with known functionally important sequences have been most well-conserved during retrovirus evolution.
9. A new strain of feline sarcoma virus (GR-FeSV) was isolated from a spontaneous sarcoma of an 8-year old domestic house cat. The virus induced sarcomas at high incidence after a short latent period in fetal

and newborn kittens and transformed cat embryo fibroblasts in vitro after 5 days. Compared to Gardner-Arnstein and the Snyder-Theilen strains of FeSV, GR-FeSV induced more pleomorphic sarcomas and larger, more rounded and discrete foci of cell transformation. GR-FeSV was shown to be defective for replication, and nonproducer transformed clones from several species were obtained at limiting GR-FeSV dilution. The defective sarcoma virus could be rescued from such transformants by superinfection with replication competent type C viruses. The primary translational product of the GR-FeSV genome is a 70,000 dalton polyprotein that contains the amino-terminal domain of the FeLV gag gene precursor protein and a sarcoma virus-specific polypeptide. These results differentiate GR-FeSV from previously isolated FeSV strains, and establish it as an independent spontaneously occurring sarcoma virus isolate of the domestic cat.

10. Recombinant viruses were generated in tissue culture between Rauscher murine leukemia virus (MuLV) temperature-sensitive (ts) mutants restricted at different steps in virus replication and a mouse endogenous xenotropic virus, BALB:virus-2. Mutants used included ts 28, a late mutant which releases noninfectious viruses at 39°C, and ts 29, a double mutant with a ts lesion in its reverse transcriptase and a late block affecting virus budding. Immunological typing of the translational products of clonal recombinant viruses made it possible to establish their partial genetic maps and localize regions of the viral genome affected by different ts lesions. Recombinants involving Rauscher-MuLV ts 28 invariably contained BALB:virus-2 p15, p12, and p30 proteins, localizing the late defect in replication by this mutant to the 5' moiety of the viral gag gene. All ts 29-derived recombinants contained the entire BALB:virus-2 gag and pol genes. Substitution of the pol gene is in agreement with the reported thermolability of Rauscher-MuLV ts 29 reverse transcriptase (Tronick et al., J. Virol. 16: 1476-1482, 1975). Substitution of the gag gene suggests that internal structural proteins are actively involved in the virus budding processing. Rauscher-MuLV recombinants were used to establish the genetic map of the Rauscher-MuLV genome by T₁ oligonucleotide fingerprinting analysis. Detection of Rauscher MuLV T₁ oligonucleotides in representative recombinant viruses, whose protein phenotypes were established by immunological techniques, permitted their assignment to specific regions of the viral genome. The genetic map of Rauscher-MuLV generated in these studies should be useful for identifying and characterizing the viral gene(s) involved in leukemogenesis.
11. We have studied the phenotype of tumors and tumor-derived cell lines induced by clonal strains of Rauscher- and Moloney-MuLV. Moloney-MuLV-induced tumors and lymphoma cell lines generally exhibited Thy.1 antigen in the absence of detectable Fc or C3 receptors, indicating their T-cell origin. In contrast, Rauscher-MuLV primary tumors and lymphoma cell lines in a variety of mouse strains invariably exhibited Fc receptors in the absence of Thy.1 antigen. These findings argue that the target specificity for transformation must be genetically determined by the virus. The majority of Rauscher-MuLV lymphoma lines expressed immunoglobulin heavy (μ) chain in the absence of detectable light (χ or λ)

chains as determined both by biosynthetic and radioimmunologic techniques. However, infrequent tumors were comprised of either immature or more differentiated cells within the B cell series. Thus, Rauscher-MuLV lymphomas reflect different stages of B cell differentiation. To investigate the basis for the target cell specificity for transformation by Moloney- and Rauscher-MuLV, we analyzed the time course of infection of target organs in the animal. The results indicated that while spleen cells were comparably susceptible to infection by both viruses, thymus cells were relatively resistant to Rauscher-MuLV infection. This resistance could not, however, be accounted for by lack of ability of Rauscher-MuLV gp70 to bind to thymus cells. The availability of a model system establishing different target cells for transformation by clonal leukemia viruses should be of continuing help in analyzing how these viruses induce malignancy.

12. The major core protein (p28) of MMC-1, an endogenous type C virus of the rhesus monkey (Macaca mulatta), was purified and subjected to structural and immunological analyses. The NH₂-terminal amino acid sequence of MC-1 p28 showed extensive homology to the sequences of the major structural protein (p30) of known mammalian type C viruses. Similarly, interspecies antigenic determinants shared by all the above viral proteins were detected in MC-1 p28. Competition radioimmunoassays, together with the results of statistical analysis of the primary structure data, provided evidence that MMC-1 p28 is not more closely related to primate type C viruses of the Papio genus than to those isolated from rodents, cats, or New World monkeys. MMC-1 p28 was found to be closely related structurally to the p30 protein of the avian reticuloendotheliosis virus (REV-A), a horizontally transmitted type C virus of putative mammalian origin. In addition, MMC-1 p28 and REV-A p30 shared a specific subset of antigenic determinants not present in any of the other avian or mammalian type C viruses studied. These findings suggest that MMC-1 and REV may have a common evolutionary origin.
13. Human DNA was analyzed for the presence of sequences homologous to the transforming gene (v-mos) of Moloney murine sarcoma virus (MSV). A single 2.5 kbp Eco RI-generated fragment of human DNA was identified 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 varying numbers of human chromosomes, as well as in subclones of such hybrids, it was possible to assign c-mos(human) to human chromosome 8.
14. The primate cell-derived transforming gene (v-sis) of simian sarcoma virus (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.

15. Polyadenylated RNAs of certain human tumor cell lines were shown to contain transcripts related to the cell-derived transforming onc genes of molecularly cloned primate, murine or avian transforming retrovirus genomes. Markedly elevated levels of single transcripts related to either SSV or MC29 onc genes were present in certain human tumor cells developed from solid tumors. These findings are consistent with transcriptional derepression of these genes in certain tumors. More complicated patterns of transcripts related to BALB-MSV and Abelson-MuLV onc genes were detected in human normal and neoplastic cells. These findings raise additional questions concerning the mode of processing of such transcripts, and their source from one or more related genes. Nonetheless, the almost universal presence of these transcripts in human cells argues strongly that these viral onc-related genes possess some important function(s).
16. Total cellular poly (A)-enriched RNA from a variety of fresh human leukemic blood cells and hematopoietic cell lines was analyzed for homology with molecularly cloned DNA probes containing the onc sequence of Abelson murine leukemia virus (Ab-MuLV), Harvey murine sarcoma virus (Ha-MuSV), simian sarcoma virus (SSV), and avian myelocytomatosis virus strain MC29. Results with the fresh blood cells paralleled those obtained with the cell lines. With Ab-MuLV and Ha-MuSV, multiple RNA bands were visualized in all cell types examined without significant variation in the relative intensities of the bands. When SSV was used as the probe, expression of related onc sequences was absent in all of the hematopoietic cell types examined except for one neoplastic T-cell line (HUT 102), which produces the human T-cell leukemia (lymphoma) retrovirus HTLV. In this cell line, a single band (4.2 kilobases) was observed. With MC29 as the probe, a single band of 2.7 kilobases was visualized in all cell types examined with only a 1- to 2-fold variation in intensity of hybridization. An exception was the promyelocytic cell line, HL60, which expressed approximately 10-fold more MC29-related onc sequences. With induction of differentiation of HL60 with either dimethyl sulfoxide or retinoic acid, a marked diminution in the amount of the MC29-related, but not the Ab-MuLV-related, onc message was observed.
17. Total cellular RNAs from a variety of fresh and culture-derived human hematopoietic neoplastic cell types at various stages of differentiation and human sarcoma, carcinoma, melanoma, and glioblastoma cell lines were enriched for poly (A)-containing sequences, fractionated by gel electrophoresis, and blot hybridized to a cloned DNA probe containing the transforming sequences (v-amv) of avian myeloblastosis virus (AMV), a virus known to cause myeloid leukemias in chickens. Expression of RNA sequences homologous to AMV was detected in all immature myeloid and lymphoid T cells in addition to the single erythroid cell lines examined, but not in mature T cells or in B cells, including lymphoblast cell lines derived from patients with Burkitt's lymphoma. In addition, induction of

the cell line, HL60, a promyelocytic leukemia line, to differentiate with dimethyl sulfoxide or retinoic acid resulted in a reduction of the level of expression of the human cellular gene c-amv homologous to v-amv. There was no detectable c-amv mRNA in any of the solid tumor cell lines examined. Thus, expression of the human c-amv gene could be correlated with the stage of differentiation of different hematopoietic cell types determined by morphologic and marker studies. Expression of c-amv could not be correlated with the extent of methylation in HL60 and HL60 induced to differentiate with dimethyl sulfoxide.

18. A transforming gene isolated from T24 human bladder carcinoma cells was found to be highly related to the BALB murine sarcoma virus (MSV) onc gene (v-bas). By restriction mapping and heteroduplex analysis, these sequences were localized to a 3.0 kbp region required for transformation by the T24 oncogene. This transforming gene was expressed as a 1.2 kbp polyadenylated transcript, which contained 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. Our demonstration that a molecular clone of the normal human homologue of v-bas is indistinguishable by restriction enzyme analysis from the T24 oncogene implies that rather subtle genetic alterations have led to its activation as a human transforming gene.

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 viral 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 viruses in human neoplasia; and 4) application of basic research advances to the investigation of mechanisms involved in malignant transformation of human cells.

Publications:

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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 gene analogues in human solid tumor cells. In Yohn, D.S. and BlakesTee, J.R. (Eds.): Advances in Comparative Leukemia Research 1981. New York/Amsterdam, Elsevier Biomedical, 1982, pp. 381-382.

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.

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Oroszlan, S., Barbacid, M., Copeland, T.D., and Aaronson, S.A.: Chemical and immunological characterization of the major structural protein (p28) of MMC-1, a rhesus monkey endogenous type C virus. J. Virol. 39: 845-854, 1981.

Pierce, J., and Aaronson, S.A.: BALB- and Harvey-MSV transformation of a novel lymphoid progenitor cell. J. Exp. Med., in press.

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Srinivasan, A., Reddy, E.P., and Aaronson, S.A.: Abelson murine leukemia virus: molecular cloning of infectious integrated proviral DNA. Proc. Natl. Acad. Sci. USA 78: 2077-2081, 1981.

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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, in press.

Westin, E.H., Gallo, R.C., Arya, S.K., Eva, A., Souza, L.M., Baluda, M.A., Aaronson, S.A., and Wong-Staal, F.: Differential expression of the amv gene in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79: 2194-2198, 1982.

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PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemical Characterization of Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	P. Reddy	Visiting Scientist	LCMB	NCI
	A. Eva-Varesio	Visiting Associate	LCMB	NCI
	K. Robbins	Expert	LCMB	NCI
	I.-M. Chiu	Visiting Fellow	LCMB	NCI
	R. Callahan	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

Dr. R. Gallo, LTCB, NCI; Dr. T. Papas, LMO, NCI;
Dr. M. Baluda, Univ. California, Los Angeles

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Molecular Biology Section

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TOTAL MANYEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to biochemically characterize retroviruses in order to understand the mechanisms by which these viruses induce cancers in their natural hosts. The role of these viruses 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; and 3) search for the presence of retroviral genes and gene products in human tumors.

Project DescriptionObjectives:

1. To biochemically characterize mammalian transforming retroviruses.
2. To biochemically characterize new isolates of retroviruses.
3. To determine the mechanisms of oncogenesis by retroviruses in their natural hosts.
4. To determine whether or not retroviruses play a role in the etiology of cancers of higher primates, including humans.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; molecular hybridization techniques using single-strand specific nuclease and hydroxyapatite chromatography 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:

In studies carried out collaboratively with LCMB investigators, human DNA sequence homologues of the onc and helper virus-related genes of retroviruses were molecularly cloned and characterized. The human cell analogue [c-mos(human)] of the onc gene of Moloney-murine sarcoma virus (MSV) was found to share a 650 bp region of continuous homology with its 1.1 kbp viral counterpart (v-mos). The homologous region was localized towards the 5' portion of v-mos. By analysis of human-rodent cell hybrids, the c-mos(human) sequences were localized to human chromosome 8, which has been shown by others to be involved in translocations observed in B-cell malignancies.

Contrasting these results were the findings that human sequences [c-amv(human)] related to the transforming gene of avian myeloblastosis virus were interrupted by a stretch of 1500 bp. The human sequences were found to represent 500-600 bp of the viral sequence (v-amv) and were localized to the 3' region of v-amv and encompassed most of the v-amv open reading frame. Human chromosome 6 was found to contain the amv-related sequences.

Three distinct human DNA segments containing MC29-related sequences were isolated and each were represented as multiple overlapping clones. Characterization of these clones is in progress.

The transforming gene (v-bas) of BALB-MSV was also used to probe human DNA. A single clone containing 19 kbp and 2 kbp Eco RI inserts was isolated. The sequences were localized to a 3.0 kbp Sst I restriction fragment by Southern blot hybridization and was shown to be interrupted by three non-bas-related sequences by heteroduplex analysis. At least 90% of the v-bas sequence was found to be represented in the human DNA clone. The c-bas(human) clone was shown to be an allele of the T24 human bladder carcinoma gene molecularly cloned by M. Barbacid and colleagues in our laboratory. The v-bas-related sequences in T24 were localized to the region required for its transforming activity. However, c-bas(human) lacked detectable transforming activity.

A human DNA clone was isolated that was found to represent the 3' 800 bp of the AMV reverse transcriptase (pol) gene. Homology to other regions of the AMV or MAV genome within the 16 kbp region of human DNA containing pol sequences could not be detected. The pol-related human DNA sequences were localized to a 1.4 kbp restriction fragment which also contains Alu-family sequences. Alu-sequences were found to flank the 1.4 kbp pol-related fragment, providing evidence that this segment of human DNA probably does not represent an endogenous retrovirus. Nucleotide sequence comparison of AMV-pol and the human DNA clone is presently underway to determine the significance of the latter.

Phage clones containing human DNA sequences related to mouse mammary tumor virus (M-MTV) were isolated in collaboration with R. Callahan. Characterization of some of these clones is now underway.

Onc probes from various transforming retroviruses were used in attempts to detect onc gene expression in human tumor cells. A variety of onc genes were found to be expressed in both normal and tumor cell lines and tissues. The results suggest that onc genes play a role in normal cell functions in addition to being present in high level in certain tumors.

Significances to Biomedical Research and the Program of the Institute:

Highly sensitive and specific biochemical probes for retroviral gene products generated in our studies have been extremely valuable in order to demonstrate the possible etiologic involvement of these viruses in human cancers and to study the role that retroviral gene products may play in normal cellular functions. The availability of molecularly cloned human gene fragments related to viral onc genes will greatly facilitate 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 is now underway.

Recombinant DNA techniques are being applied and further developed to study other mammalian transforming viruses and cellular genes involved in transformation.

Publications:

Andersen, P.R., Devare, S.G., Tronick, S.R., Ellis, R.W., Aaronson, S.A., and Scolnick, E.M.: Generation of BALB-MuSV and Ha-MuSV by type C virus transduction of homologous transforming genes from different species. Cell 26: 129-134, 1981.

Andersen, P.R., Tronick, S.R., and Aaronson, S.A.: Molecular cloning and structural organization of BALB/c mouse sarcoma virus (BALB-MSV). J. Virology 40: 431-439, 1981.

Devare, S.G., Reddy, E.P., Robbins, K.C., Andersen, P.R., Tronick, S.R., and Aaronson, S.A.: Nucleotide sequence of the transforming gene of simian sarcoma virus. Proc. Natl. Acad. Sci. USA 79: 3179-3182, 1982.

Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.R., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C. and Aaronson, S.A.: Cellular genes analogous to retroviral onc genes are transcribed in human tumor cells. Nature 295: 116-119, 1982.

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, in press.

Santos, E., Tronick, S., Aaronson, S., 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, in press.

Westin, E.H., Wong-Staal, F., Gelmann, E.P., Dalla Favera, R., Papas, T.S., Lautenberger, J.A., Eva, A., Reddy, E.P., Tronick, S.R., Aaronson, S.A., and Gallo, R.C.: Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79: 2490-2494, 1982.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Transforming Genes from Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Barbacid	Visiting Scientist	LCMB NCI
OTHERS:	S. Pulciani	Visiting Fellow	LCMB NCI
	E. Santos	Visiting Fellow	LCMB NCI
	V. Notario	Visiting Associate	LCMB NCI
	K.C. Robbins	Expert	LCMB NCI
	S.A. Aaronson	Chief	LCMB NCI
	S. Tronick	Microbiologist	LCMB NCI
	P. Reddy	Visiting Scientist	LCMB NCI

COOPERATING UNITS (if any)

R. Huebner, LCMB, NCI; J. Rhim, LCMB, NCI; U. Rapp, LVC, NCI; S. Rasheed, USC

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SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goals of this project are: (1) to identify transforming genes in human tumor cells by DNA-mediated gene transfer techniques; (2) to isolate these human oncogenes by molecular cloning techniques; (3) to isolate the normal homologues of these human oncogenes; (4) to biochemically compare the transforming genes and their respective normal homologues to establish the molecular changes that lead to oncogenesis; (5) to characterize the transcriptional and translational products of these human oncogenes to understand the mechanisms by which they induce malignant transformation.

Project DescriptionObjectives:

1. To test DNAs isolated from a variety of human tumor cell lines and from naturally occurring tumors for transformation of NIH/3T3 mouse cells.
2. To determine whether cells other than NIH/3T3 are also susceptible to transformation by human tumor DNAs.
3. To molecularly clone human oncogenes in their biologically active form.
4. To determine the oncogenic properties of these genes by testing their ability to transform a variety of normal cells including those of human origin.
5. To compare these genes with their normal homologues at the molecular level to identify the genetic changes involved in oncogenesis.
6. To characterize the transcriptional and translational products of the molecularly cloned human oncogenes.
7. To study the relationship of human oncogenes with other well-characterized transforming genes of nonhuman origin.
8. To investigate whether these human oncogenes are either responsible for or etiologically involved in certain forms of human neoplasia.

Methods Employed:

The development of gene transfer and genetic engineering technologies has permitted the designing of experimental protocols aimed at detecting human tumor cells possessing dominant transforming genes. High molecular weight DNAs were isolated from a variety of human tumor cell lines, as well as from tumored tissues. These DNAs were transfected into normal NIH/3T3 mouse fibroblasts by the calcium phosphate precipitation technique. After two weeks of incubation, discrete areas of transformed cells could be detected. The presence of human DNA sequences in these NIH/3T3 transformants was detected by submitting their DNAs to Southern blot analysis utilizing as a probe the Alu family of highly repetitive human sequences.

Human DNA sequences that specifically co-segregated with the transformed phenotype after several cycles of transfection were cleaved with Eco RI, purified by sucrose gradient centrifugation and ligated to Eco RI-cleaved λ Charon 9 phage DNA at 1:1 molar ratio. The ligation mixture was then packaged in vitro into phage particles and recombinant phages carrying human sequences were identified by the method of Benton and Davis using the Alu family of human repetitive sequences as a probe.

Major Findings:

1. High molecular weight DNAs isolated from cell lines established from carcinomas and sarcomas of various organs as well as from a glioblastoma and two melanomas were utilized to transfect NIH/3T3 mouse fibroblasts. The DNAs of T24 and A2182, two cell lines derived from a bladder and a lung carcinoma, respectively, and of HT-1080, a cell line established from a fibrosarcoma, were able to transform recipient NIH/3T3 cells. First-cycle transformants exhibited anchorage-independent growth and were tumorigenic in athymic and immunocompetent mice. Moreover, they contained human DNA sequences and were able to transmit their malignant phenotype in additional cycles of transfection. Southern blot analysis of T24-derived transformants showed that a single fragment of human DNA specifically cosegregated with the malignant phenotype, suggesting that it contained the T24 oncogene. Therefore, these human sequences were molecularly cloned with λ Charon 9A as the cloning vector. The resulting recombinant DNA molecule, designated λ T24-15A, was shown to contain a 15-kilobase pair Eco RI insert of human cellular DNA. λ T24-15A DNA (either intact or Eco RI digested) transformed NIH/3T3 fibroblasts with a specific activity of 20,000 focus-forming units per pmol of cloned DNA. These results indicate that we have molecularly cloned a biologically active oncogene present in T24 human bladder carcinoma cells.
2. A transforming gene isolated from T24 human bladder carcinoma cells was found to be highly related to onc gene (v-bas) of BALB murine sarcoma virus (MSV). By restriction mapping and heteroduplex analysis, these sequences were localized to a 3.0 kbp region required for transformation by the T24 oncogene. This transforming gene was expressed as a 1.2 kbp polyadenylated transcript which contained 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. Our demonstration that a molecular clone of the normal human homologue of v-bas is indistinguishable by restriction enzyme analysis from the T24 onc gene implies that rather subtle genetic alterations have led to its activation as a human transforming gene.

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 by molecular cloning techniques will allow us to identify and characterize their transcriptional and translational products as well as also to establish the molecular events that led to the acquisition of their malignant properties.

Proposed Course:

1. To continue our efforts to detect transforming genes in human tumors in which they have not yet been found.
2. To isolate by molecular cloning techniques human oncogenes present in a variety of human tumors including carcinomas of the lung,

gall bladder, colon and pancreas, as well as from a fibrosarcoma and rhabdomyosarcoma.

3. To establish what genetic changes led to the activation of the T24 oncogene.
4. To investigate the role that these oncogenes play in human neoplasias.

Publications:

Barbacid, M., Donner, L., Ruscetti, S.K., and Sherr, C.J.: Transformation defective mutants of Snyder-Theilen feline sarcoma virus lack tyrosine-specific protein kinase activity. J. Virol. 39: 246-254, 1981.

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Pulciani, S., Santos, E., Lauer, A.V., Long, L.K., and Barbacid, M.: Transforming genes in human tumors. J. Cell. Biochem., in press.

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Rasheed, S., Barbacid, M., Aaronson, S.A., and Gardner, M.B.: Origin and biological properties of a new feline sarcoma virus. Virology 117: 238-244, 1982.

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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, in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Transforming Genes of Mammalian Retroviruses and their Cellular Analogues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	S.R. Tronick	Microbiologist	LCMB	NCI
	E.P. Reddy	Visiting Scientist	LCMB	NCI
	A. Srinivasan	Visiting Associate	LCMB	NCI
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	K. Prakash	Visiting Fellow	LCMB	NCI
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COOPERATING UNITS (if any)

NONE

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Simian sarcoma virus (SSV) is the only known transforming retrovirus of primate origin. The nucleotide sequence analysis of the cloned transforming gene of SSV revealed a 675 nucleotide long open reading frame with a coding capacity for a protein of around 27,000 molecular weight, commencing 19 bases in the helper viral sequences. Thus the helper virus provided the regulatory elements for the expression of transforming gene. Based on data obtained from sequence analysis, antibodies were developed against a synthetic peptide of the predicted SSV transforming protein. These antibodies specifically precipitated a protein of about 28,000 MW in cells transformed by SSV, indicating it to be the product of the SSV transforming gene. The nucleotide sequence of the long terminal repeat (LTR) of SSV revealed functional regulatory elements like promoter, mRNA capping site and polyadenylation signal. A comparison of the SSV LTR sequence with LTRs of other type C retroviruses demonstrated that functionally important domains of LTRs have been conserved during evolution.

Project Description

Objectives:

(1) To study genomic organization of replication-defective mammalian retroviruses in order to evaluate the role of their acquired oncogenic sequences in malignant transformation; (2) identification of regulatory signals in transforming retroviral genomes to evaluate molecular mechanisms of oncogenesis; and (3) isolation and characterization of cellular analogues of retroviral transforming genes to define their involvement in transformation of normal cells.

Methods Employed:

Primary nucleotide sequence analysis of molecularly cloned retroviral transforming genes to identify regulatory elements involved in their expression; heteroduplex analysis for comparison and characterization of cellular homologues of retroviral transforming genes; and immunologic techniques for detection of retrovirus-encoded translational products.

Major Findings:

1. Nucleotide sequence of the transforming gene of simian sarcoma virus. Studies to date have indicated that the cell-derived sequences of transforming retroviruses are responsible for their transforming function(s). In our attempt to better understand the structural organization and possible molecular mechanisms involved in transformation by simian sarcoma virus (SSV), the primary sequence of the transforming region of the molecularly cloned SSV DNA was determined. This region encompassed the 1.0 kilobase pair (kbp) 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. These results demonstrate that helper viral sequences provide regulatory elements for the expression of the SSV-transforming gene v-sis.

2. Translational products of simian sarcoma virus transforming gene. One of the indispensable factors in studies to elucidate molecular mechanisms of transformation by a virus is identification of the translational product(s) encoded by its transforming gene. Use of standard immunological methods was unsuccessful in identifying SSV transforming proteins. The nucleotide sequence analysis of the SSV transforming gene provided an approach for identifying its product. A synthetic peptide from predicted transforming gene product based on the nucleotide sequence was used to elicit antibodies after conjugating it to thyroglobulin. These antibodies detected a protein around 28,000 molecular weight (p28) in normal rat kidney and marmoset cells transformed by SSV. The absence of an analogous protein in uninfected cells demonstrated the specificity of immunoprecipitation. The transforming protein predicted by nucleotide

sequence analysis would have an apparent molecular weight of about 27,000. Thus p28 seems to be a suitable candidate for the SSV transforming protein. It should now be possible to study the functional properties of this transforming protein in the process of oncogenesis by SSV.

3. Nucleotide sequence analysis of the long terminal repeat of simian sarcoma virus. An important structural feature of the retroviral genome is the appearance of two long terminal repeats (LTRs) at both 5' and 3' ends of the proviral genome. To date, detailed analysis of the structure of the LTR has been limited to a few avian and mammalian retroviruses. In view of the known evolutionary relationships among mammalian type C retroviruses, as well as the important functional role of the LTR in the virus life cycle, it was of interest to analyze and compare primary sequences of different mammalian type C retroviral LTRs. Nucleotide sequence analysis of the LTR of the integrated SSV showed that SSV LTR was comprised of 504 nucleotides with an inverted repeat of seven bases at its 5' and 3' termini. At the site of SSV integration, cellular flanking sequences adjacent to the proviral LTR contained a direct repeat of four bases. A 13-base sequence after the 5' LTR was found to be complementary to prolyl tRNA, suggesting that prolyl tRNA may serve as the primer for reverse transcription of SSV RNA. The U₃ and R regions, derived respectively from the 5' end and terminal redundant sequences of the viral RNA, were found to have organization and sequence homology close to that of the Moloney murine sarcoma virus or Moloney murine leukemia virus. These results indicate that regions within LTRs with known functionally important sequences have been well conserved during retrovirus evolution.

4. A human cellular analogue of the Moloney murine sarcoma virus transforming gene. The studies to evaluate the role of retroviral transforming genes in normal and malignant cells using the transforming gene-specific probes provide a potentially important approach to assess the role of such sequences in the etiology of human cancer. A 2.5 kilobase pair (kbp) DNA fragment was identified in human placental DNA using the Moloney murine sarcoma virus transforming gene (*v-mos*)-specific probe and molecularly cloned in a λ vector. By heteroduplex and restriction enzyme analysis, it was demonstrated that this 2.5 kbp fragment contained a 0.65 kbp region of continuous homology with *v-mos*. The role of these sequences in transformation or in normal cellular processes is yet to be defined. However, in view of the fact that entire 1.15 kbp *v-mos* sequences are essential for its transforming function, the *c-mos*(human) sequences which lack 3' end sequences may not be biologically active. Thus, *c-mos*(human) may represent a conserved cellular gene which has diverged significantly during evolution with loss of part of the active gene.

Significance to Biomedical Research and the Program of the Institute:

Nucleotide sequence analysis of the transforming gene of SSV revealed an open reading frame of 675 nucleotides commencing 19 bases within the helper virus and terminating in acquired cellular sequences. The recombinational event that led to the generation of SSV occurred in the middle of two functional codons, indicating that the helper virus provided the regulatory elements for transcription as well as the initiation codon for translation of SSV cell-derived transforming sequences. These results are of importance

to our understanding of the structural organization and, thus, the molecular mechanism of transformation by a primate retrovirus. Since the transforming gene sequences are conserved among vertebrates, including humans, these studies provide a potential approach to assess the role of such sequences in the etiology of human cancer.

The identification of the SSV transforming gene product using antibodies made against a synthetic peptide predicted from the nucleotide sequence analysis and availability of specific antiserum against the SSV transforming protein provide important tools for future research to understand the molecular mechanisms of transformation. These studies are of special significance in view of the fact that SSV-specific transcripts have been detected in human fibrosarcomas and osteosarcoma cells.

Proposed Course:

Studies evaluating the molecular organization of mammalian transforming retroviruses and their cellular analogues will be continued. The major emphasis of these investigations will be directed toward understanding the regulatory mechanism(s) which govern expression of transforming gene(s) of retroviral as well as of cellular origin, and functional properties of transforming proteins in the process of oncogenesis.

Publications:

Andersen, P.R., Devare, S.G., Tronick, S.R., Ellis, R.W., Aaronson, S.A., and Scolnick, E.M.: Generation of BALB-MuSV and Harvey-MuSV by type C virus transduction of homologous transforming genes from different species. Cell 26: 129-134, 1981.

Devare, S.G., Reddy, E.P., Law, J.D., and Aaronson, S.A.: Nucleotide sequence analysis of the long terminal repeat (LTR) of integrated simian sarcoma virus: evolutionary relationship with other mammalian retroviral LTRs. J. Virol. 42: 1108-1113, 1982.

Devare, S.G., Reddy, E.P., Robbins, K.C., Andersen, P.R., Tronick, S.R., and Aaronson, S.A.: Nucleotide sequence of the transforming gene of simian sarcoma virus. Proc. Natl. Acad. Sci. USA 79: 3179-3182, 1982.

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, in press.

Robbins, K.C., Devare, S.G., and Aaronson, S.A.: Molecular cloning of integrated simian sarcoma virus: genome organization of infectious DNA clones. Proc. Natl. Acad. Sci. USA 78: 2918-2922, 1981.

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, in press.

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunological Characterization of Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. Dahlberg Microbiologist NCI LCMB

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hybridoma technology has been utilized to produce 34 monoclonal antibodies which immunoprecipitate viriion proteins of the transforming primate herpesvirus, Herpes virus saimiri. This battery of antibodies, which collectively recognize virtually all of the major structural antigens of the virus, offers a unique opportunity to analyse the replication and mode of transformation of this virus. Sensitive competition radioimmunoassays which detect nanogram levels of the major structural proteins of two interesting retroviruses, caprine arthritis-encephalitis virus (CAEV) and equine infectious anemia virus (EIAV) have been developed. To date, it has been possible to measure the degree of immunological relatedness between CAEV and the prototype lentivirus, visna virus. Further characterization of immunological relationship between these viruses and other retroviruses is currently underway.

Project DescriptionObjectives:

1. To utilize hybridoma technology to develop monoclonal antibodies useful in characterizing the expression of viral antigens. To develop monoclonal antibodies to cellular antigens which can be used as genetic markers and/or to define differentiation antigens and antigens associated with neoplastic transformation.
2. To further characterize, primarily by immunological means, retroviruses of the lentivirus group, and their immunological relatedness to each other and to other groups of retroviruses. To analyse the means by which these viruses are naturally transmitted and are involved in the pathogenesis of disease in their natural hosts.

Methods Employed:

Light and electron microscopy, cell culture, virus growth and purification, protein purification, electrophoresis, radioimmunoassays, and other preparative and analytical procedures.

Major Findings:

1. The development of hybridoma technology, and the ability to obtain monoclonal antibodies to antigens of interest, allows an immunological analysis of problems which otherwise would be nearly impossible. One such application of hybridoma technology is currently being conducted in this laboratory.

BALB/c mice were immunized with purified preparations of the oncogenic primate herpesvirus, Herpesvirus saimiri (HVS). Immune spleen cells were fused with the P3 x 63 Ag8 myeloma cell line using conventional procedures, and hybrid cells were selected in HAT medium. Clones of cells were screened for reactivity to HVS by a solid phase immunoassay. Positive clones were grown further, screened against an extract of uninfected cells to eliminate the bulk of false positive clones, and then tested for their ability to immunoprecipitate ³⁵S-methionine labeled extracts of HVS-infected cells. Cultures which produced antibodies which precipitated viral antigens were cloned to ensure stable expression of the antibody and cryopreserved. To date, 34 monoclonal antibodies, all of which are IgG's, as defined by their capacity to be bound by protein A, have been obtained.

At this time, little is known about these antibodies except for their specificity in terms of the viral antigen(s) which they recognize. Approximately 20 different antigens are precipitated by this battery of sera. It was frequently observed that several different antibodies precipitated the same antigen, while in several cases, different antibodies precipitated the same group of antigens, i.e., a monoclonal identified from 2-5 different antigens. Since monoclonal antibodies recognize only a single epitope, the set of antigens recognized by a single antibody must share that epitope. One explanation for this would involve some of the antigens being breakdown products of others. Experiments

to investigate the nature of these complex precipitation patterns are currently underway. Also being initiated are studies to determine if any of these HVS specific antibodies recognize antigens of other herpesviruses. Since HVS can transform T cells of various primate species as well as certain strains of rabbits, the antibodies will be used to investigate whether any viral antigens are expressed in transformed nonproducer cells, since to date, no "T antigen," characteristic of cells transformed by other DNA viruses, has been identified in HVS transformed cells.

2. Caprine arthritis-encephalitis virus (CAEV) is a member of the lentivirus subgroup of the retroviridae. Other members, all of which appear to cause widespread and economically significant animal diseases, include visna virus of sheep and equine infectious anemia virus (EIAV). I have previously shown that CAEV, which is a relatively recent isolate, is very similar to visna virus in terms of morphogenesis, preference for Mg^{++} in a reverse transcriptase assay, and electrophoretic mobility of its major structural proteins. The ability of anti-visna serum to immunoprecipitate CAEV antigens indicated that the two viruses, while not identical, were closely related.

Recently, more precise evidence regarding the relatedness of CAEV to visna has been obtained. When CAEV cDNA is hybridized to visna RNA, the maximum hybridization is 30% compared to a control value of 70% hybridization of CAEV cDNA to CAEV viral RNA. In addition, CAEV p28 has been purified and used to establish a competition radioimmunoassay capable of detecting less than one nanogram of antigen. Visna virus competes very poorly in this immunoassay, while in a heterologous assay established by using anti-visna serum to precipitate CAEV p28, both CAEV and visna compete equally well. These findings verify that CAEV and visna viruses are distinctly different, and that, although both viruses can infect and cause disease in both sheep and goats, they have diverged substantially.

A radioimmunoassay has also been developed recently to the p28 of EIAV. Efforts are currently being concentrated on a search for heterologous sera which would allow the detection of immunological relatedness between CAEV, EIAV, and possibly additional retroviruses. The apparently rapid rate of divergence between visna and CAEV suggests that this search may prove to be difficult. The immunoassays which detect EIAV p28 and CAEV p28 can also prove useful in determining, in a far more sensitive way than previously, the virological status of animals in infected herds, and can be used to investigate modes of transmission of the virus.

Significance to Biomedical Research and the Program of the Institute:

1. A battery of monoclonal antibodies which recognizes most of the proteins of a transforming primate herpesvirus allows an analysis of many aspects of HVS replication and its mechanism of transformation. It is also possible that some of these antibodies may recognize antigens present in other herpes viruses, including those of man. If so, they may be of diagnostic value.

2. Lentiviruses are retroviruses which cause widespread morbidity and mortality in domestic animal populations. The development of sensitive RIAs to detect

viral antigens will allow an investigation of how these viruses are related to each other and to other retroviruses, and will lead to a better understanding of how these viruses are transmitted and participate in the development of diseases which are excellent models for slow virus diseases of man.

Proposed Course:

1. The monoclonal antibodies to Herpesvirus saimiri will be further characterized and will be used to investigate aspects of HVS replication and transformation. Hybridoma technology will then be utilized to investigate other problems which are of interest to this laboratory. Of great importance is the recent identification of human genes involved in the transformation of normal cells to a malignant state. The most critical objective is the identification of the role that these genes play in both normal and transformed cells, and it is likely that antisera will be essential in detecting and evaluating the gene products involved. An effort will be made to develop monoclonal antibodies to these putative gene products.

2. The RIAs developed to detect the p28 proteins of CAEV and EIAV will be the basis for searching for immunological relatedness between these and other retroviruses. The assays will also be used to study the mode of transmission of CAEV particularly, since so little is known. Since antigenic drift has already been documented in these viruses, conventional approaches to control involving vaccines have been frustrating, and it may be that accurate detection and understanding of how the virus is transmitted may prove to be the optimal way of producing virus-free herds. At the same time, these diseases represent excellent models of slow virus diseases of man, and the assays will be used to aid in the analysis of the clinical and pathological progression of disease. Of great interest is the progression of disease in the face of a competent immune system and circulating antibody.

Publications:

Dahlberg, J.E., Gaskin, J.M., and Perk, K.: Morphological and immunological comparison of caprine arthritis encephalitis and ovine progressive pneumonia viruses. J. Virol. 39: 914-919, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Role of Genetic and Physiological Factors in Ontogeny and Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P.T. Allen Microbiologist LCMB NCI
OTHER: A.K. Fowler Chief, Experimental Ontogeny Section LCMB NCI

COOPERATING UNITS (if any)

Litton Bionetics, Frederick Cancer Research Facility,
Frederick, MD 21701; Genentech, Inc., San Francisco, CA 94080

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Experimental Ontogeny Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The properties of bioregulatory substances associated with embryogenesis and fetal development are under investigation. Growth stimulatory, as well as anti-proliferative and anti-viral factors, have been solubilized from mouse placental tissues taken after the 12th day of gestation, with primary emphasis on tissues taken at term. A rapid reliable photometric microassay was developed to quantitate murine interferon (IFN) utilizing an EIA reader. Aqueous placental extracts contained an acid-sensitive anti-viral activity which was specific for mouse cells. Under conditions of relaxed stringency, this IFN could be neutralized by antiserum to mouse fibroblast IFN, but not by antiserum to mouse immune IFN. The same placental extracts contained a trypsin-sensitive growth factor (PIGF) active in cultured fibroblasts. Anchorage independent growth of mouse and rat cells, mitogenesis of quiescent mouse and rat cells, and sustained replication of mouse and hamster cells was induced by PIGF. In contradistinction to epidermal and tumor growth factors, PIGF was stable in thiol reagent, and unstable at pH 3 and 80°. The mitogenic activity was inhibited in the presence of mouse fibroblast IFN, as reported for other protein growth factors.

Project Description

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:

The induction of cellular proliferation by tissue-associated growth factors was examined by analyzing tissue-extracts for mitogenic activity. Extracts were prepared by homogenization in 4 ml of buffer per gram of tissue, followed by low speed centrifugation. A semi-micro assay was used to analyze mitogenic activity in which indicator cells were propagated in microplates. Serial dilutions of tissue homogenate were tested by addition of 11 microtiter volumes to quiescent microplate cultures and scored for incorporation of ³H-thymidine into an acid-insoluble form. Rate zonal sedimentation studies were done in gradients of 10% to 30% (V/V) glycerol in phosphate buffered saline centrifuged at 53,000 revolutions per minute in the SW 60 Ti rotor. Gel exclusion chromatography was done on Bio-Gel P-100 in 0.05 M acetic acid.

Mouse interferons were assayed on L929 indicator cells, challenged with the MM strain of encephalomyocarditis virus. Anti-viral activity was measured by the inhibition of viral cytopathogenic effects, evaluated either visually under the light microscope or photometrically in a dye-binding assay using an EIA microplate reader. Neutralization of interferons was performed by two methods. In the initial studies, neutralization of interferon was evaluated by a constant interferon, variable antiserum procedure in which serial dilutions of antisera were mixed with interferons, incubated at 37°C, and the surviving interferon titer was determined by serial dilution using the photometric assay. This procedure has the disadvantage that the interferon-antibody complex tends to dissociate when the concentration of unreacted components is reduced during serial dilution. In the second procedure, the method of Kawade (J. Interferon Res. 1: 61-70, 1980) was used in which the antibody concentration is held constant, the interferon concentration is varied, and the antigen-antibody interaction takes place in the culture itself. The antiserum titer is computed from the concentration of interferon it neutralizes.

Major Findings:

Placenta-associated bioregulatory substances are under investigation. A precise, sensitive, rapid, semi-micro bioassay for quantitating the interferon-like component of placental tissues, as well as interferons from other sources, was developed. The method is based on the photometric quantitation of dye bound by cells rendered virus-resistant by serially

diluted interferon preparations utilizing a titer-tray culture system and an EIA reader. Quantitation of interferon preparations containing as little as 10 international units per milliliter of interferon consumes no more than 0.06 ml of the specimen. Preparations known to be of higher titer may be assayed using correspondingly smaller volumes of sample. This method has been used extensively in studies of the placental interferon (P1 IFN).

Serological studies of P1 IFN utilizing antisera prepared in rabbits against mouse immune interferon and mouse fibroblast interferon have revealed no detectable antigenic cross-reactivity between P1 IFN and immune interferon, while demonstrating a weak antigenic relatedness between P1 IFN and fibroblast interferon. P1 IFN is neutralized by anti-fibroblast interferon but the serum concentration required for comparable interferon titer reduction differs by two orders of magnitude between the homologous interferon and P1 IFN. It is concluded that P1 IFN is distinct from immune interferon and from fibroblast (or beta) interferon. The partial cross-reactivity with fibroblast interferon antiserum may be due to the presence of a shared minor antigenic determinant of P1 IFN and beta interferon. However, the possible presence in the antiserum of antibodies to leucocyte (alpha) interferon has not been ruled out at this time due to the paucity of type-specific serological reagents for the murine interferons.

The specificity of P1 IFN for mouse cells was demonstrated in studies utilizing cells of human, rat, dog, bovine and hamster origin. P1 IFN protected mouse cells from viral cytopathogenesis, but failed to protect cells of heterologous origin, even at IFN concentrations up to 100 times greater than that providing protection to mouse cells. Species specificity is a widely observed property of interferons, though a few examples of cross-species response are known to occur. This property of P1 IFN is of major importance in verifying the identity of P1 IFN as a true interferon.

Characterization of the placenta-associated growth factor (PIGF) has disclosed the following information about this agent. It exhibits mitogenic activity in a variety of cell lines derived from mouse and rat tissues. PIGF induced anchorage-independent growth in cells of mouse and rat origin. The mitogenic activity was equally stable in dialysis in membranes having exclusion limits of 3,500 and 12,000. It was trypsin sensitive, indicating the active component is a protein. It was stable on exposure to thiol reagent, indicating the absence of essential disulfide bonds; it was unstable in 1 M acetic acid (pH 3) but moderately stable in 0.05 M acetic acid (pH 3.5); and it was stable at 56°C but not at 80°C. These latter properties appear to distinguish PIGF from epidermal and tumor growth factors. Rate zonal sedimentation of PIGF at neutral pH indicated its molecular weight to be in the 25,000 to 30,000 range. However, on gel exclusion chromatography at pH 3.5 the apparent molecular weight was approximately 14,000. These data suggest PIGF may be composed of 14,000 molecular weight subunits which occur as dimers under native conditions. The addition of mouse fibroblast interferon to a mouse cell/PIGF system inhibited the mitogenic activity of the PIGF as has been reported for other protein growth factors.

Significance to Biomedical Research and the Program of the Institute:

The effects of exogenous growth factors on the proliferation and differentiation of fetal tissues under experimental conditions has been recognized for a number of years. More recently, EGF has been detected in fetal tissues and receptors for EGF have been reported on placental membranes. Cultured tumor cells are reported to release growth factors which reduce their requirement for serum in the medium. The precise role that any of these factors play under natural conditions in ontogeny or in oncogenesis remains poorly understood. The goal of this project is to investigate the occurrence and interaction of these bioregulatory molecules. To this end, growth factor(s) and a growth factor antagonist (interferon) have been detected in placental and fetal tissues. Progress toward characterizing these factors and their occurrence during gestation has been made. With continued study, the role of these factors in the control of cell proliferation and differentiation may be elucidated.

Proposed Course:

Studies to purify and further characterize the placental growth factors are in progress. Characterization will include comparison to other factors such as sarcoma growth factor, the somatomedins, fibroblast growth factor (FGF), and platelet-derived growth factor. A study of the receptor for placental growth factor is anticipated to characterize their nature in comparison to growth factor receptors under study in other laboratories. Further purification and characterization of the placenta-associated interferon will also be done, with a view to establishing its relationship to the other better characterized mouse interferons.

Publication:

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. International Agency for Research on Cancer Monograph. Lyon, France, in press.

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Carcinogenesis of Mammalian Cells in Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
OTHERS:	F.M. Price	Biologist	LCMB	NCI
	G.M. Jones	Microbiologist	LCMB	NCI
	R. Gantt	Research Chemist	LCMB	NCI
	W.G. Taylor	Research Biologist	LCMB	NCI

COOPERATING UNITS (if any)

R. Parshad, Howard University College of Medicine; R.E. Tarone, Biometry Branch, NCI; J.R. Frost, Johns Hopkins Hospital, Baltimore, MD.; C.W. Boone, Al Hada Hospital, Saudi Arabia; B. Trus, Division of Computer Research & Technology.

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

4.0

PROFESSIONAL:

1.5

OTHER:

2.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to determine the mechanisms of malignant neoplastic transformation in cultured mammalian cells with emphasis on the use of human cells, particularly those of epithelial origin. The project this year consists of the following four studies:

1. Repair of chromosome damage induced by x-irradiation during G₂ phase in a line of normal human fibroblasts and its malignant derivative.
2. Chromatid damage induced by light exposure in human cells from normal and cancer-prone individuals.
3. Development of a human epidermal cell system for carcinogenesis studies.
4. Cytomorphologic characteristics of neoplastic transformation.

Project Description

Study 1: Repair of chromatid damage induced by x-irradiation during G₂ phase in a line of normal human fibroblasts and its malignant derivative. Collaboration with Drs. Parshad and Gantt.

Objectives:

To determine whether a line of normal human skin fibroblasts (KD) differs from its malignant derivative (Hut-14) in repair of chromosome DNA damage induced by low-level x-irradiation. This study is unique in that normal cells and their malignant derivatives were compared following x-irradiation exclusively in the G₂ phase of the cell cycle or within 1-1.5 hr of metaphase.

Methods Employed:

Leighton tubes, each containing a coverslip, were inoculated with cells. After 48 hr of incubation, cultures were x-irradiated (25, 50, 100 rad). After x-irradiation, culture fluid was renewed and colcemid added for 1 hr to arrest cells in metaphase. Cells were processed *in situ* on the coverslips and only metaphase cells were examined for number of chromatid breaks or gaps per cell. Abnormalities scored as breaks showed distinct dislocation and non-alignment, whereas gaps were achromatic lesions showing no dislocation. DNA repair inhibitors, caffeine and cytosine arabinoside (ara-C) were used following irradiation to analyze mechanisms; catalase and mannitol were used during and following x-irradiation to scavenge or destroy •OH and H₂O₂ formed by ionizing radiation.

Major Findings:

The malignant cells had significantly more chromatid breaks and gaps after exposure to 25, 50 or 100 rad than their normal counterparts. Results from alkaline elution of cellular DNA immediately after irradiation showed that normal and malignant cells were equally sensitive to DNA single-strand breakage by x-irradiation. Caffeine or ara-C, inhibitors of DNA repair, when added directly following G₂ exposure, significantly increased the incidence of radiation-induced chromatid damage in the normal cells. In contrast, similar treatment of the malignant cells had little influence. Ara-C differed from caffeine in its effects; whereas both agents increased the frequency of chromatid breaks and gaps, only ara-C increased the frequency of gaps to the level observed in the irradiated malignant cells. Addition of catalase, which destroys H₂O₂ or mannitol, a scavenger of the derivative-free hydroxyl radical •OH, to the cultures of malignant cells during irradiation significantly reduced the chromatid damage, and catalase prevented formation of chromatid gaps. The DNA damage induced by x-ray during G₂ phase in the normal KD cells is apparently repaired by a caffeine and ara-C sensitive mechanism(s) that is deficient or absent in their malignant derivatives.

Significance to Biomedical Research and the Program of the Institute:

A chromatid (half chromosome) at metaphase is thought to contain a single, continuous DNA double helix. A chromatid break observed in the first metaphase

following x-irradiation would, therefore, represent an unrepaired DNA double-strand break. Gaps are interpreted as single-strand breaks. The significance of the above results is that a defect(s) in DNA repair capacity has been revealed in human cells following their malignant transformation in culture. This study is currently being extended to 11 lines of human tumor cells compared with 7 lines of normal cells. All human tumor cell lines are also apparently deficient in the repair of strand breaks produced by x-irradiation during the G₂ period of the cell cycle. Our findings of an association between malignant neoplastic potential and deficient DNA repair in human cells suggest that loss of DNA repair capacity is an essential step in neoplastic conversion. This deficiency may also explain the development of genetic heterogeneity within tumor cell populations that renders chemotherapy so difficult.

Proposed Course:

This study will be continued to characterize further the DNA repair defects and to understand their relevance to malignant transformation of human cells.

Study 2: Chromatid damage induced by G₁ and late S-G₂ light exposure in human cells from normal and cancer-prone individuals. Collaboration with Drs. Parshad and Gantt.

Objective:

To determine whether lines of human skin fibroblasts derived from individuals genetically predisposed to a high risk of cancer show more chromatid breaks following exposure to low-intensity (4.6 W/m²) fluorescent light (cool-white) than six lines of skin fibroblasts from normal donors.

Methods Employed:

Since the repair of light-induced lesions is influenced by the stage of the cell cycle at the time of insult, cells were exposed to light during G₁, S, and late S-G₂ and examined at the subsequent post-treatment metaphase. To study cells under physiologic conditions, agents to induce synchrony were not used. Exposure of cells (grown on coverslips in Leighton tubes) during late S-G₂ or G₁ was achieved by illuminating for 5 hr or for 5 hr followed by 15 hr in the dark, respectively. Since the chromatid damage is scored in metaphase cells, those cells examined after 5 hr of light would presumably be in late S-G₂ at the time of illumination, while those exposed for 5 hr followed by 15 hr in the dark would be in G₁. To illuminate during S phase, cells were exposed for 5 hr followed by 3 hr in the dark.

Major Findings:

Lines of human skin fibroblasts derived from ataxia telangiectasia, Gardner's syndrome, Fanconi's anemia, Bloom's syndrome and xeroderma pigmentosum (XP-A, C, E, Var) donors, all genetically predisposed to a high risk of cancer, differ (except XP-A) from lines of skin fibroblasts from normal donors in showing a significant increase in chromatid breaks following late S-G₂ light exposure. They do not differ from normal cells if the light treatment is given during G₁

or S phase. The late S-G₂ light-induced chromatid damage can be prevented by mannitol, a finding that implicates the free hydroxyl radical ($\cdot\text{OH}$) as the causative agent.

Significance to Biomedical Research and the Program of the Institute:

The failure of late S-G₂ light exposure to produce chromatid breaks in XP-A cells, which are known to be deficient in endonuclease activity, suggests that endonuclease incision of DNA base damage is a necessary step in chromatid breakage induced by light exposure during late S-G₂. One explanation of these observations is that cells from genetic variants with a high risk of cancer, as compared with those from normal donors are deficient in a mechanism of excision repair operative at the late S-G₂ stage of the cell cycle. This study discloses for the first time a defect common to all of these genetic variants.

Proposed Course:

This study will be continued to characterize further the DNA repair defects and to understand their relevance to malignant transformation of human cells.

Study 3: Development of an epithelial cell system for carcinogenesis studies. Collaboration with Mr. Price and Dr. Taylor (see also report by Dr. W. Taylor - Z01 CP 4978-05 LCMB).

Objectives:

1. To evaluate by quantitative techniques the calcium and other requirements for rapid proliferation of human epidermal cells in culture.
2. To develop a strategy for transforming human skin epithelial cells by chemical carcinogens.
3. To evaluate the role of added catalase and retinoic acid in preventing karyotypic instability and spontaneous malignant transformation in mouse epidermal cells as compared with fibroblasts.

Methods Employed:

A culture medium for human epithelial cells together with general procedures for initiating primary culture has been described (Price, et al., *In Vitro* 16: 147-158, 1980) and are modified as required. Mouse epithelial cells are handled in a similar fashion but with different culture media. Carcinogens and retinoic acid are solubilized in DMSO. Karyotypic analyses are performed as described for Study 1 above.

Major Findings:

1. Methods for preparing replicate cultures of cells from human foreskin and quantifying cell proliferation by enumeration of cell nuclei have been successfully applied.

2. The highest yield of human epidermal cells was attained with a calcium concentration of 1 mM in our medium as compared with the low concentration of 0.02 mM reported by other investigators with medium MCDB 151.

3. Medium NCTC 168 formulated in this laboratory produced a significantly higher cell yield than medium MCDB 151 designed for clonal growth; however, raising the $[Ca^{++}]$ concentration in MCDB 151 increased cell yield.

4. Medium MCDB 151 proved a superior plating medium than NCTC 168, and by initiating cultures in MCDB 151 and transferring to NCTC 168 after one week, we can now provide reproducible high yields of proliferating human epidermal cells for accomplishing objective 2.

5. Addition of catalase to mouse epidermal cell cultures reduces the karyotypic instability.

Significance to Biomedical Research and the Program of the Institute:

Since most human cancers are carcinomas, it is important to develop a transformation system with epithelial cells. We are concentrating on human epidermal cells because of availability of tissue and because the epidermal basal cell is an important target for environmental carcinogens.

Proposed Course:

These studies will be continued to meet the above objectives. A manuscript is ready for submission entitled "Approaches to enhance the proliferation of human epidermal cells in culture."

Study 4: Cytomorphic characteristics of neoplastic transformation. Collaboration with C.W. Boone, J.K. Frost and B. Trus.

Objectives:

1. To determine whether standard cytological procedures used by practicing cytopathologists can be used to determine different degrees of neoplastic potential of cultured cells growing attached to coverslips.

2. To determine whether living cells in culture show quantifiable manifestations of neoplastic transformation.

Methods Employed:

Paired normal and tumorigenic rodent and human cells have been grown on coverslips and fixed here, and then Papanicolaou stained in the laboratory of Dr. Frost at Hopkins Hospital. A kit of unknowns was evaluated by a group of outstanding cytopathologists in the country.

Major Findings:

Four cytopathologists have completed their evaluations. A review indicates that Papanicolaou stained preparations of cultured cells can be accurately diagnosed. Living preparations of the cells were also photographed. Photographs were digitized and are currently subject to computerized image analysis.

Significance to Biomedical Research and the Program of the Institute:

With increasing emphasis in our program on human epithelial cells for carcinogenesis studies, it is important to establish cytologic and other criteria of malignant transformation in human epithelial cells and to attempt to analyze the underlying molecular basis for the morphologic changes.

Proposed Course:

The first aspect of this study is being prepared for publication. The analysis of photographs of living cells will be continued to meet the second objective.

Publications:

Camalier, R.F., Gantt, R., Price, F.M., Stephens, E.V., Baeck, A.E., Taylor, W.G., and Sanford, K.K.: Effect of visible light on benzo(α)pyrene binding to DNA of cultured human skin epithelial cells. Cancer Res. 41: 1789-1793, 1981.

Parshad, R., Gantt, R., Sanford, K.K., Jones, G.M., and Camalier, R.F.: Light-induced chromatid damage in human skin fibroblasts in culture in relation to their neoplastic potential. Int. J. Cancer 28: 335-340, 1981.

Parshad, R., Gantt, R., Sanford, K.K., Jones, G.M., and Tarone, R.E.: Repair of chromosome damage by x-irradiation during G₂ phase in a line of normal human fibroblasts and its malignant derivative. J. Natl. Cancer Inst., in press.

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., in press.

Tucker, R.W., Meltzer, M.S., and Sanford, K.K.: Susceptibility to killing by BCG-activated macrophages associated with "spontaneous" neoplastic transformation in culture. Int. J. Cancer 27: 555-562, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 04977-05 LCMB

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Primary Biochemical Alterations Leading to Neoplastic Conversion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.R. Gantt	Research Chemist	LCMB	NCI
OTHERS:	K.K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
	W.G. Taylor	Research Biologist	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular & Molecular Biology

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In Vitro Carcinogenesis Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

3.0

PROFESSIONAL:

1.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This program is designed to identify primary chemical and structural alterations in DNA leading to neoplastic transformation in human cells and rodent cells and quantitate the repair response of the cells to agents which induce the primary alterations. The immediate objectives are to develop methods to transform human epithelial cells and to compare the type of lesions caused by low level (nontoxic) insults to DNA with high level (toxic) insults and measure the cell repair response to these lesions. The lesions currently of most interest are DNA-protein crosslinks, including both their induction and repair.

Project Description

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 it is 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-Pt (trans-diaminodichloroplatinum) 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. Previous results showed mouse L1210 cells repaired 20 μm trans-Pt-induced lesions in 24 hours and that their repair was inhibited by cycloheximide and conditioned medium, suggesting cell cycling is required for repair and that S-phase DNA synthesis may be essential. These results have now been extended and indicate there are two pathways for repair of DNA-protein crosslinks, based on the following results. (1) Kinetic studies in rapidly dividing cells (12 hour doubling time) show the rate of crosslink repair is the same as the rate of DNA replication in the cell population. This strongly suggests DNA repair is coupled to DNA synthesis. (2) Cycloheximide inhibition (1 $\mu\text{m}/\text{ml}$) of repair is not complete; about 15-30% of the crosslinks are repaired in 24 hours, but DNA replication of the cell population is also 15-30%. These results suggest repair is coupled to S-phase synthesis. (3) However, complete crosslink repair takes place, though more slowly, in the presence of aphidicolin (10 $\mu\text{m}/\text{ml}$), an effective inhibitor of S-phase DNA synthesis (>90%). These experiments unequivocally show that DNA synthesis is not essential for repair of crosslinks, but indicate that cycloheximide inhibition is due to protein synthesis inhibition. (4) When doubling time of L1210 cells is increased from 12 hours (20% serum supplement) to 30 hours (1% serum) the DNA-protein crosslink repair is twice the rate of DNA synthesis but only one-half the repair rate of the more rapidly growing cells. (5) Repair of DNA-protein crosslinks in human normal fibroblasts, which are known to be far more competent in excision repair than rodent cells, is also inhibited by cycloheximide and proceeds in the presence of complete inhibition of DNA replication (aphidicolin, 10 $\mu\text{m}/\text{ml}$), though again more slowly. (6) α -Amanitin, an inhibitor of RNA polymerase, inhibits crosslink repair similar to the inhibition by cycloheximide. This indicates induction of a required function(s) is necessary for complete repair. Taken together, these results indicate the existence of two repair mechanisms. One mechanism

is coupled with DNA replication and the rate of repair is limited by the rate of DNA synthesis in the population. The other mechanism is independent of DNA synthesis and apparently needs induction of a required function(s).

Significance to Biomedical Research and the Program of the Institute:

Reports of others show that DNA-protein crosslinks (trans-Pt induced) increase sister chromatid exchanges and transform 10T-1/2 mouse cells, observations which indicate important perturbations of DNA. However, DNA-protein crosslinks are reported to be mutagenic. 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, it strengthens the idea 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 or induction, particularly if the recognition system for induction is insensitive. 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 induction of protein synthesis and production of messenger RNA; a general decline of organ response would ensue.

Proposed Course:

- A. Determine whether other types of DNA lesions are repaired by the two pathways detailed in this report.
- B. Establish clearly whether an inducible system is required for crosslink repair in human and mouse cells.
- C. Simplify the assay procedure and develop alternate modes of crosslink production.
- D. Identify, isolate and characterize proteins which may be induced by crosslink formation.
- E. Look for the accumulation of DNA-protein crosslinks in animals as a function of age or "deterioration."

Publications:

Camalier, R.F., Gantt, R., Price, F.M., Stephens, E.V., Baeck, A.E., Taylor, W.G., and Sanford, K.K.: Effect of visible light on benzo(α) pyrene binding to DNA of cultured human skin epithelial cells. Cancer Res. 41: 1789-1793, 1981.

Parshad, R., Gantt, R., Sanford, K.K., Jones, G.M., and Camalier, R.F.: Light-induced chromatid damage in human skin fibroblasts in culture in relation to their neoplastic potential. Int. J. Cancer, in press.

Parshad, R., Gantt, R., Sanford, K.K., Jones, G.M., and Tarone, R.E.: Repair of chromosome damage induced by x-irradiation during G₂ in normal human fibroblasts and their malignant derivatives. J. Natl. Cancer Inst., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04978-05 LCMB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Growth, Nutrition, and Neoplastic Transformation of Mammalian Cells In Vivo

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W.G. Taylor	Research Biologist	LCMB	NCI
OTHERS:	K.K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
	R.R. Gantt	Research Chemist	LCMB	NCI

COOPERATING UNITS (if any)

None

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2.0

PROFESSIONAL:

1.0

OTHER:

1.0

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(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-term objective of this program is to understand the mechanism(s) of neoplastic transformation in cultured mammalian cells, particularly human cells. Studies described in this report address these intermediate goals: (1) modulation of proliferation and functional activity in epithelial cell cultures at determined calcium or dissolved oxygen concentrations; (2) assessment of the undersurface morphology of nonneoplastic and neoplastic cells by interference-reflexion microscopy; and (3) repair of DNA-protein crosslinks by normal human fibroblasts during inhibition of protein and DNA synthesis. Dissolved oxygen concentration is essential for continued proliferation and hemicyst formation by monkey kidney epithelial cells. Epithelial cells utilize oxygen rapidly and a means for maintenance of an adequate dissolved oxygen supply without a potentially deleterious excess is needed. Comparison of the undersurface of several rodent and human cells is in progress. Studies on the repair of nonlethal DNA-protein crosslinks reveal the potential for more than one pathway or mechanism of repair.

Project Description

Study 1: Modification of epithelial cell proliferation by calcium or oxygen concentration.

Objectives:

Most solid human tumors are carcinomas which arise in tissues rich in epithelium. Thus, the use of epithelial cells is preferred for experimental carcinogenesis studies. Our strategy for transformation of human epithelial cells includes temporary alteration of the cellular environment to maximize the impact of potential transforming agents. One objective was to reassess proliferation rate and population density as a function of calcium concentration and medium formulation. In addition, calcium-free medium NCTC 168 was tested as a "staging" medium for initiating DNA-synthesis in primary cultures of human skin epithelium. The second objective was to define the role of dissolved oxygen in the proliferation and function of NCTC 8939.

Methods Employed:

Two epithelial cell systems are used: primary or low passage human keratinocytes which can be grown in medium NCTC 168 without a feeder layer, and line NCTC 8939, a derivative of LLC-MK₂ rhesus monkey kidney cells, which can be grown in serum-free medium. Standard culture procedures are used or are modified as required, as are methods for quantification. Dissolved oxygen is measured with a radiometer blood gas analyzer. Population density is determined by enumeration of nuclei extracted from keratinocytes or counting of suspensions of NCTC 8939 with a cytograf 6300A counter.

Major Findings:

1. Calcium and epithelial cell proliferation. This is a collaborative study with Dr. K. Sanford and a complete description of the findings and significance is found in her report (Z01 CP 04976-05 LCMB).
2. Use of calcium-free NCTC 168 for staging human skin epithelium. Milo and coworkers have proposed that human epithelium is most susceptible to transformation by chemical carcinogen if cells are treated during S-phase. Moreover, they reported that human keratinocytes differ from fibroblasts in that incorporation of thymidine begins and peaks soon after introduction of "stress" (i.e., depleted) medium. We also see a peak of incorporation 1.5 - 2 hr after replacement of complete medium with Ca⁺⁺-free NCTC 168, but the amount of label incorporated suggests repair rather than S-phase synthesis. Further studies to characterize this response in precisely the same system used by Milo are planned.
3. Dissolved oxygen. Several studies in this laboratory indicate that epithelial cell proliferation and function is modulated by dissolved oxygen. The growth rate and final cell density of primary human skin epithelium and of a continuous line of rhesus monkey kidney cells grown in serum-free medium (NCTC 8939) is dependent on dissolved oxygen concentration. Proliferation rates and saturation densities are not significantly altered when the gas phase oxygen

concentration is 10%, 18%, or 30% v/v. But, a gas phase containing 1% O₂ v/v yields a dissolved oxygen concentration of ~ 40 mm Hg and proliferation is retarded; neither diffusion gradient formation nor defective cellular adhesion explains this response. In addition, several studies show that vectorial ion and fluid transport by essentially nonmitotic, postconfluent monolayers of NCTC 8939 is reduced either by cellular or artificial depletion of dissolved oxygen, as reflected by a dramatic decline in the frequency of three-dimensional hemicyst formation in living cultures.

Significance to Biomedical Research and the Program of the Institute:

The significance of these findings are two-fold. First, although there is little evidence that mitosis itself is oxygen-dependent, these findings show that both proliferation and expression of functional characteristics can be markedly influenced by oxygen dissolved in the cellular microenvironment. The cessation of either proliferation or functional activity even with physiologic levels of dissolved oxygen implies that epithelial cells or epithelial population can reversibly respond to the microenvironment and that dissolved oxygen, one essential metabolite for epithelial cells, may function in a fashion analogous to the cell-cycle restriction point requirement of fibroblasts. Second, all studies indicate rapid oxygen utilization which necessitates, ideally, maintenance of this metabolite within physiologic limits, since deliberately increased concentration (e.g., 30% O₂ v/v) does not correct for ultimate depletion and increases the risk of cellular damage resulting from deleterious oxygen intermediates, free radicals, and hydroperoxides. This type of chronic, nonlethal oxidative cell injury may profoundly influence the progression of carcinogenic events both in vivo and in vitro.

Proposed Course:

The first important question is whether the observed oxygen sensitivity is a more general response which can be detected with epithelium from other tissues. Second, a more precise analysis of the cellular and subcellular responses to limiting dissolved oxygen concentration is needed since: (a) this may be the condition(s) under which all epithelial cells presently grow or function; (b) little or nothing is known regarding the kinetics of utilization by cells in culture; (c) identification of functions and pathways which require dissolved oxygen will determine whether these requirements can be satisfied indirectly, or alternatively, blocked.

Study 2. Evaluation of cell undersurface adhesion sites as a marker for tumorigenic potential.

Objectives:

Cell shape changes characterize responses to deleterious agents or growth conditions and frequently accompany the acquisition of neoplastic potential by cells in culture. Following cell retraction due to injury or the morphologic alterations associated with transformation, the total area of cell membrane which remains in apposition to the growth surface is reduced. An earlier study showed that neoplastic mouse fibroblasts have higher rates of locomotion and

exhibit a more random pattern of movement when compared to nonneoplastic cells from the same clone (Sanford et al., ECR 109: 454, 1977). One possible explanation is that neoplastic fibroblasts have a reduced number of sites with which they adhere to the growth surface or, alternatively, that the sites of attachment on the undersurface of a neoplastic cell are less adhesive. Conceivably, this would contribute to the transformed cell's ability to metastasize and develop invasive neoplasms. To evaluate this hypothesis, we will examine the undersurface of paired neoplastic and nonneoplastic cells by interference-reflexion.

Methods Employed:

A Zeiss inverted microscope has been fitted for both phase and interference-reflexion microscopy. Dvorak chambers are inoculated with no more than 500 cells, incubated overnight, and individual cells photographed with both sets of optics. Film, filters, lighting, etc., have been optimized for maximum contrast with additional improvements incorporated as needed.

Major Findings:

Three pairs of normal and tumorigenic fibroblasts (mouse, rat, and human) have been evaluated to date. Preliminary analyses suggest that within a species, clear differences in the number of adhesion sites can be seen between the nonneoplastic and tumorigenic lines.

Significance to Biomedical Research and the Program of the Institute:

This form of microscopy allows visualization of the undersurface of the cell and its sites of attachment to the growth surface. As such, it represents a tool for mapping the basal surface of nonneoplastic and tumorigenic cells. The interrelationships between microfilament bundle attachment to the cell membrane, locomotion morphology, and virus infection are beginning to emerge, and all are related to the way in which cells attach to a growth surface of other cells and how cell growth is controlled.

Proposed Course:

Additional analyses of data are planned to determine if similarities in the distribution of adhesion sites can be seen as a function of cell cycle phase or between species as a function of tumorigenic potential. Efforts will be made (a) to use interference-reflexion microscopy in conjunction with fluorescent microscopy to characterize cell adhesion sites of anchorage-dependent and independent cells and (b) to determine if some form of computerized image analysis can be used to quantify differences between normal and neoplastic cells.

Study 3:

Repair of DNA-protein crosslinks during inhibition of protein and DNA synthesis in normal human fibroblasts. This is a collaborative study with Dr. R. Gantt. See his report for complete details (Z01 CP 04977-05 LCMB).

Publications:

Taylor, W.G.: Electrolytes, dissolved gases, buffers and pH changes in tissue culture systems: an overview. In Waymouth, C., Ham R. G., and Chapple, P.J. (Eds.): The Growth Requirements of Vertebrate Cells In Vitro. New York, Cambridge University Press, 1981, pp. 94-104.

Taylor, W.G.: Studies on the chemical nature of a growth promoting agent, bactopeptone dialysate. In Waymouth, C., Ham R. G., and Chapple, P.J. (Eds.): The Growth Requirements of Vertebrate Cells In Vitro. New York, Cambridge University Press, 1981, pp. 258-276.

Taylor, W.G., and Camalier, R.F.: Modulation of epithelial cell proliferation in culture by dissolved oxygen. J. Cell. Physiol. 111: 21-27, 1982.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Studies of RNA Tumor Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHERS:	S. Aaronson	Chief	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	A. Srinivasan	Visiting Associate	LCMB	NCI
	A. Habara	Visiting Fellow	LCMB	NCI
	Y. Yuasa	Visiting Fellow	LCMB	NCI
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COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:

3.0

PROFESSIONAL:

1.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Biochemical organization of several retroviral onc genes was studied by nucleotide sequence analysis. The transforming genes of BALB-MSV, Abelson-MuLV, SSV, MC-29 and AMV have been sequenced.

2. Several deletion mutants were constructed using the proviral genome of A-MuLV to understand the structural requirements for transforming gene function and to delineate the regulatory signals present in retroviral long terminal repeats.

3. Using cloned proviral DNAs of Rauscher and Moloney murine leukemia viruses, several recombinants were constructed between the two viruses to map the regions in the leukemia viral genome responsible for target cell specificity.

Project DescriptionObjectives:

Project 1. To study the biochemical organization and molecular mechanisms involved in the transformation of fibroblasts and lymphoid cells by Abelson murine leukemia virus.

Project 2. To study the biochemical organization of myelocytomatosis virus (MC29).

Project 3. To study tissue specificity and mechanisms of leukemogenesis of replication-competent Moloney and Rauscher murine leukemia viruses.

Project 4. Nucleotide sequence analysis of transforming gene of BALB-MSV and its human analog.

Methods Employed:

Molecular cloning, DNA sequence analysis, site specific mutagenesis and DNA transfection.

Major Findings:Project 1.

A. Structural requirements for transforming gene functions of A-MuLV genome. The integrated proviral genome of Abelson murine leukemia virus was molecularly cloned in *E. coli* and its physical map determined. Cloned A-MuLV DNA was shown to transform NIH/3T3 cells at high efficiency. In order to localize the region of A-MuLV required for transformation, we measured the infectivity of the proviral genome following exposure to different restriction enzymes. Comparison of the biological activity of cloned A-MuLV genomic and subgenomic fragments revealed that subgenomic clones which lacked the 5' LTR and adjoining sequences (30 bp downstream of LTR) were not biologically active. In contrast, subgenomic clones, which lacked the 3' LTR and as much as 1.3 kbp of the A-MuLV cell-derived *abl* gene, were as efficient as wild type virus DNA in transformation. The A-MuLV encoded polyprotein p120 and its associated protein kinase activity were detected in the 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.

B. In vitro mutagenesis experiments to delineate the regulatory signals present in retroviral long terminal repeats. The LTR of A-MuLV contains a 72 bp tandem repeat and putative signals for promotion, initiation and termination of transcription. We utilized site-specific mutagenesis to define regions of the 5' LTR required for A-MuLV transforming gene function in vivo. We constructed a

series of mutants encompassing deletions of different domains and assayed for viral gene expression (transforming activity) following transfection of NIH/3T3 cells. Removal of one or both 72 bp repeated units did not abolish transforming ability. Deletion of either TATA and CCAAT sequences or sequences upstream from the 72 bp repeat reduced but did not totally abolish transforming activity. Finally, deletions which encompassed TATA, CCAAT and poly A signals completely abolished transformation. The results indicate that both TATA and some sequences localized toward the 5' end of the LTR are required, but that the 72 base pair repeat does not play a major role in efficient A-MuLV transformation.

C. Biochemical organization of A-MuLV genome. The complete nucleotide sequence of the integrated proviral genome of A-MuLV has been determined. By comparison of this sequence with that of Mo-MuLV genome, it was possible to localize the junction points between helper viral and v-abl sequences. These studies demonstrate that the viral genome has the coding capacity for p15, p12 and the first 21 amino acids of p30 sequences. The viral genome has deleted the rest of the gag, pol and env sequences. The recombination between viral and cellular sequences occurred such that the open reading frame extends for an additional stretch of 2613 bases, resulting in a gag-abl hybrid protein. The open reading frame is terminated within v-abl region, 485 bases upstream of v-abl-helper viral junction.

Project 2. The complete 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 junction points between helper viral and v-myc sequences. These studies demonstrate that (1) the LTR sequence of MC29 is very similar to that of RSV, (2) the viral genome has suffered extensive deletions in the gag, pol and env genes, (3) the gag region can code for p19 and part of p27, (4) 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 1278 bp, resulting in a gag-myc hybrid protein, (5) the open reading frame terminated within the v-myc region 300 bases upstream of v-myc-helper viral junction, and (6) the v-myc-helper viral junction at the 3' end occurred in the middle of env gene, rendering it defective.

Project 3. In an attempt to develop model systems for understanding the mechanisms of type C virus-induced mouse leukemias, we have analyzed the target cell specificity for transformation by different viruses. It has been possible to show that clonal strains of M-MuLV and R-MuLV induce tumors of T and B lymphoid cells, respectively. Since these viruses have similar genomic structure and code for related components, we reasoned that a genetic approach might make it feasible to localize the region of the viral genome responsible for target cell specificity. In efforts aimed at construction of molecular recombinants between Rauscher and Moloney leukemia virus genomes, we cloned the integrated proviral DNA of R-MuLV. Taking advantage of the common restriction sites that occur in the two viral genomes, we constructed several recombinants between the two proviral DNA molecules. These recombinant DNA molecules, when transfected into NIH/3T3 cells, yielded retroviruses that contained different regions of Moloney and Rauscher leukemia viruses.

Project 4. BALB-MSV is a spontaneously occurring transforming retrovirus of mouse origin. The integrated form of the viral genome was cloned from the DNA of BALB-MSV-transformed nonproducer NRK cell line. The 6.8 kb proviral genome was found to contain a segment of approximately 0.6 kbp of cellular insertion sequences (v-bas) which were essential for the viral transforming activity. Nucleotide sequence analysis of this region and its flanking MuLV-associated sequences revealed an open reading frame that could code for 21 K protein. This open reading frame commenced within the helper viral region and terminated within v-bas itself.

Significance to Biomedical Research and the Program of the Institute:

These studies have provided a large amount of information leading to a better understanding of the biochemical organization of mammalian onc genes. The nucleotide sequence studies have provided approaches to isolate and characterize the transforming proteins coded by these onc genes. These studies combined with mutagenesis studies are expected to provide further information on the mechanisms that lead to the transformation of a normal gene to an onc gene, which will lead to developing approaches toward cancer prevention.

Proposed Course:

Project 1. The nucleotide sequence data will be utilized to develop site-specific mutants in the transforming region of the viral genome in an effort to elucidate the molecular mechanisms involved in the transformation process.

Project 2. Synthetic peptides will be generated from the predicted amino acid sequence of the transforming proteins and will be used to prepare antisera against the onc protein. These antisera will be used to study the expression of this onc protein in naturally occurring lymphoid tumors.

Project 3. The recombinant viruses generated by the above approach will be injected into animals and the tumors tested for T and B cell types in an attempt to map the region within the viral genome responsible for tissue specificity. These regions also will be sequenced to understand the molecular mechanisms involved in determining such specificity.

Project 4. Using a probe derived from the v-bas region, a human analog of these sequences was obtained from a human library. The structure of this human homolog with respect to v-bas, as well as pertinent features of its nucleotide sequence, are currently being investigated.

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 Fox, C.F., and Burger, M.M. (Eds.): Progress in Clinical and Biological Research: Differentiation and Function of Hematopoietic Cell Surfaces. New York, Allen R. Liss, Inc., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05060-04 LCMB
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PERIOD COVERED October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies of Oncogenic Expressions in Experimental Animal and Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.S. Rhim	Research Microbiologist	LCMB	NCI
OTHERS:	R.J. Huebner	Chief, Viral Immunology Section	LCMB	NCI
	M. Kraus	Guest Researcher	LCMB	NCI
	R. Trimmer	Bio. Lab. Tech.	LCMB	NCI
	A. Scheib	Bio. Lab. Tech.	LCMB	NCI
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COOPERATING UNITS (if any)
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S. Sieber-Fabro, LCHPH, NCI; M. Essex, Harvard U.; M. Gardner & S. Rasheed,
USC; S. Kalter & R. Heberling, S.W. Fdn. for Res. & Educ.; C. Borek, Columbia U.

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Viral Immunology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4.0	PROFESSIONAL: 1.0	OTHER: 3.0
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CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 DescriptionObjectives:

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 RNA-dependent DNA polymerase, radio-immunoprecipitation and I125 protein A assay.

Major Findings:

1. Human skin fibroblasts derived from cancer family syndromes, such as adenomatosis of the colon and rectum (ACR) and Gardner's, furnished and tested by Drs. L. Kopelovich and M.G. Gardner, were tested for focus formation by the Kirsten mouse sarcoma virus (Ki-MSV). The results confirmed the genetic differential susceptibility of cultured human skin fibroblasts to transformation by Ki-MSV. Thus tests for identification of high risk individuals in families carrying autosomal dominant genes for colorectal cancer were confirmed in three laboratory test systems. In order to establish further its accuracy, coded skin samples received from Drs. Eldon Gardner and Randy Moon are currently being tested for their susceptibility to Ki-MSV. The specificity of this skin transformation assay will be determined when tests are complete and the code is broken.
2. All transformed human skin fibroblast cultures were Ki-MSV producers and contained murine leukemia virus p30 antigen. Tumors were produced in nude athymic mice by some of the morphologically altered human skin fibroblast cultures. However, such tumors were always characterized karyologically as murine and not human. In addition, the Ki-MSV-transformed human skin fibroblasts were difficult to maintain since with serial cultivation the proportion of altered cells decreased or disappeared. Thus, Ki-MSV-transformed human skin fibroblasts failed to become established lines, had limited life-spans, and were unable to produce tumors when transplanted into nude mice.

3. DNA hybrid adeno 12-SV40 virus induced neoplastic transformation of human skin cultures derived from high risk autosomal dominant ACR cells. Increased transformation efficiency of adeno 12-SV40 was observed in these mutant cells. Transformation was evident by morphological alteration and presence of adeno 12 and SV40 large tumor antigens in the complement fixation test. In contrast to Ki-MSV transformed human skin cells, the line established from the hybrid virus transformed ACR fibroblasts became stable, and although initially virus-productive, gradually became nonproductive over serial passages. Cultured skin fibroblasts from a normal control infected with adeno 12-SV40 also underwent morphological transformation and was virus-productive. Although these cells had a prolonged life compared to uninfected controls, they eventually died. All of the transformed cells formed large cell aggregates, grew in liquid growth medium above an agar base and formed colonies in soft agar. The altered ACR cells became heteroploid and produced tumors when transplanted subcutaneously into nude mice but the tumors regressed. The results support the conclusion that cells derived from ACR individuals are more susceptible to transformation by oncogenic viruses.

4. Human skin fibroblasts from ACR and Gardner's syndrome individuals: susceptibility to transformation by Ki-MSV but not to the Schmidt-Ruppin strain of the Rous sarcoma virus (RSV-SR). In contrast, the human osteosarcoma (HOS) clonal cells (TE-85 clone F-5), known to be sensitive to Ki-MSV transformation, were readily transformed by RSV-SR. The RSV-SR transformed cells formed large cell aggregates and grew in this aggregate form when suspended in liquid growth medium above an agar base, formed colonies in soft agar with high efficiency, and grew to high saturation densities, while the uninfected cells did not. The transformed cells produced tumors when transplanted subcutaneously into nude mice. The morphologically altered cells produced neither infectious virus nor type C virus particles. However, they contained RSV-RS complement-fixing antigen, RSV transformation-specific protein (p60^{SPC})-associated kinase activity, and a rescuable infectious virus. Thus, the transformation of human cells in vitro by RSV-SR from a continuous cell line has been demonstrated. This is the first documentation of the establishment of RSV-transformed human cell lines. These cell lines should be useful in studying the interaction of RSV and human cells.

It is interesting to note that ACR-derived skin fibroblasts were sensitive to Ki-MSV transformation but not to RSV transformation. These results indicate that each type of virus requires its own specific cell surface receptor for adsorption and infection.

5. Sensitivity of chimpanzee skin fibroblasts to transformation by both RNA and DNA tumor viruses. The DNA hybrid virus adeno 12-SV40 produced productive and nonproductive transformation of chimpanzee skin fibroblasts in vitro. The transformed cells contained both adenovirus 12 and SV40 large tumor antigens and became permanent lines. One of the nonproducer lines produced tumors when transplanted subcutaneously into nude mice, confirming its malignant potential. Thus, adeno 12-SV40 virus is capable of inducing neoplastic transformation of chimpanzee fibroblasts in vitro. Several RNA tumor viruses, including the

fibroblasts. However, the RNA tumor virus-transformed cells had a limited lifespan, failed to become established lines and were usually unable to produce tumors in nude mice.

6. Attempts to induce tumors in chimpanzees by oncogenic tumor viruses, chemical carcinogens and oncogenic RNA tumor virus-producing human cancer cells. To date, it has been possible to induce a chimpanzee-specific sarcoma in an infant chimpanzee following subcutaneous inoculation with a nonproducer human osteosarcoma line (KHOS) infected with Ki-MSV(BaEV). By contrast, no tumors were observed following inoculations with cell-free Ki-MSV(BaEV) alone or with human foreskin cells infected with the pseudotype virus. The tumor was histopathologically diagnosed as a sarcoma. The cells reestablished in culture were characterized as chimpanzee by karyological analysis. The tumor cell line became stable and was demonstrated to be a low virus producer. The tumor cells contained BaEV p30 antigen but attempts to rescue the Ki-MSV genome were unsuccessful. The sera of the tumor bearing chimpanzee demonstrated high titered complement-fixing antibodies against the BaEV when tested two weeks after inoculation. These tumor induction studies were done in collaboration with Drs. S. Kalter and R. Heberling and were undertaken based on preliminary evidence that Ki-MSV(BaEV) was capable of inducing metastatic disease in a variety of animal species, including the chimpanzee.

In collaboration with Dr. Sieber-Fabro, liver tumors were induced in an adolescent chimpanzee following treatment with a chemical carcinogen (DENA). Tumor lines were established and characterization studies are in progress.

7. 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 human epithelial cells are very difficult to culture. Therefore, there are few reports describing the viral transformation of human epithelial cells. Recently 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 culture of human skin 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 infected cells did not grow. Confirmatory studies are in progress.

8. A cell aggregation assay for evaluating in vitro transformation. Transformed cells formed larger cell aggregates than counterpart normal cells when suspended in liquid media above an agar base, a property which correlates with growth in soft agar and tumorigenicity. Using a human osteosarcoma cell line transformed by viruses and chemicals, we found increased cell growth of the transformed aggregates over untransformed cells. Moreover, certain aggregation properties (size/survival of aggregates) of transformed rat, mouse, hamster, human, dog, cat, chimpanzee and sheep cell lines were all found to be correlated with tumor potential regardless of the method of transformation (spontaneous, chemical or virus-induced). Certain lines derived from cell aggregates growing in liquid medium above an agar layer were tumorigenic in nude mice, whereas the

parent transformed line was not. Therefore, this assay can be utilized not only to evaluate tumorigenic potential, but also for selection of neoplastically altered cells.

9. Neoplastic transformation of BALB/c 3T3 cells by metabolically activated estrogens. In collaboration with Dr. Purdy, it was found that diethylstilbestrol, estradiol and ethynylestradiol induced transformation of two subcloned lines of BALB/c 3T3 embryo-derived mouse fibroblast cells in vitro. The Type III foci produced by these estrogens were morphologically indistinguishable from those induced by 3-methylcholanthrene. Transformed cells from these Type III foci grew in soft agar, formed anchorage-independent aggregates, grew as multicellular tumor spheroids, and were tumorigenic in nude mice. Transformation was not observed where metabolic activation of the estrogen was inhibited by a neighboring substituent, as in the cases of 11 β -methoxyethynylestradiol (moxestrol) and 11 β -ethylestradiol, which are potent estrogens in vitro and in vivo. This provides the first documented evidence that neoplastic transformation is associated with the metabolically activated intermediates of some types of estrogens.

Significance to Biomedical Research and the Program of the Institute:

1. Development of testing systems for identification of genetic susceptibilities to cancer is much needed in cancer diagnosis.
2. Definition of factors associated with cell transformation will 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. Further definition of the Ki-MSV human skin transformation system and establishment of its validity and parameters as possible diagnostic indices for detection of hereditary cancer in man.
2. Further characterization of RNA and DNA tumor virus-transformed human skin fibroblasts derived from genetically abnormal individuals, including efforts to determine the molecular bases of these disorders.
3. The role of the tumor promoter, TPA, as well as nontransforming retrovirus in MNNG transformation of a human skin cell system will be studied in efforts to develop a model for cancer promotion in vitro.

4. Continuation of studies to elucidate the various factors (with emphasis on the effect of hormones) regulating cellular transformation induced by chemical carcinogens and oncogenic viruses.
5. Further characterization of chimpanzee tumors induced by a chemical carcinogen and human tumor cell.
6. Continuation of efforts to rescue src expressions specific for primate and human tumors. The following efforts will be pursued:
 - A. Isolation and characterization of variants from transformed human and subhuman primate cells.
 - B. Isolation and characterization of revertants from transformed human cells which may provide a potentially important model for understanding the mechanisms involved in cellular gene expression and tumorigenicity.
 - C. Attempts to demonstrate the transforming activity of human and primate tumors by the DNA transfection assay.
7. Continued characterization of adeno 12-SV40 transformed human epithelial cells, and attempts to transform human epithelial cells by chemicals or chemicals plus virus.

Publications:

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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Study of Abelson Murine Leukemia Virus Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	J. Pierce	Staff Fellow	LCMB	NCI
	A. Eva-Varesio	Visiting Associate	LCMB	NCI
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LAB/BRANCH

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SECTION

Molecular Biology Section

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TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The integrated Abelson murine leukemia virus (A-MuLV) genome cloned in bacteriophage vector was used to localize viral genetic sequences required for transformation. Comparison of the biological activity of cloned A-MuLV genomic and subgenomic fragments revealed that subgenomic clones which lacked 5' LTR and adjoining sequences were not biologically active. In contrast, subgenomic clones which lacked 3' LTR and as much as 1.3 kbp of the A-MuLV-specific abl gene, were as efficient as wild-type virus DNA in transformation. Further, site-specific mutagenesis was utilized to define regions of the 5' LTR required for A-MuLV transforming gene function in vivo. Removal of one or both 72 bp repeated units did not impair transforming ability. Deletion of either TATA or CAAT sequences, or sequences upstream from the 72 bp repeat, reduced but did not abolish transforming activity. Finally, deletions which encompassed TATA, CAAT and poly A signals completely abolished transformation.

Project Description

Objectives:

1. To study the structural requirements for the transforming gene function of A-MuLV.
2. To study the functional domains present in an A-MuLV LTR by in vitro mutagenesis.

Methods Employed:

1. Molecular cloning techniques, transfection assay, immunoprecipitation and SDS-PAGE.
2. Molecular cloning, DNA transfection and sequencing.

Major Findings:

1. The integrated proviral genome of A-MuLV was molecularly cloned in E. coli using bacteriophage vector λ gtWES. λ B. Further, mapping studies of cloned DNA (λ AM-1) with several restriction enzymes and heteroduplex analysis showed that A-MuLV shares homologous sequences of about 1.7 kbp and 0.7 kbp at 5' and 3' ends, respectively, with its parental M-MuLV genome, defining the length of A-MuLV-specific abl gene as 3.1 kbp.

Transformation by cloned whole and subgenomic A-MuLV fragments. In order to identify the minimal A-MuLV DNA sequences required for transformation, we monitored the biologic activity of λ AM-1 DNA after exposure to different restriction enzymes. Restriction enzymes Ava I, Sma I, Pst I, Sac I, Bgl I and Bgl II, which cleave at multiple sites within the viral genome, completely abolished transforming activity. Xba I and Kpn I each cleave the A-MuLV genome only within the long terminal repeat (LTR). Xba I significantly reduced and Kpn I completely abolished transforming activity. Pvu I, which cleaves at a unique site 300 bp downstream from the 5' LTR, also abolished A-MuLV biologic activity.

Cleavage with enzymes that cut at unique sites towards the 3' end of the viral genome made it possible to localize the region of abl required for transforming activity. Thus, Bam HI, Hind III, and Cla I, which cut at unique sites towards the 3' end of the genome, did not appreciably alter the biologic activity of λ AM-1 DNA. Even cleavage at a unique Sal I site within abl sequences resulted in transforming activity comparable to that of the parental A-MuLV DNA. In contrast, Acc I, which cleaves approximately 700 bp upstream from the Sal I site, completely abolished A-MuLV biologic activity. These results suggested that not more than 56% of abl was required for fibroblast transformation.

Comparison of transforming activities of A-MuLV subgenomic DNA clones. In an effort to provide more quantitative comparisons of their transforming activities, several A-MuLV DNA fragments were subcloned in plasmid vector. In most cases it was possible to utilize the same restriction sites in

pBR322 for construction of the subclones. However, for Xba I, Acc I and Kpn I subclones, it was necessary to utilize Eco RI linkers. A-MuLV DNA subcloned in pBR322 demonstrated 1.7×10^2 ffu/ μ g DNA when utilized to transfect NIH/3T3 cells. Cla I, Bam HI, Hind III, and Sal I DNA subclones each showed comparable transforming efficiencies, while the Acc I subclone was completely inactive under the same assay conditions. These findings directly established that the distal 44% of abl was not essential for efficient transformation of fibroblasts.

Analysis of A-MuLV DNA subclones constructed from the Pvu I, Xba I, or Kpn I restricted wild-type genome provided information concerning the requirements for transformation of sequences at the 5' terminal portion of the A-MuLV genome. Both Pvu I and Kpn I subclones lacked detectable biologic activity. The transforming efficiency of the Xba I subclone was around 3 to 10% that of wild-type A-MuLV DNA. These latter findings demonstrate that while the 5' viral LTR is critical to transformation by A-MuLV, the entire LTR is not absolutely essential.

Identification of gene products coded by A-MuLV subgenomic DNAs. A-MuLV is known to code for expression of a 120,000 molecular weight protein, which contains gag gene products p15, p12, and a small portion of p30, as well as a region unrelated to known MuLV proteins. Since transformation by A-MuLV is thought to be mediated by P120, it was of interest to analyze A-MuLV-specific proteins present in cells transformed by subgenomic A-MuLV DNAs. Cells were metabolically labelled with [35 S]-methionine and viral proteins analyzed by immunoprecipitation with anti-M-MuLV sera followed by SDS-PAGE. Transformants induced by A-MuLV genomic DNA, as well as by Cla I, Bam HI and Hind III subgenomic clones were found to express proteins whose sizes were indistinguishable from P120 coded by wild type A-MuLV. These results indicate that the A-MuLV sequence coding for P120 terminates prior to the Hind III site. Transformants obtained with the Sal I DNA subclone demonstrated anti-M-MuLV precipitable proteins, whose sizes varied from 82,000 to 95,000 daltons among individual transformants tested.

Previous studies have indicated that wild type A-MuLV coded P120 possesses an associated protein kinase activity. Analysis of transformants induced by different A-MuLV DNA subclones in each case revealed A-MuLV-specific proteins with this activity. Moreover, the sizes of the proteins corresponded to those of the A-MuLV-specific proteins precipitable with anti-MuLV serum in each transformant. Protein kinase activity was even preserved in the Sal I subclone coded 82,000 daltons protein. These results help to localize the coding region for the A-MuLV transforming protein responsible for its kinase activity to sequences within the proximal 40% of abl.

Expression of an A-MuLV abl gene homologue in human cells. In collaboration with A. Eva-Varesio, expression of the cellular homologue of the abl gene in human cells was analyzed. Normal fibroblasts revealed only two abl-related transcripts, whereas a complex pattern of transcripts was observed in human tumor cell lines derived from sarcoma, carcinoma, melanoma and astrocytoma. The widespread presence in tumors and even normal fibroblasts of multiple transcripts related to the onc gene of A-MuLV suggests that expression of related human genes may be associated with an important cellular function(s).

Also, whether the multiple RNAs observed with the abl probe reflect RNA processing or transcription of more than one member of a family of related onc genes remains to be determined.

2. Proviral DNA of A-MuLV, like other RNA-containing animal viruses, has characteristic directly repeated sequences designated as LTR at either end of the genome. The LTR has sequence homologies to the 5' noncoding and 3' noncoding regions of eukaryotic genes, which indicates that these sequences may have a functional role in the expression of the viral genome. Site-specific mutagenesis was utilized to define regions of the 5' LTR required for the A-MuLV transforming gene function in vivo. A series of mutants, encompassing deletions of different domains, was constructed and assayed for viral gene expression (transforming activity) following transfection of NIH/3T3 cells. Removal of sequences upstream from the Xba I site in the LTR reduced considerably the transforming activity of proviral DNA. Deletion of one 72 bp repeat did not affect the transforming activity, and extension of this into the second 72 bp repeat did not seem to have any drastic effect. Recombinants lacking CAAT, TATA and cap site showed reduced levels of transformation. Recombinants lacking CAAT, TATA, cap site and poly A signals 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 understanding the basic processes underlying transformation. Results of these experiments are providing insights and approaches to a better understanding of human neoplasia.

Proposed Course:

1. 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.
2. The complete nucleotide sequence of the A-MuLV genome is being determined by P. Reddy in our laboratory. Since the abl gene codes for a protein (P120) which is essential for transformation of cells, an attempt will be made to alter P120 to study the functional domains. Oligonucleotide-directed mutagenesis, using the available sequence data, provides a powerful method for creating such redesigned proteins.

Publications:

Balachandran, R., and Srinivasan, A.: Caffeine inhibits DNA polymerase I from Escherichia coli: studies in vitro. Carcinogenesis 3: 151-153, 1982.

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.H., Wong-Staal, F., Gallo, R.C., and Aaronson, S.A.: Cellular genes analogous to retroviral onc genes are transcribed in human tumor cells. Nature 295: 116-119, 1982.

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.H., Wong-Staal, F., Gallo, R.C., and Aaronson, S.A.: Transcription of retrovirus onc gene analogues in human solid tumor cells. In Yohn, D. (Ed.): Comparative Research on Leukemia and Related Diseases. New York, Elsevier/North Holland, 1982, 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, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05062-04 LCMB		
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>				
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Transforming Genes Associated with Naturally Occurring and Virus-Induced Tumors</p>				
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT				
PI: OTHERS:	A. Eva-Varesio S.A. Aaronson M. Barbacid K.C. Robbins S.R. Tronick	Visiting Associate Chief Visiting Scientist Expert Microbiologist	LCMB LCMB LCMB LCMB LCMB	NCI NCI NCI NCI 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: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0		
CHECK APPROPRIATE BOX(ES)				
<input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER				
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS				
SUMMARY OF WORK (200 words or less - underline keywords)				
<p>The goals of this project are (1) to study the <u>mechanism of tumor induction</u> by mouse leukemia viruses and to identify the <u>possible transforming genes</u> involved in this virus-induced neoplastic transformation, and (2) to investigate the regulation of expression of retroviral transforming gene analogues in human cells with the aim of elucidating the possible role of these genes in <u>human neoplasia</u>.</p>				

Project Description

Objectives:

1. To investigate whether DNA analogues of retroviral transforming genes are actively transcribed in human cancer cells in order to analyze the possible role of these genes in human neoplasia.
2. To investigate the mechanism by which mouse leukemia viruses cause neoplasia.

Methods Employed:

Standard and developmental techniques in cell biology, biochemistry, and recombinant DNA.

Major Findings:

1. We have shown that polyadenylated RNAs of certain human tumor cell lines contain transcripts related to the cell-derived transforming onc genes of molecularly cloned primate, murine or avian transforming retrovirus genomes. We found that analogues of retroviral transforming genes are both present and frequently expressed in neoplastic cells.
2. We analyzed mouse cell lines established from murine leukemia virus induced by lymphoma for the specific activation of several retrovirus onc gene analogues, viral DNA integration, and viral transcript structure. Our data suggest that a mechanism different from the one described in the avian leukemia virus-induced tumor is probably involved in the MuLV-induced neoplasia.

Significance to Biomedical Research and the Program of the Institute:

Since many transforming RNA tumor viruses are known to cause sarcomas and hematopoietic malignancies through the expression of certain genes derived from a set of well-conserved cellular genes, it is of interest to investigate whether and how these genes are specifically expressed in naturally occurring and virus-mediated malignancies.

Proposed Course:

1. To extend the study of expression of virus transforming gene analogues in human tumor cells and tissues in order to better define their quantitative and/or qualitative specificity of expression in an attempt to establish their involvement in human neoplastic transformation.
2. Analysis of MuLV-induced tumor DNAs for transforming activity in transfection assays in order to identify the transforming cellular gene associated with mouse leukemia virus-induced leukemias.

Publications:

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, S., Westin, E.H., Wong-Staal, F., Gallo, R.C., and Aaronson, S.A.: Cellular genes analogous to retroviral onc genes are transcribed in human tumor cells. Nature 295: 116-119, 1982.

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 gene 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.

Westin, E.H., Gallo, R.C., Arya, S.K., Eva, A., Souza, L.M., Baluda, M.A., Aaronson, S.A., and Wong-Staal, F.: Differential expression of the amv gene in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79: 2194-2198, 1982.

Westin, E., Wong-Staal, F., Gelmann, E., Baluda, M., Papas, T.S., Eva, A., Reddy, E.P., Tronick, S.R., 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.

Westin, E.H., Wong-Staal, F., Gelmann, E.P., Dalla Favera, R., Papas, T.S., Lautenberger, J.A., Eva, A., Reddy, E.P., Tronick, S.R., Aaronson, S.A., and Gallo, R.C.: Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79: 2194-2198, 1982.

PERIOD COVERED October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
 Studies on Epstein-Barr Virus and Herpesvirus saimiri

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Ablashi	Coordinator of DNA Virus Studies	LCMB	NCI
OTHERS:	P. Levine	Chief, Clinical Studies Section	LVC	NCI
	S.K. Sundar	Visiting Fellow	LVC	NCI
	A.M. Faggioni	Visiting Fellow	LCMB	NCI
	S. Straus	Chief, Clinical Virology Section	LCI	NIAID
	D. Longo	Medical Officer	MB	NCI

COOPERATING UNITS (if any) G. Krueger, Pathology Inst., U. Cologne, West Germany;
 G. Pearson, Mayo Clinic, Rochester, Minnesota; U. Prasad, U. Malaya, Kuala Lumpur, Malaysia; S. Baron, U. Texas, Galveston, Texas

LAB/BRANCH Laboratory of Cellular and Molecular Biology

SECTION Office of the Chief

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.0	OTHER: 2.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) (1) The sera of undifferentiated nasopharyngeal carcinoma (NPC) patients in active disease but not in remission contained a lymphocyte stimulation inhibitor (LSI) which abrogated the response of sensitized lymphocytes from Epstein-Barr virus (EBV) seropositive individuals to EBV antigens. LSI was not detected in sera from EBV-seropositive or negative healthy individuals, nor in sera from patients with non-NPC carcinomas of the head and neck, and thus may prove an NPC marker of clinical, diagnostic and prognostic significance. (2) The role of EBV in immunodeficiency was explored in chronic lymphocytic leukemia (CLL) and recurrent or chronic infectious mononucleosis (IM). Elevated EBV levels were demonstrated in 6/13 CLL and most IM patients, suggesting EBV involvement in CLL as well as IM. They also suggest that an increase in EBV levels in immunocompromised patients may presage increasing risk of lymphoproliferative disorders and malignancies, and signal the need for aggressive antiviral and supportive therapy. (3) A human lymphoblastoid B cell line, replicating high levels of transfer factor, was found also to produce high levels of leukocyte interferon which exhibited broad antiviral activity.

Project DescriptionObjectives:

To study the EBV association in NPC and in certain immunodeficiency disorders which may predispose individuals to risk of developing EBV-induced lymphoproliferative disorders and malignancies. Secondly, to use EBV-related serology for long-term prognosis in NPC patients, particularly those with undifferentiated NPC. Thirdly, to explore antiviral therapy to prevent reactivation of EBV and Herpesvirus saimiri (HVS)-induced pathogenesis.

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.

Herpesvirus saimiri and EBV virus-infected cell lines were used to study the effects of antiviral therapy or interferon.

Monoclonal antibodies to transforming EBV membrane glycoproteins (i.e., 320K), were prepared by hybridoma technology.

Major Findings:

A unique association of EBV with undifferentiated NPC is an acknowledged phenomenon. IgA antibody to EBV virus capsid antigen (VCA) was demonstrated to aid in NPC diagnosis. A factor, the lymphocyte stimulation inhibitor (LSI), was detected in the sera of NPC patients. LSI inhibited the blastogenic response of lymphocytes from EBV seropositive individuals to UV-treated EBV or to soluble antigen from EBV-nonproducer Raji cells. The LSI was associated with the IgA fraction of the serum immunoglobulins. No inhibitory activity was detected in the sera or immunoglobulins derived from 10 healthy controls or from 20 patients with carcinomas other than NPC of the head and neck regions. Interestingly, the IgA-LSI was absent in 4 sera obtained from NPC patients in remission, but was readily detectable in 2 sera from patients in relapse. The LSI-positive IgA fractions did not inhibit the mitogenic response of lymphocytes to phytohemagglutinin, thus suggesting specificity of the inhibitor to EBV antigens. LSI activity was further correlated with NPC patient status to determine the applicability of this clinical marker. The LSI data was based on serial serum samples of 8 NPC patients followed for a period of 3 years at the University of Cologne, West Germany. These data correlated the rise and fall in LSI titers with active tumor burden, relapse, response or lack of response to radiation or other therapy, and remission. Thus, LSI represents a marker of clinical and epidemiological significance regarding undifferentiated NPC.

NPC is rare in Indians, and ethnic Malay males are considered to be at intermediate to low risk. Three patients (1 Indian adult male; and 2 adult Malays, 1 male and 1 female, who had swellings behind their ears and lumps on both sides of their necks), were diagnosed as having carcinomas non-NPC in origin. Sera and tumor cells from these patients were tested for the presence of EBV nuclear antigen (EBNA) at the hospital clinic of the University of Malaya and were found to have elevated levels of EBV-IgG antibodies to VCA and early antigen (EA), and IgA antibodies to VCA, thereby establishing their tumors as nasopharyngeal carcinomas. Moreover, the presence of EBNA in the tumor cells, and later histological diagnosis, confirmed these tumors to be NPC of the undifferentiated type. Thus, EBV serology was valuable for correcting the diagnoses and also for localization of the tumors for radiotherapy. Reacting monoclonal antibody against 2F5.6, an EBV membrane protein, with peripheral blood lymphocytes from 1 of the 3 patients confirmed the presence of EBV.

Although the etiological role of EBV in NPC and Burkitt's lymphoma (BL) is well documented, recent investigations indicate that the elevated levels of EBV in immunodeficient and immunosuppressed patients may be involved in their increased risk of fatal lymphoproliferative disorders, lymphomas and other cancers. Examples of such groups include recipients of renal allografts and cardiac transplants, and individuals with X-linked lymphoproliferative syndrome, Duncan's disease and CLL. Two patients at the Mayo Clinic showed EBV serologic rises 15 months prior to developing CLL, suggesting EBV involvement. Fresh peripheral blood lymphocytes from 4/11 CLL patients from the Clinical Center, NIH, reacted to EBV 2F5.6 monoclonal antibodies (3-17% membrane immunofluorescence) and had EBV titers in serology similar to those observed in BL or NPC. In addition, cell-free preparations from uncultured lymphocytes of these patients transformed human cord blood lymphocytes and the immortalized cells were shown to contain EBNA. The finding of elevated EBV-VCA IgA was unique, since the latter is predominantly associated with NPC. The absence of reactivity to 2F5.6 monoclonal antibody following Acyclovir treatment suggested EBV activation. In view of this evidence, efforts to control EBV activation and expression by vigorous immuno- or antiviral therapies should be pursued in high risk immunocompromised populations.

Similarly, chronic and recurrent IM illness, characterized by fever, pharyngitis, hepatosplenomegaly and atypical lymphocytosis was studied for EBV involvement and possible antiviral therapy. The data revealed increased levels of EBV antibodies, particularly to IgM and EA-R. Secondly, transforming EBV could be isolated from throat and peripheral lymphocytes, and viral isolation correlated with the presence of 2F5.6 monoclonal antibody-positive and EBNA-positive cells. Treatment with Acyclovir prevented the reactivity of 2F5.6 antibody for 3-4 weeks following treatment; however, no immediate change in EBV antibodies to VCA or EA were observed. The persistence of VCA-IgM for longer periods of time in some IM patients suggested either early infection, reinfection or reactivation of virus; the titers of anti-EA-R support the latter.

A human lymphoblastoid B cell line, LDV/7, capable of replicating significant levels of transfer factor and immuno-RNA, was found to produce high quantities of interferon continuously. The maximum yield of interferon (600 international units/ml) was detected on the 7th day. The interferon was characterized as the human leukocyte(α) type. It exhibited broad antiviral activity against vesicu-

lar stomatitis virus, poliovirus, Sindbis virus, Herpesvirus saimiri and EBV. The treatment of LDV/7 cells with the tumor-promoting agents PHA or LPS failed to enhance interferon levels, but infection with Sendai virus markedly enhanced interferon production. Production levels correlated best with cell proliferation.

Significance to Biomedical Research and the Program of the Institute:

The detection of LSI in sera of NPC patients and not from patients with non-NPC head and neck tumors or healthy individuals points up the specificity and potential importance of this test in diagnosis and disease management, particularly with regard to response to therapy or early indication of relapse. In 8 NPC patients in which serial serum samples were only 3-5 months apart, LSI titers correlated with the clinical status of the patient. To our knowledge, no similar markers for measuring subtle clinical changes over short periods of time are available.

The EBV serology data demonstrate its diagnostic value for clinical oncologists and radiotherapists, particularly in equivocal cases. The use of EBV serology or monoclonal antibody assays in monitoring recurrent or chronic IM or CLL promises to provide important aids in diagnosis, prevention and treatment of individuals at risk for life-threatening EBV-induced disorders. The detection of EBV in CLL raises the possibility of its having a contributory role in the etiology of that disease. However, it is not yet clear whether patients with CLL have defects in T- or B- cell immunity which might render them more susceptible to EBV infection. Thus, it remains to be determined whether EBV is the cause or consequence of this and other lymphoproliferative disorders. T and B cell deficiencies may predispose to activation of a latent EBV genome in host cells. Certainly this has been observed in at least some patients with immunodeficiencies who develop B-cell lymphomas.

Lastly, the spontaneous production of interferon by LDV/7 suggests that this human cell line could prove an excellent and inexpensive method of producing large amounts of human leukocyte interferon, as well as for cloning the interferon genome and for studies of gene expression. Interferon produced by the LDV/7 can also be studied to determine whether it has properties distinct from those of virus-induced lymphoblastoid interferon and for its comparative antitumor activity.

Proposed Course: This project will be followed up, taking advantage of new developments described above, for as long as the Principal Investigator remains in laboratory research.

Publications:

Ablashi, D.V., Allal, L., Armstrong, G., Bouguermouh, A., Pearson, G.R., Levine, P.H., Bengali, Z., Easton, J.M., Zaghouni, S., Chouiter, A., Allouache, A., and Ghouadli, R.: Some characteristics of nasopharyngeal carcinoma in Algeria. In Grundmann, E., Krueger, G., and Ablashi, D. (Eds.): Cancer Campaign Series Vol. 5 on Nasopharyngeal Carcinoma. New York/Stuttgart, Gustav Fischer Verlag, 1981, pp. 157-167.

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Faggioni, A., Ablashi, D.V., Armstrong, G., Sundar, S., Loeb, W., Martin, D., Valerio, M., Parker, G., and Fox, R.: Herpesvirus saimiri induced malignant lymphoma of poorly and well differentiated types in three inbred strains of New Zealand white rabbits. In Yohn, D. (Ed.): Comparative Research in Leukemia and Related Diseases. New York, Elsevier/North Holland, 1981, pp. 383-384.

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., in press.

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Hass, W., Pearson, G.R., Ablashi, D.V., Krueger, G.R.F., Bertram, G., and Sesterhenn, K.: Serological and immunological data indicating the prognosis of NPC. In Grundmann, E., Krueger, G., and Ablashi, D. (Eds.): Cancer Campaign Series Vol. 5, Nasopharyngeal Carcinoma. New York/Stuttgart, Gustav Fischer Verlag, 1981, pp. 213-218.

Karpinski, A., Krueger, G.R.F., Wustrow, J., Ablashi, D.V., Sesterhenn, K., Bertram, G., and Pearson, G.: EBV antibody titers in various histological types of NPC. In Grundmann, E., Krueger, G., and Ablashi, D. (Eds.): Cancer Campaign Series Vol. 5, Nasopharyngeal Carcinoma. New York/Stuttgart, Gustav Fischer Verlag, 1981, pp. 95-99.

Krueger, G.R., Kottaridis, S.D., Wolf, H., Ablashi, D.V., Sesterhenn, K., and Bertram, G.: Comparison of various histologic types of nasopharyngeal carcinoma with Epstein-Barr virus serological data and its implications. Anticancer Res. 1: 187-194, 1981.

Levine, P.H., Pizza, G., Cannon, G., Ablashi, D.V., and Armstrong, G.R.: Cell mediated immunity to Epstein-Barr virus associated membrane antigen in patients with nasopharyngeal carcinoma. In Grundmann, E., Krueger, G., and Ablashi, D. (Eds.): Cancer Campaign Series Vol. 5, Nasopharyngeal Carcinoma. New York/Stuttgart, Gustav Fischer Verlag, 1981, pp. 137-144.

Lvovsky, E., Levine, P.H., Fuccillo, D., Ablashi, D.V., Bengali, Z.H., Armstrong, G.R., and Levy, H.B.: Epstein-Barr virus and Herpesvirus saimiri sensitivity to interferon and interferon inducers. J. Natl. Cancer Inst. 66: 1013-1019, 1981.

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., in press.

Pizza, G., Viza, D., Ablashi, D.V., Jerome, L., Armstrong, G.R., and Levine, P.H.: The possible use of specific transfer factor in the treatment of patients with nasopharyngeal carcinoma. In Grundmann, E., Krueger, G., and Ablashi, D. (Eds.): Cancer Campaign Series Vol. 5, Nasopharyngeal Carcinoma, New York/Stuttgart, Gustav Fischer Verlag, 1981, pp. 301-307.

Sundar, S.K., Ablashi, D.V., Armstrong, G.R., Zipkin, M., Faggioni, A., and Levine, P.H.: Steroids inhibit tumor promoting agent induced Epstein-Barr virus early antigens in Raji cells. Int. J. Cancer 28: 503-507, 1981.

Sundar, S.K., Ablashi, D.V., Kamaraju, L.S., Levine, P.H., Faggioni, A., Armstrong, G., Pearson, G.R., Krueger, G.R.F., Hewetson, J.F., Bertram, G., Sesterhenn, K., and Menezes, J.: Sera from patients with undifferentiated nasopharyngeal carcinoma contain a factor which abrogates specific Epstein-Barr virus antigen-induced lymphocyte response. Int. J. Cancer 29: 407-412, 1982.

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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, in press.

Whitmann, J.E., Lemp, J.F., Hung, C., Ablashi, D.V., and Crowley, G.M.: Purified human interferons: prospects for therapy in NPC. In Grundmann, E., Krueger, G., and Ablashi, D. (Eds.): Cancer Campaign Series Vol. 5, Nasopharyngeal Carcinoma: New York/Stuttgart, Gustav Fischer Verlag, 1981, pp. 137-144.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05112-03 LCMB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Murine Leukemia Viruses: Mechanism of Leukemogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Storch	Research Associate	LCMB	NCI
OTHERS:	S.A. Aaronson	Chief	LCMB	NCI
	P. Arnstein	Veterinary Officer	LCMB	NCI

COOPERATING UNITS (if any)

T.M. Chused, LMI, NIAID

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have sought to understand the mechanisms by which murine leukemia viruses induce tumors by identifying 1) preleukemic changes in the lymphocyte compartment of MuLV-infected mice using fluorescent reagents and flow cytometry; 2) specific defects in lymphocyte ontogeny and function that confer resistance to or alter the target for transformation using inbred mouse strains; and 3) the region of the MuLV genome responsible for transforming specific lymphoid cells by generating recombinants between MuLVs with different targets.

Project DescriptionObjective:

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 3 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- and null cells. Most of the null cells express determinants associated with an early cell in T lymphocyte ontogeny. 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 in association with a delay in the appearance of lymphoma. This relative resistance of CBA/N 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 2 autosomal genes, one of which controls dualtropic virus replication. In addition, an X-linked resistance gene can be demonstrated in crosses to certain susceptible strains. MuLV recombinants containing a Moloney gag and a Rauscher env region induce a high proportion of T cell lymphomas.

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:

1. Identify the sequence of changes in the composition and function of the lymphoid compartment that terminates in malignancies of specific lineages of lymphocytes.
2. Correlate these changes with the appearance of viral recombinants and specific chromosomal alterations.

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 Fox, C.F. and Burger, M.M. (Eds.): Progress in Clinical and Biological Research: Differentiation and Function of Hematopoietic Cell Surfaces. New York, Allen R. Liss, Inc., in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Human Monoclonal Antibodies Reactive with Human Mammary Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jeffrey Schlom	Chief, Experimental Oncology Section	LCMB	NCI
OTHER:	David Wunderlich	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

C. Alford, Dept. of Surgery, George Washington Univ.,
Washington, D.C.

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

0.2

OTHER:

0.8

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lymphocytes from lymph nodes obtained at mastectomy from breast cancer patients have been fused with murine non-immunoglobulin (Ig) secreting myeloma cells to obtain human-mouse hybridoma cultures that synthesize human monoclonal antibodies. Several assays were used to demonstrate that the Ig's produced by the interspecies hybridomas were indeed human and not murine. The immunologic reactivities of the human Ig's were assayed with the immunoperoxidase method using tissue sections of the primary tumor from the patient whose lymphocytes were used for fusion, as well as using tissue sections from other primary tumors. One human IgM monoclonal was used to discriminate between mammary carcinoma cells (from 55 of 59 patients) and normal mammary epithelial cells, stroma, or lymphocytes of the same breast. This same antibody reacted with selected non-breast carcinomas and metastatic mammary carcinoma cells in lymph nodes and at distal sites. Experiments are in progress to develop nonhuman primate monoclonal antibodies using lymphocytes from old world monkeys and chimpanzees immunized with membrane enriched extracts of human mammary tumors.

Project DescriptionObjectives:

To characterize human monoclonal antibodies generated by the fusions of human lymphocytes from axillary lymph nodes of mastectomy patients with murine and human myeloma cells. In addition, to characterize nonhuman primate monoclonal antibodies generated by the fusions of lymphocytes from chimpanzees and Old World monkeys immunized with membrane-enriched extracts of human mammary cancer. The human and nonhuman primate monoclonal antibodies may ultimately be useful in the in situ diagnosis and/or therapy of human mammary cancer.

Methods Employed:

Human-mouse, human-human, nonhuman primate-mouse, and nonhuman primate-human hybridomas are generated using some modifications of standard fusion and hybridoma techniques. Human and nonhuman primate immunoglobulin production by the hybridomas is detected using specific solid phase and immunobead competition radioimmunoassays. The immunoreactivities of the human and nonhuman primate monoclonal antibodies are elucidated by employing solid phase radioimmunoassays and the immunoperoxidase technique.

Major Findings:I. Human monoclonal antibodies.

(A) Generation and characterization of human-mouse hybridoma cultures. In our first series of experiments, 1460 microtiter wells have been seeded with fusion products of mouse NS-1 myeloma cells and lymphocytes from 16 patients. Of these, 301 human-mouse hybridoma cultures have been successfully propagated. All of the patients had infiltrating duct or lobular carcinoma. The percentage of wells planted that yielded viable hybridoma cultures varied among patients; the reasons for this variability are unknown at present. Replicating human-mouse hybridoma cultures were obtained from lymph node segments of 81% of patients.

Hybridoma cultures were first tested for synthesis of human IgG or IgM 14-28 days after fusion. Of the 301 replicating hybridoma cultures, 17% synthesized either human IgG or human IgM. The duration of human Ig synthesis ranged from 14 to greater than 300 days. The level of human Ig production, as measured by liquid immunobead competition and solid phase radioimmunoassays, were comparable to those detected in our laboratory with both mouse-mouse and mouse-rat hybridomas. Many of the cloned human-mouse hybridoma cultures were unstable for Ig production. For example, only 7 of 21 primary clones of hybridomas from patient MB, shown to be positive for Ig production on day 71, were still positive when assayed on day 94. Upon recloning of the three clones highest for Ig synthesis, however, 126 of 129 secondary clones remained positive for human Ig synthesis for greater than the 300-day observation period.

(B) Characterization of human Ig's. Several assays were used to determine if the Ig's secreted by the human-mouse hybridoma cultures were indeed human and not murine. These included: (a) immunobead liquid competition radioimmunoassays, (b) solid phase linker radioimmunoassays, and (c) Ouchterlony double-diffusion. The

immunobead and solid-phase radioimmunoassays could also be used to quantitate human IgG or IgM synthesis. Mouse Ig's did not react in these assays. Two additional anti-murine Ig assays were used to determine if the human-mouse hybridoma supernatant fluids displayed any evidence of murine Ig's; both assays gave negative results. Several of the human-mouse hybridoma supernatant fluids were analyzed by SDS polyacrylamide gel electrophoresis and found to possess complete Ig (i.e., both heavy and light chains). Monoclonal MBE6 also demonstrated a sedimentation coefficient of approximately 19 S, corresponding to pentameric IgM.

(C) Immunoreactivity of human monoclonal antibodies. To define the immunologic reactivities of the human Ig's secreted by human-mouse hybridoma cultures, supernatant fluids were analyzed on tissue sections of human mammary tumors using the immunoperoxidase technique. Supernatant fluids from hybridoma cultures derived from five different patients showed differential reactivity with mammary carcinoma cells in sections of their own mammary tumors. One monoclonal antibody, MBE6, from double-cloned cultures from the fusion of lymphocytes from patient MB, was chosen for further study. Monoclonal MBE6 was first tested by immunoperoxidase for reactivity with tissue sections of the primary breast tumor mass (infiltrating duct carcinoma) of patient MB, and demonstrated marked cytoplasmic staining in most of the mammary tumor cells. Differences in both intensity of staining and percentage of cells stained were observed in various areas of the tumor. Antibody MBE6 could distinguish clearly between malignant mammary cells and normal mammary epithelium or stromal cells of the same breast. MBE6 was then tested for its ability to detect metastatic mammary carcinoma cells in the lymph nodes of patient MB (13 of 16 lymph nodes were histologically positive for tumor); it could clearly distinguish between metastatic breast cells and adjacent lymphocytes. MBE6 was also tested for its ability to bind to primary and metastatic mammary carcinoma cells of patients other than MB. MBE6 bound to primary breast tumors from 55 of 59 patients and also detected metastatic breast lesions, in lymph nodes and at distal sites, from 20 of 20 patients. Preliminary studies also indicate a cross-reactivity between MBE6 and cells of selected non-breast carcinomas, such as bronchial alveolar carcinoma and medullary carcinoma of the thyroid. MBE6 did not stain smooth muscle, arteries, veins, and nerve bundles of various breasts and did not react with normal colon, thyroid, lung, or cartilage.

All five monoclonal antibodies obtained in our initial series of experiments that showed preferential reactivity for human mammary tumor cells were of the IgM isotype. All proved difficult to utilize in RIA's due to the high degree of nonspecific "sticking" to substrates such as plastic. Hybridomas from too few patients' nodes have been assayed thus far to determine if the lack of immunoreactive IgGs is just a chance occurrence, or if there is a biologic basis for only IgM production. To date, lymph nodes from 39 additional patients have been used in fusions with murine NS-1 cells; 41 IgG and 25 IgM secreting cultures have been generated. They are currently being assayed for selective binding to mammary carcinoma cells using solid phase RIAs. Lymphocytes obtained from recently acquired nodes are also now being divided into two aliquots: one part being fused with the murine NS-1 non-Ig secretor and the other with human myeloma cells.

II. Nonhuman primate (NHP) monoclonal antibodies.

We have developed solid phase RIAs for the detection of NHP Ig's. Two Patas and two Cynomolgous (cyno) monkeys (both Old World) were immunized with membrane-enriched fractions from either a human breast metastatic lesion or the MCF-7 mammary carcinoma cell line. Pre- and post-immunization bleeds showed that the monkeys were making Ig's which are preferentially reactive with the immunogen. Using standard hybridoma technology, lymphocytes from lymph node biopsies (draining immunization sites) were fused with murine NS-1 myeloma cells. Preliminary results indicate that some of the NHP-hybridoma cultures are synthesizing immunoglobulins that are preferentially reactive (in initial screens) with the immunogen.

Significance to Biomedical Research and the Program of the Institute:

The development of a human or NHP monoclonal antibody that is preferentially reactive with human mammary carcinoma cells would have an obvious advantage in in situ diagnosis (gamma scanning for distal metastasis or in lymphangiography), and/or ultimately in antibody-mediated therapy of human mammary cancer. In particular, the generation of Fab or Fab'₂ fragments of human or NHP monoclonals in these studies would minimize any host anti-allotype response. It should be pointed out that a recent study from the NIH laboratory of Dr. Fauci has utilized our protocols and succeeded in the development of a human monoclonal antibody to KLH (keyhole limpet hemocyanin) following fusion of human lymphocytes with murine NS-1 myeloma cells.

Proposed Course:

We plan to continue to fuse lymphocytes from lymph nodes of mastectomy patients with the murine and human myeloma lines. We also plan to alter conditions of fusion in an attempt to enhance efficiency of immunoglobulin production. Parameters such as ratios of lymphocytes to myeloma cells and *in vitro* immunization with sterile purified antigen preparations will also be investigated.

We are in the process of characterizing the nonhuman primate (NHP) IgG's produced by hybridomas resulting from the fusion of murine myeloma cells and lymphocytes from monkeys immunized with tumor fractions. Solid phase RIAs for human and NHP Ig's have already been developed in our laboratory. Chimpanzees have recently been inoculated with membrane enriched fractions of human breast tumor cells. As with the Old World monkey experiments, peripheral blood and node lymphocytes will be fused with both the murine myeloma and human myeloma lines. We have already determined that chimpanzee Ig's react in assays developed for either NHP or human Ig's.

Publications:

Teramoto, Y.A., Mariani, R., Wunderlich, D., and Schlom, J.: The immunohistochemical reactivity of a human monoclonal antibody with tissue sections of human mammary tumors. Cancer, in press.

Wunderlich, D., Teramoto, Y.A., Alford, C., and Schlom, J.: The use of lymphocytes from axillary lymph nodes of mastectomy patients to generate human monoclonal antibodies. Eur. J. Cancer Clin. Oncol. 17: 719-730, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Study of Neoplasias of Outbred Colonies of Feral Species of the Genus Mus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert Callahan	Microbiologist	LCMB	NCI
OTHERS:	Jeffrey Schlom	Chief, Experimental Oncology Section	LCMB	NCI
	Michael Potter	Chief, Immunochemistry Section	LCBGY	NCI
	Steven Tronick	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0-0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINDS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

High stringency blot hybridization analysis of restricted cellular DNA from the different species of mice, using cloned MMTV proviral DNA as a probe, has led to the identification of M. musculus, M. caroli, and M. pahari breeding colonies which lack the genetically transmitted MMTV genome (designated MMTV-negative mice). One other M. musculus colony was found to contain only the MMTV long terminal repeat region of the viral genome in its germline. By lowering the stringency of blot hybridization, we have identified a new class of highly diverged MMTV-related sequences (designated MMTV-B) in the cellular DNA of all species of the genus Mus, including MMTV-negative mice. Sequences related to the MMTV genome have also been detected in human cellular DNA using low stringency blot hybridization conditions. Several recombinant clones of human cellular DNA containing MMTV-related sequences have been isolated. The major region of homology corresponds to the gag-pol genes of the MMTV genome.

Project Description

Objective:

To develop colonies of feral species of *Mus* to identify new endogenous retroviral genes and genetic elements which affect their expression and/or involvement in neoplastic disease.

Methods Employed:

Retroviral related sequences were detected in restriction endonuclease digested recombinant and cellular DNAs by the Southern transfer-blot hybridization technique. Recombinant clones containing MMTV-related sequences were isolated from libraries of cellular DNA in Charon lambda phage. The pedigreed breeding colonies of feral mice have been described in the 1981 project report and in Callahan et al., Proc. Natl. Acad. Sci. USA, 1982, in press.

Major Findings:

Identification of a pedigreed breeding colony of feral *M. musculus* which lack MMTV proviral DNA in their germline. Restriction endonuclease digests of cellular DNA from individuals of feral *M. musculus* and *M. spretus* breeding colonies were initially tested for the presence of MMTV-related DNA fragments. Most striking are the number of the *M. musculus* colonies in which individuals carry either one to two copies of the viral genome or lack the viral genome. Thus, approximately 50% of the *M. m. brevisrostris* colony contains a single MMTV genome in their cellular DNA, the remainder lack the viral genome. Similarly, the *M. m. musculus* Czech I mice appear to be segregating two copies of the MMTV genome, while others lack the MMTV genome in their cellular DNA. A partially inbred strain of *M. m. molossinus* (line 3I) sent to us in 1977 by Dr. T. Roderick (Jackson Laboratory) represents a third population of mice which, in this case, are segregating a single MMTV genome. A survey of the *M. m. musculus* Czech II colony showed that this colony is completely free of the genetically transmitted MMTV genome. The *M. m. praetextus*, *M. m. musculus* Skive, and *M. m. molossinus* NIH appear to be homozygous for one to three copies of the MMTV genome at nonhomologous sites of the cellular genome. The colonies of the closely related species *M. spretus* contain a significantly greater genetic burden of MMTV genomes. Although this species in its natural habitat is reproductively separate from *M. musculus*, interspecies breeding has been successful in our laboratory.

We have also analyzed mice from two geographically isolated demes (natural breeding populations) from the eastern shore of Maryland for the frequency of MMTV-negative mice. The results of this study are consistent with those obtained with the breeding colonies of feral mice and lead us to conclude that natural demes of mice are heterozygous for a limited number of genetically transmitted genomes and that mice which lack them arose as a result of random chromosomal segregation.

Identification of a feral *M. musculus domesticus* colony which contains only the long terminal repeat (LTR) portion of a MMTV proviral genome in its germline. As a part of the study in the previous section, we have examined restriction endonuclease digested cellular DNA from feral *M. musculus domesticus* breeding colonies for the presence of genetically transmitted MMTV proviral genomes. These colonies

were established with mice trapped by Dr. M. Potter (NCI) on the eastern shore of Maryland. One of these colonies, designated Centerville Lights, contains only the long terminal repeat region of the MMTV viral genome. We conclude that the structural genes of the provirus were lost during an aberrant integration event or a reciprocal recombination event in the germline.

Detection of MMTV-related sequences in the cellular DNA of mice using relaxed conditions of hybridization. We have considered the possibility that by using stringent hybridization conditions partially related MMTV sequences could have gone undetected in the cellular DNA from the MMTV-negative mice. To test this possibility, the stringency of blot-hybridization was decreased so that DNA hybrids having a ΔT_m of 35°C or less (22% base pair mismatch) would be thermally stable. In PstI restricted cellular DNA, seven common MMTV-related fragments were observed (7.0, 6.4, 4.6, 3.7, 2.8, 2.6 and 1.5 kbp). The restricted cellular DNA from the inbred strains of mice contain additional MMTV-related fragments which correspond to those observed using stringent hybridization conditions. Although we have not determined the organization of these novel sequences in the cellular DNA, the relative intensity and size of the new bands suggest that they represent multiple copies of a family of highly mismatched MMTV-related sequences. On the basis of these results, we can distinguish two classes of MMTV related sequences in M. musculus cellular DNA which are defined by the stringency of the hybridization conditions (designated MMTV- α , high stringency; and MMTV- β low stringency).

In order to gain an insight into the evolutionary significance of this result, the scope of this analysis was broadened to include other species of the genus Mus. We have previously shown that the non-repetitive cellular DNA from members of this genus exhibits a broad spectrum of sequence homology. PstI digests of cellular DNA from several different species of mice were compared using high and low stringency hybridization conditions. Using high stringency blot-hybridization conditions, cloned MMTV proviral DNA reacts with distinct fragments of restricted cellular DNA from M. cervicolor cervicolor, M. cookii, M. dunni and M. short-ridgei. M. caroli- and M. pahari-restricted cellular DNAs do not react with the MMTV probe using these hybridization conditions. However, by relaxing the hybridization conditions, several new species-specific MMTV-related DNA fragments (MMTV- β) are detected in each of these cellular DNAs. In other experiments using low stringency hybridization conditions, the MMTV proviral DNA did not react with salmon sperm DNA or Moloney MuLV recombinant DNA. By comparing digests of cellular DNA from individual mice of several Mus species, major differences in the pattern of MMTV- β fragments are observed only between species. These observations suggest that the MMTV- β sequences may represent an accumulative "fossil" record of insertions of infectious MMTV proviral genomes into the murine germline. Alternatively, the lack of variation in the pattern of MMTV- β -related fragments in restricted cellular DNA from members of a given species suggests these sequences predate the MMTV- α proviral genomes.

Detection and molecular cloning of human MMTV-related DNA sequences. Using low stringency conditions of blot hybridization and cloned MMTV proviral DNA as a probe, we have detected MMTV-related sequences in restricted cellular DNA from a variety of mammals including humans. Sequences related to the gag-pol and env regions of the MMTV genome were detected in human cellular DNA. Using similar conditions of blot-hybridization, we have isolated several recombinant clones from

a phage lambda library of human fetal liver cellular DNA. Our preliminary characterization of the recombinant clones shows: (1) A 3.7 kbp restriction fragment hybridizes with restriction fragments of the MMTV genome which correspond to the pol gene. (2) The pattern of MMTV-related fragments in restricted human cellular DNA, probed with MMTV proviral DNA, using low stringency hybridization conditions is similar to that observed using high stringency hybridization conditions with the cloned 3.7 kbp human related fragment as a probe.

Significance to Biomedical Research and the Program of the Institute:

The development of pedigreed breeding colonies from an evolutionarily diverse group of species from the genus Mus provides a rich source of new genetic material as well as model systems for studying tumorigenesis. The generally low incidence of spontaneous tumors in these colonies suggests that they will be useful in future studies directed at the retrieval of induced transformation-specific genetic elements. In addition, the breeding colony of the MMTV-negative mice (M. m. musculus Czech II) will allow us to dissect the genetic and molecular interaction between genetically transmitted MMTV proviral genomes and exogenous carcinogens. Our recent efforts have led to the identification of a new class of MMTV related sequences (MMTV- β) which are found in all species of Mus as well as other mammalian species, including humans.

The role of endogenous retroviral genes in the etiology of human neoplasias is at present unknown. Significantly, however, human cellular genes related to the transforming genes of several mammalian and avian sarcoma retroviruses have been identified and molecularly cloned. An analogous transforming gene for MMTV, however, has not yet been identified. Attempts to identify components of retroviral particles by biochemical and immunological techniques in human breast tumors have met with some success. The isolation of recombinant clones containing human MMTV related sequences presents us with an opportunity to determine if a relationship exists between their expression and human mammary neoplasia.

Proposed Course:

We have recently initiated studies to determine the effect of chemical and biological carcinogens on the spontaneous incidence of mammary tumors in MMTV-negative M. musculus musculus Czech II mice. By selective breeding, we have also begun, in collaboration with Dr. M. Potter, NCI, to introduce specific endogenous MMTV- α proviral genomes from C3H and GR inbred strains onto the MMTV-negative background of the Czech II mice to definitively assess their role in the development of spontaneous and induced mammary tumors.

Restriction fragments of cellular DNA from M. musculus domesticus Centerville Lights, which contain only the MMTV- α LTR sequences, are currently being molecularly cloned. These recombinant DNAs will be used to determine: (1) the effect of the LTR on the expression of the host flanking sequences in lactating females by transfection of NIH/3T3 tissue culture cells; (2) if expression is detected, determine whether infectious MMTV can package and transmit the LTR-linked cellular sequences; (3) determine the effect of chemical carcinogen treatment on the incidence of mammary tumors and on the expression of the LTR sequences.

We have recently obtained molecular clones of MMTV- β sequences from a phage lambda library of BALB/c embryo cellular DNA. We are currently characterizing these clones by restriction enzyme mapping and comparing them with MMTV- α proviral DNA by heteroduplex mapping (in collaboration with Dr. S. Tronick, NCI). Using these clones, we plan to focus our efforts on the following questions: (1) determine the organization and genetic stability of these sequences in cellular DNA from different species of mice; (2) determine whether MMTV- β sequences are expressed in normal or neoplastic mouse tissues; (3) assess the biological activity of MMTV- β sequences by transfection of NIH/3T3 tissue culture cells with recombinant DNA.

Currently we are comparing the recombinant clones of MMTV-related human cellular DNA by restriction enzyme mapping and heteroduplex analysis (in collaboration with Dr. S. Tronick, NCI) with murine MMTV- α and MMTV- β proviral DNA. Upon completion of these studies, we anticipate addressing the following questions: (1) determine whether related RNA sequences are expressed in human mammary tumors; (2) determine the organization of the sequences in normal human cellular DNA and whether there are tumor-specific changes in their organization; and (3) assess the biological activity of the human MMTV related clones by transfection into E. coli and eucaryotic tissue culture cells.

Publications:

Callahan, R., Drohan, W., Gallahan, D., D'Hoostelaere, L., and Potter, M.: A novel class of MMTV related DNA sequences found in all species of Mus including mice lacking the MMTV proviral genome. Proc. Natl. Acad. Sci. USA, in press.

Callahan, R. Drohan, W., Tronick, S., and Schlom, J.: Identification and molecular cloning of human cellular DNA sequences related to the mouse mammary tumor virus. Proc. Natl. Acad. Sci. USA, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05159-02 LCMB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Immunoglobulin Gene Arrangement in Cells Transformed by Murine Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Balachandran	Visiting Fellow	LCMB	NCI
OTHERS:	S.A. Aaronson	Chief	LCMB	NCI
	E.P. Reddy	Visiting Scientist	LCMB	NCI
	D. Swan	Expert	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

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Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were carried out to understand cellular targets for in vivo transformation by two clonal replication competent retroviruses, Moloney- and Rauscher-MuLV. Moloney-MuLV was found to induce T-cell tumors in several strains of mice tested, while Rauscher-MuLV produced tumors of B-cell origin in the same strains of mice. The majority of Rauscher-MuLV lymphoma cell lines expressed immunoglobulin heavy (μ) chain in the absence of detectable light (κ or λ) chains. However, 10-20% of the tumors were comprised of immature cells making no Ig-related products or more differentiated cells producing both μ and κ chains. Studies on the structure of Ig-related genes revealed the rearrangement of μ -genes in essentially all cell lines, irrespective of μ -synthesis, while rearrangement of κ -genes was restricted only to producer cell lines.

Project DescriptionObjectives:

To study tissue specificity and mechanisms of leukemogenesis of replication competent Moloney- and Rauscher-murine leukemia viruses.

Methods Employed:

Tissue culturing of lymphoid cells, biosynthesis studies, radioimmunologic studies, restriction enzyme analysis of cellular DNAs, and hybridization studies with immunoglobulin gene-related probes.

Major Findings:

Studies were carried out to understand the cellular targets for in vivo transformation by two clonal replication-competent type C viruses, Moloney- and Rauscher-MuLV. Moloney-MuLV-induced tumors and lymphoma cell lines derived from NIH Swiss mice exhibited Thy-1 antigen in the absence of detectable Fc and C3 receptors, indicating their T-cell origin. However, Rauscher-MuLV primary tumors and lymphoma cell lines of the same mouse strain invariably exhibited Fc receptors in the absence of Thy-1 antigen, suggesting that the tumors were of the B lymphoid lineage. The pattern of immunoglobulin synthesis by these various cell lines further confirmed the above conclusion.

To examine whether the differences in target cells for transformation by the two viruses were due to genetic properties of the viruses as opposed to the properties of the host, we analyzed a large number of tumors induced by each virus in a variety of mouse strains. The results showed that almost all Rauscher-MuLV-induced tumors and tumor cell lines obtained with each strain exhibited the Fc⁺ Thy-1⁻ phenotype, while the great majority of tumors induced by Moloney-MuLV possessed T-cell markers. Such findings argued that this specificity must be genetically determined by virus-coded functions.

A detailed analysis of immunoglobulin synthesis in Rauscher-MuLV-transformed lymphomas was undertaken to determine whether a specific sub-population of B-cells are the targets for this virus-induced neoplasia. Thus, the expression of μ , and κ -chains was studied using biosynthetic and radioimmunologic techniques. These experiments revealed several different patterns of immunoglobulin production among these lymphomas. Approximately 10% of the cell lines lacked detectable amounts of μ and κ chains. The great majority of the cell lines tested contained μ in the absence of κ -chains with variable levels of μ -chain synthesis. In approximately 5-10% of the Rauscher-MuLV lymphoma cell lines analyzed, both μ and κ -chain synthesis was observed. Among more than 40 Rauscher MuLV lymphoma cell lines analyzed, none synthesized κ -chain in the absence of μ -chain. Thus, Rauscher-MuLV lymphomas reflect different stages of B lymphoid cell differentiation and offer a unique model system to study the progression of biochemical events that occur during the differentiation of a B lymphoid cell population.

To study the organization and reorganization of immunoglobulin genes in such a differentiating population of clonal cell lines, we analyzed the structure of immunoglobulin-related genes in individual Rauscher-MuLV-transformed lymphoid cell lines. Essentially all of these cell lines, whether immunoglobulin producing or not, had DNA rearrangements in the vicinity of the J_H regions of both chromosomes. In contrast, detectable rearrangement of κ -chains was observed only in those cell lines which were found to express κ -chains while there was no κ -chain gene arrangement in all the κ -nonproducer cell lines analyzed. The results appear to indicate that heavy chain gene rearrangement precedes that of light chain gene rearrangement and synthesis of μ -chain does not necessarily follow the rearrangement of these genes.

Significance to Biomedical Research and the Program of the Institute:

The Rauscher and Moloney-induced lymphomas provide a model system (a) to study the mechanisms involved in tissue-specificity of replication-competent leukemia viruses, and (b) to study the gene rearrangements involved in the differentiation of a B lymphoid cell.

Proposed Course:

Attempts will be made to understand the mechanisms involved in the differentiation of B lymphoid cells by studying the effect of mitogens on various sub-populations of B lymphoid cells.

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 Fox, C.F., and Burger, M.M. (Eds.): Progress in Clinical and Biological Research: Differentiation and Function of Hematopoietic Cell Surfaces. New York, Allen R. Liss, Inc., in press.

Balachandran, R., and Srinivasan, A.: Caffeine inhibits DNA polymerase I from Escherichia coli: studies in vitro. Carcinogenesis 3: 151-153, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05162-02 LCMB

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Oncogenic Herpesviruses of Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Faggioni	Visiting Fellow	LCMB	NCI
OTHERS:	D.V. Ablashi	Microbiologist	LCMB	NCI
	P.H. Levine	Head, Clinical Study Section	LVC	NCI

COOPERATING UNITS (if any)

H. Robin and W. Loeb, Litton Bionetics; S.K. Sundar, Hospital St. Justine, Montreal, Canada; G. Pearson, Mayo Clinic, Rochester, MN

LAB/BRANCH

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TOTAL MANYEARS:

1.0

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1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. A cell line established from the spleen of a III/J strain inbred New Zealand white rabbit, inoculated with Herpesvirus saimiri (HVS) and a tumor promoting agent, has been characterized. The cells (7710) are of T origin, are HVS nonproducer, but have been shown by hybridization that they are HVS DNA positive. The cells grow in tissue culture either in large clumps or as single cells only in the presence of a T-cell growth factor. These cells consistently induced a malignant T-cell lymphoma when transplanted in other III/J inbred rabbits.

2. The ability of an in vitro replicated transfer factor against Herpesvirus saimiri to transfer the immunity in negative hosts has been studied in several owl and rhesus monkeys.

Project Description

Objectives:

1. Characterization of a cell line consistently able to induce T cell malignant lymphomas when transplanted in New Zealand white rabbits.
2. Test whether the oncogenicity of the cell line is due to the virus released by the cells or by the cells themselves proliferating into the host.
3. Try to prolong the duration of the disease by inoculating a smaller amount of cells or using non-inbred rabbits, in order to have a reliable and stable model for evaluation of chemotherapy or immunotherapy of T cell lymphomas.
4. To test whether specific transfer factor against Herpesvirus saimiri is consistently able to transfer immunity.

Methods Employed:

1. The 7710 cells have been grown in culture for more than 30 passages in the presence of a T cell growth factor obtained from the supernatants of a baboon cell line, MLA 144. Various doses of cells, ranging from 10^2 to 10^8 , have been inoculated subcutaneously or intraperitoneally into various New Zealand white inbred rabbits of strains III/J or Ax. The rabbits have been checked weekly for presence of antibodies against Herpesvirus saimiri with indirect immunofluorescence technique. Virus isolation has been attempted periodically by cocultivation of rabbit's peripheral blood or lymphocytes with owl monkey kidney cells.
2. Several rhesus and owl monkeys have been inoculated with transfer factor directed against Herpesvirus saimiri and their cell mediated immunity tested with the direct leukocyte migration inhibition test.

Major Findings:

1. A nonproducer lymphoblastoid cell line (7710) containing the Herpesvirus saimiri genome was established from the spleen of a male inbred New Zealand white rabbit (III/J strain) which developed a well-differentiated lymphoma after inoculation of HVS and a tumor promoting agent (TPA). The cell line was characterized to be of T-cell origin, HVS nonproducer, not superinfectable with HVS but inducible for HVS early antigens with TPA. The cells failed to induce tumors in athymic nude mice. Five rabbits inoculated with these cells developed acute lymphoma, fatal in 12-18 days. Chromosomal analysis showed that both the inoculum cells and the cells recovered from the inoculation site of a female rabbit were of male origin, confirming that the tumors were induced directly by the donor cells. Chromosomal abnormalities were observed in 20% of the 7710 cells, and more frequently in the cells of a 7710-induced lymphoma. Chromosomes 6 and 15 were most frequently involved in abnormalities. Blot hybridization has shown that 7710 cells contain circular HVS DNA with deletions which are different from those found in nonproducer marmoset cell lines. These cells, therefore, represent a useful model for molecular studies of HVS-induced tumors in different species.

2. Ten rhesus monkeys and six owl monkeys have been tested for their cellular immune response specific against Herpesvirus saimiri. All the animals found to be non-immune were inoculated with 5×10^8 units of dializable transfer factor against HVS replicated in vitro by a lymphoblastoid cell line (LDV/7). Transfer of immunity was demonstrated by the leukocyte migration inhibition assay in three rhesus and two owl monkeys. Thus, it has been demonstrated that transfer factor replicated in vitro can transfer immunity to oncogenic viruses.

Significance to Biomedical Research and the Program of the Institute:

The 7710 cells-rabbit system is the first report where HVS nonproducer cells have been shown to be tumorigenic. For its reliability, low cost and reproducibility, this cell tumor system could be a very useful model for the study of lymphoblastic lymphoma of man. Moreover, this system may allow several in vivo viral, molecular and cell mediated investigations.

Transfer factor can be prepared against OMK membrane antigens whether or not associated with the oncogenic lymphotropic HVS. The present studies in animals may be relevant to the treatment of virus induced diseases in man. In particular, transfer factor against Epstein-Barr virus could be used in clinical trials for therapy of patients with Burkitt's lymphoma or nasopharyngeal carcinoma.

Proposed Course:

Termination of project.

Publications:

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). Proc. Soc. Exp. Biol. Med., in press.

Ablashi, D.V., Glaser, R., Faggioni, A., Easton, J.M., and Armstrong, G.: Animal models for the study of nasopharyngeal carcinoma. In Proceedings of the UICC Workshop on Nasopharyngeal Carcinoma, in press.

Bertram, G., Pearson, G.R., Faggioni, A., Armstrong, G., Krueger, G.R.F., and Ablashi, D.V.: Das Nasopharyngeale Karzinom (NPC): Prognostische Aussagekraft des Antikörperabhängigen Zellulären Zytotoxizitätstestes (ADCC). Arch. Oto-rhinolaryngol 231: 768-773, 1981.

Faggioni, A., Ablashi, D., Armstrong, G., Sundar, S.K., Merrill, R., Martin, D., Valerio, M., Parker, G. and Fox, R.: Herpesvirus saimiri induced malignant lymphoma of the poorly and well differentiated types in three inbred strains of New Zealand white rabbit. In Yohn, D. (Ed.): Comparative Research on Leukemia and Related Diseases, New York, Elsevier/North Holland, 1981, pp. 383-384.

Sundar, S.K., Ablashi, D.V., Armstrong, G.R., Zipkin, M., Faggioni, A., and Levine, P.H.: Steroids inhibit tumor promoting agent induced Epstein-Barr virus early antigens in Raji Cells. Int. J. Cancer 28: 503-507, 1981.

Sundar, S.K., Ablashi, D.V., Kamaraju, L.S., Levine, P.H., Faggioni, A., Armstrong, G.R., Pearson, G.R., Krueger, G., Hewetson, J.F., Bertram, G., Sesterhenn, K., and Menezes, J.: Sera from patients with undifferentiated nasopharyngeal carcinoma contain a factor which abrogates specific Epstein-Barr virus antigen-induced lymphocyte response. Int. J. Cancer 29: 407-412, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05163-02 LCMB
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanisms of Leukemogenesis by Rauscher and Moloney Murine Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Habara	Visiting Fellow	LCMB	NCI
OTHERS:	P. Reddy	Visiting Scientist	LCMB	NCI
	S. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In an attempt to develop model systems for understanding the mechanisms of type C virus-induced mouse leukemias, we have analyzed target cell specificities for transformation by different viruses. It has been possible to show that clonal strains of M-MuLV and R-MuLV induce tumors of T and B lymphoid cells, respectively. Since these viruses have similar genomic structures and code for related components, we reasoned that a genetic approach might make it feasible to localize the region of the viral genome responsible for target cell specificity. In efforts aimed at construction of molecular recombinants between Rauscher and Moloney leukemia virus genomes, we cloned the integrated proviral DNA of R-MuLV. Taking advantage of the common restriction sites that occur in the two viral genomes, we constructed several recombinants between the two proviral DNA molecules. These recombinant DNA molecules, when transfected into NIH/3T3 cells, yielded retroviruses that contained different regions of M-MuLV and R-MuLV DNA.

Project DescriptionObjectives:

To study mechanisms of oncogenesis of Rauscher (R-MuLV) and Moloney murine leukemia viruses (M-MuLV) which induce tumors in B and T lymphoid cells, respectively.

Methods Employed:

Restriction enzyme digestions (double digestion, partial digestion), Southern blotting, molecular cloning, transfection.

Major Findings:

1. R-MuLV was cloned in its integrated form as follows: cellular DNA was isolated from normal rat kidney (NRK) cells after infection with clonal R-MuLV and was digested with Eco RI which has already been shown not to cleave unintegrated viral genome. An Eco RI fragment containing R-MuLV-specific DNA was enriched by RPC-5 column chromatography and sucrose density gradient and cloned in lambda bacteriophage Charon 4A and finally recloned in bacteria plasmid pBR322.
2. From the restriction map and heteroduplex analysis, it was estimated that the cloned DNA is 12.5 ± 1.25 kbp in length and consists of more than 9 kbp proviral DNA and adjacent host flanking sequences of approximately 23 kbp and 0.3 kbp at 5' and 3' ends, respectively.
3. The cloned DNA induced virus production in NIH/3T3 cells upon transfection.
4. Unintegrated linear R-MuLV DNA fraction was isolated by gene machine preparative gel electrophoresis from cellular supernatant of R-MuLV-inoculated NRK cells and a preliminary restriction map developed. The cloned DNA was shown to have the same restriction map as that of unintegrated linear R-MuLV DNA.
5. M-MuLV was also cloned at its Hind III site from unintegrated form. Physical map of this clone was consistent with that previously published.
6. Recombinant DNAs were constructed between R-MuLV and M-MuLV, taking advantage of common restriction cleavage sites. These recombinants were demonstrated to possess biological activity in tissue culture.

Significance to Biomedical Research and the Program of the Institute:

Unlike acute retroviruses which have discrete transforming genes, R-MuLV and M-MuLV cause tumors only after a long latent period. The elucidation of their mechanisms of oncogenesis should provide insights into more general mechanisms of carcinogenesis under natural conditions.

Proposed Course:

This project will be continued by Dr. R. Narayanan, a Visiting Fellow with the LCMB. Dr. A. Habara has returned to Japan.

Publications:

Habara, A., Reddy, E.P., and Aaronson, S.A.: Rauscher murine leukemia virus: molecular cloning of infectious integrated proviral DNA. J. Virol., in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Interaction of Hematopoietic Cells and Mammalian Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Pierce	Staff Fellow	LCMB	NCI
OTHERS:	S.A. Aaronson	Chief	LCMB	NCI
	P.E. Reddy	Visiting Scientist	LCMB	NCI
	Y. Yuasa	Visiting Fellow	LCMB	NCI
	R. Balachandran	Visiting Fellow	LCMB	NCI
	A. Eva-Varesio	Visiting Associate	LCMB	NCI
	D. Swan	Expert	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

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 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. A hematopoietic colony forming assay that detects transformation of lymphoid cells was utilized to demonstrate that BALB- and Harvey-MSV transform a novel lymphoid progenitor cell both in vitro and in vivo.
2. The ST strain of feline sarcoma virus (ST-FeSV) was shown to transform murine hematopoietic cells of the pre-B-cell phenotype in vitro.
3. Deletion mutants of Abelson-MuLV were constructed and utilized to locate the region of the viral genome that is required for fibroblast and lymphoid cell transformation as well as the ability to induce disease in infected animals.

Project DescriptionObjectives:

1. Investigate the ability of defective mammalian retroviruses other than Abelson-MuLV to induce in vitro transformation of mouse hematopoietic cells utilizing an in vitro soft agar colony method.
2. To determine which regions of the Abelson-MuLV genome are required for transformation of fibroblast and lymphoid target cells utilizing defined deletion mutants constructed in our laboratory.

Methods Employed:

Standard hematopoietic cell culture techniques included a hematopoietic colony formation assay developed to detect retroviral 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 techniques and biochemical assays such as radiomunoprecipitation and enzyme assays.

A quantitative tissue culture assay was also developed to detect the presence of transformed hematopoietic cells after in vivo infection with mammalian retroviruses. Tumorigenicity experiments were also employed utilizing inbred strains to determine the leukemogenicity of established lines.

Major Findings:

1. BALB- and Harvey-murine sarcoma viruses (MSVs) 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 Abelson-murine leukemia virus was utilized 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 which 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, 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 Abelson-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.

2. ST-FeSV was shown to transform murine lymphoid cells in vitro at a low efficiency utilizing an hematopoietic cell colony forming assay. Clonal lines of ST-FeSV-transformed hematopoietic cells were established and characterized with respect to their hematopoietic phenotype. Many of the ST-FeSV transformants were shown to synthesize immunoglobulin μ chain and to possess Fc receptors. This pre-B-cell phenotype is analogous to lymphoid target cells of Abelson-MuLV. These viruses also show similarities in their genomic structure and associated protein kinase activity. However, no genetic relationship between their onc genes could be detected by molecular hybridization.
3. To analyze the regions of the Abelson-MuLV (A-MuLV) genome that are required for fibroblast and lymphoid cell transformation, deletion mutants of an infectious A-MuLV molecular clone were constructed in our laboratory. Two fragments, 1.6 kilobase pairs (kbp) at the center and 0.7 kbp at the 3' end of the A-MuLV cell-derived sequence abl, are produced by Bgl II digestion. A-MuLV mutant DNAs with the 1.6 kbp fragment deleted lacked the ability to transform fibroblasts in the 3T3 transfection assay indicating that this fragment is essential for fibroblast transformation. Abelson-MuLV DNA which lacked a Bgl II 0.7 kbp fragment at the 3' end of abl gene (pAB-B16 DNA) transformed NIH/3T3 cells less efficiently than wt A-MuLV DNA. The Abelson-specific polyprotein synthesized by foci derived from transfection with pAB-B16 DNA had a molecular weight of 90,000 daltons and retained protein kinase activity. Virus rescued from the pAB-B16 DNA transformants was completely deficient in its ability to induce Abelson disease in mice. Moreover, the pAB-B16 mutant virus did not induce the expected number of transformed lymphoid colonies in vitro based on its fibroblast transforming titer. However, transformed cell lines established from hematopoietic colonies that did arise from pAB-B16 mutant infection were similar to cells transformed by wt A-MuLV with respect to levels of Abelson-specific protein kinase activity, expression of lymphoid differentiation markers, growth properties, and tumorigenicity. These results indicate that the 0.7 kbp sequences deleted in the pAB-B16 mutant reduce its ability to initiate cellular transformation while the maintenance of the transformed state does not appear to be affected.

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 of retrovirus transformation and, in particular, the relationship of the differentiated state of the cell to its susceptibility to onc gene action.

Proposed Course:

1. We are interested in determining whether BALB- or Harvey-MSV 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. If so, these transformants should provide a useful approach for analysis of lymphoid cell ontogeny and the possible function of TdT in normal lymphocyte development. We also plan to investigate the extent of the hematopoietic target cell diversity for transformation by BALB- and Harvey-MSV under different assay conditions.

2. Efforts are underway to construct site-specific mutations in the BALB-MSV or Abelson-MuLV genome that will lead to the isolation of temperature-sensitive mutants. These conditional mutants will be utilized to determine whether the mechanism of retrovirus transformation in the hematopoietic cell system is due to a block in differentiation induced by their retroviral onc gene products.
3. Several other mammalian retroviruses that have not been previously characterized for their ability to induce transformation of murine hematopoietic cells will be analyzed for their ability to cause malignant alteration of hematopoietic cells.
4. The ability of amphotropic-MuLV or primate helper virus pseudotypes of BALB- and Harvey-MSV and A-MuLV to induce transformation of normal human cord blood or bone marrow in vitro will be analyzed.

Publications:

Pierce, J., and Aaronson, S.A.: BALB- and Harvey-MSV transformation of a novel lymphoid progenitor cell. J. Exp. Med., in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies of Human onc Sequences

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. Prakash	Visiting Fellow	LCMB NCI
OTHERS:	S.A. Aaronson	Chief	LCMB NCI
	S. Tronick	Microbiologist	LCMB NCI
	D.C. Swan	Expert	LCMB NCI
	O.W. McBride	Biochemist	LB NCI
	S.G. Devare	Visiting Associate	LCMB NCI

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sequences homologous to the Moloney-murine sarcoma virus (MSV) transforming gene (v-mos) were found to be present within a unique 2.5 kbp Eco RI fragment of human DNA. This fragment [designated c-mos(human)] was molecularly cloned from human placental DNA using a bacteriophage vector. Comparison of v-mos and c-mos(human) by restriction enzyme and heteroduplex mapping techniques revealed that c-mos(human) possesses a stretch of 650 nucleotides of continuous homology related to v-mos (1100 nucleotides). Attempts to demonstrate biological activity of c-mos(human) have thus far been unsuccessful. C-mos(human) was used as a probe to localize mos sequences within the human genome to chromosome 8.

Project Description

Objective:

Characterization of human analogs of transforming genes of murine retroviruses and elucidation of their role in human malignancy.

Methods Employed:

Standard gene enrichment techniques, molecular cloning using phage and plasmid systems, restriction enzyme analysis, heteroduplex mapping, and molecular hybridization on membrane filters.

Major Findings:

1. Identification and cloning of an Eco RI generated (2.5 kbp fragment) human placenta DNA analog of the M-MSV onc gene in bacteriophage λ Charon 16A.
2. By both restriction enzyme and heteroduplex analysis, the human analog was shown to possess an uninterrupted region of homology of 650 base pairs with the M-MSV onc gene in the 5' region only. No introns were present.
3. The absence of the 3' region of M-MSV onc gene in the human analog suggests deletion of this region from the human genome or striking divergence of this region.
4. The human analog was unequivocally shown to be present on human chromosome 8. This finding becomes significant due to the fact that chromosome 8 has been known to be involved in a reciprocal translocation with chromosome 14, a condition observed in Burkitt's lymphoma. Less well-noted translocations have been between chromosome 8 and chromosomes 2 and 22.
5. Since only one marker, glutathione reductase, has been assigned to chromosome 8 so far, this human analog would serve as a good marker for human chromosome 8.

Significance to Biomedical Research and the Program of the Institute:

The systematic characterization of human analogs of various transforming genes of retroviruses will allow us to assess their tumorigenic potential and involvement in human malignancies.

Proposed Course:

Detailed analysis of RNAs from various human tumors and established tumor cell lines with the cloned human analog as a probe is being done to assess the extent of expression of these DNAs in malignancies. The exact locus of the human analog on human chromosome 8 is being determined using the in situ hybridization technique. It will be of interest to see whether it maps near the breakpoint involved in chromosomal translocations between chromosomes 8, 14, 22 and 2.

Publications:

Prakash, K., McBride, O.W., Swan, D.C., Devare, S.G., Tronick, S., 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, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05166-02 LCMB		
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>				
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Isolation of Oncogenic Cellular Genes</p>				
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT				
PI: OTHERS:	S. Pulciani S.A. Aaronson K.C. Robbins S. Tronick M. Barbacid	Visiting Fellow Chief Expert Microbiologist Visiting Scientist	LCMB LCMB LCMB LCMB LCMB	NCI NCI NCI NCI NCI
COOPERATING UNITS (if any)				
LAB/BRANCH <p style="text-align: center;">Laboratory of Cellular and Molecular Biology</p>				
SECTION <p style="text-align: center;">Molecular Biology Section</p>				
INSTITUTE AND LOCATION <p style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</p>				
TOTAL MANYEARS: <p style="text-align: center;">1.0</p>	PROFESSIONAL: <p style="text-align: center;">1.0</p>	OTHER: <p style="text-align: center;">0.0</p>		
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<input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER				
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS				
SUMMARY OF WORK (200 words or less - underline keywords)				
<p>We have investigated the presence of <u>dominant transforming genes</u> in human tumors by <u>DNA-mediated gene transfer</u> experiments. High molecular weight DNAs from carcinomas and sarcomas were tested in transfection assays. Nine of these DNAs, including those isolated from pancreatic and colon carcinomas and from an embryonic rhabdomyosarcoma, induced morphologic transformation in recipient mouse NIH/3T3 fibroblasts. Our goal is to determine whether these transforming genes are etiologically involved in <u>human neoplasias</u>.</p>				

Project DescriptionObjectives:

To detect transforming activity in human tumor DNAs.

Methods Employed:

The techniques utilized are DNA extraction and DNA transfection. DNAs are extracted from a variety of malignant transformed cells and human tumors, then are added as calcium phosphate coprecipitates to NIH/3T3. The foci of transformed cells are then analyzed by Southern blotting techniques to score for human genetic information. The oncogenes involved in that process can then be cloned by standard molecular cloning techniques.

Major Findings:

1. We were able to induce foci of transformed cells with human DNA extracted from a variety of cell lines, including those established from bladder, lung and gall bladder carcinomas, as well as from fibrosarcoma cells. We also tested DNAs isolated from a variety of naturally occurring tumors. DNAs extracted from pancreatic and colon carcinomas and from a rhabdomyosarcoma were positive for inducing malignant transformation in recipient NIH/3T3 mouse cells. Transfectants show anchorage-independent growth and cause tumors when injected into athymic and immunocompetent mice. Further, their DNA transmitted the transformed phenotype in subsequent transfection assays. Southern blotting analysis revealed the presence of human DNA sequences in all the primary transformants. Some of these human sequences cosegregate in additional cycles of transfection with the transformed phenotype.
2. We have isolated by molecular cloning techniques a transforming gene present in T24 bladder carcinoma cells. Further, we have shown that the T24 oncogene is related to the transforming gene (v-bas) of BALB-MSV tumor virus. Moreover, elevated levels of an immunologically related protein of onc gene products of BALB- and Harvey-MSVs have been detected in T24 cells. We have also isolated the human sequences (c-bas) related to BALB-MSV transforming gene (v-bas) and have shown them to be the normal homologue of the T24 oncogene.

Significance to Biomedical Research and the Program of the Institute:

Identification and characterization of transforming genes present in human tumor cells is an important step towards the understanding of human neoplasias.

Proposed Course:

To improve experimental transfection conditions and to use a wider variety of recipient cells in order to ascertain whether human tumors whose DNA have not transformed NIH/3T3 cells, as yet, may also contain transforming genes.

Publications:

Pulciani, S., Santos, E., Lauver, A., Long, L.K., and Barbacid, M.: Transforming genes in human tumors. J. Cell. Biochem., in press.

Pulciani, S., Santos, E., Lauver, A., 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.

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., 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, in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Analysis of the Transforming Gene of Simian Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.C. Robbins	Expert	LCMB	NCI
OTHERS:	S.A. Aaronson	Chief	LCMB	NCI
	S.R. Tronick	Microbiologist	LCMB	NCI
	M. Barbacid	Visiting Scientist	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

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

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TOTAL YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Analysis of a biologically active molecular clone of simian sarcoma virus (SSV) has shown that the 5.1 kbp genome contains a 1.0 kbp segment (v-sis) which is derived from a unique woolly monkey cellular gene. Utilizing SSV deletion mutants constructed in vitro, we have demonstrated that v-sis is required for the induction and maintenance of transformation. Analysis of the primary nucleotide sequence of v-sis has revealed a 675 bp open reading frame commencing 19 nucleotides upstream of the v-sis helper virus junction and terminating within v-sis. This open reading frame has the capacity to code for a 28,000 dalton protein. A small peptide selected from the amino acid sequence of the predicted v-sis gene product was used as an immunogen to prepare antiserum. The peptide antibody detected a v-sis coded protein of 28,000 daltons (p28^{sis}) expressed specifically in SSV transformed cells. Preliminary characterization of p28^{sis} indicates that properties of this onc gene product differ from those of other described retrovirus transforming proteins. Other studies have described the chromosomal localization of a gene analogous to v-sis within the human genome and have demonstrated activation of this gene in certain types of human tumor cells.

Project Description

Objectives:

1. To examine the interaction of the SSV transforming protein (p28sis) 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 simian sarcoma virus. 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. Whether expression of the human c-sis gene is directly related to the malignant state or to specific stages of cellular differentiation remains to be determined.

3. The primate cell-derived transforming gene (v-sis) of simian sarcoma virus (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 translocation 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 simian sarcoma virus (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 indicates that p28^{STS} differs from other onc-gene products.

Significance of Biomedical Research to 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 transformation.
3. To continue to assess the involvement of retrovirus onc-related genes in naturally occurring human malignancies.

Publications:

Devare, S.G., Reddy, E.P., Robbins, K.C., Andersen, P.R., Tronick, S.R., and Aaronson, S.A.: Nucleotide sequence of the transforming gene of simian sarcoma virus. Proc. Natl. Acad. Sci. USA 79: 3179-3182, 1982.

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.H., Wong-Staal, F., Gallo, R.C., and Aaronson, S.A.: Cellular genes analogous to retroviral onc genes are transcribed in human tumor cells. Nature 295: 116-119, 1982.

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 gene 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.

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.

Pulciani, S., Santos, E., Lauer, A., 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.

Robbins, K.C., Devare, S.G., and Aaronson, S.A.: Molecular cloning of simian sarcoma virus: genome organization of infectious DNA clones. Proc. Natl. Acad. Sci. USA 78: 2918-2922, 1981.

Robbins, K.C., Hill, R.L., and Aaronson, S.A.: Primate origin of the cell-derived sequences of simian sarcoma virus. J. Virol. 41: 721-725, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05168-02 LCMB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Chromosomal Localization of Human Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. Swan	Expert	LCMB	NCI
OTHERS:	S. Tronick	Research Microbiologist	LCMB	NCI
	P. Reddy	Visiting Scientist	LCMB	NCI
	K. Prakash	Visiting Fellow	LCMB	NCI
	R. Balachandran	Visiting Fellow	LCMB	NCI

COOPERATING UNITS (if any)

P. Leder, Department of Genetics, Harvard University; O.W. McBride, Laboratory of Biochemistry, DCBD, NCI

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

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Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chromosomal mapping of human immunoglobulin and several onc gene analogues has been carried out using somatic cell hybrids. It has been shown that the expressed immunoglobulin genes are on three different human chromosomes. This, and the finding that at least one human onc gene is on chromosome 8, has given an insight into a potential mechanism for transformation in Burkitt's lymphoma.

Projection Description

Objectives:

1. Chromosomal mapping of human immunoglobulin genes.
2. Chromosomal mapping of human onc genes.
3. Establish relationship in Burkitt's lymphoma between type of immunoglobulin synthesized and onc gene expressed.

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 DNA's 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 the case of λ immunoglobulin, SSV onc gene, and MSV onc gene it was necessary to further subclone some of the positive cell lines in order to allow further segregation of human chromosomes.

Major Findings:

The following genes have been mapped during fiscal year 1982:

- Human κ immunoglobulin to chromosome 2
- Human λ immunoglobulin to chromosome 22
- Human SSV onc gene to chromosome 22
- Human MSV onc gene to chromosome 8
- Human AMV onc gene to chromosome 6
- Mouse MSV onc gene to chromosome 4
- Mouse Abelson onc gene to chromosome 2

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 on chromosomes 2 and 22, respectively, it has become clearer how the translocations might lead to the specific activation of one of the two light chain loci. In situ hybridization using a λ light chain probe has shown it to be located precisely at the break point on chromosome 22 (22q11). Further, the fact that chromosome 8 is always involved and the recent finding that the MSV onc gene is found on this chromosome allows us to speculate that activation of this gene or some other as yet unmapped onc gene on chromosome 8 might be responsible for the transformation of these cells.

Proposed Course:

1. All the remaining known human onc gene analogues will be mapped.
2. In situ hybridization will be performed with certain onc genes where their precise location on the chromosome is required.
3. In situ hybridization and cloning of certain human onc gene analogues will be carried out on Burkitt cell DNA in order to establish the relative locations of the immunoglobulin and onc genes after the translocation event.

Publications:

Batley, 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, in press.

Hollis, G.F., Hieter, P.A., McBride, O.W., Swan, D., and Leder, P.: Processed genes: a dispersed human immunoglobulin gene bearing evidence of RNA type processing. Nature 296: 321-325, 1982.

Leder, A., Swan, D., Ruddle, F., D'Eustachio, P., and Leder, P.: Dispersion of α -like globin genes of the mouse to three different chromosomes. Nature 293: 196-200, 1981.

McBride, O.W., Hieter, P.A., Hollis, G.F., Swan, D., Otey, M.C., and Leder, P.: Chromosomal location of human kappa and lambda immunoglobulin light chain constant region genes. J. Exp. Med. 155: 1480-1490, 1982.

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, in press.

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, in press.

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., in press.

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.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Study of Human Tumor Cells and Mouse Virus (Abelson) Transforming Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. Yuasa	Visiting Fellow	LCMB	NCI
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	P.E. Reddy	Visiting Scientist	LCMB	NCI
	A. Srinivasan	Visiting Associate	LCMB	NCI
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	J. Rhim	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

NONE

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TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

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 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. To clone and characterize transforming DNA sequences responsible for acquisition of tumor-forming activity, DNAs from various human tumor cells were transfected onto NIH/3T3 cells. Distinct foci were induced by DNA from two out of ten lung carcinoma cell lines. Human repetitive sequences (Alu) were detected in all of the transformed cells by Southern blot hybridization.

2. A-MuLV mutants, which deleted the Bgl II 1.6 kbp fragment, lacked the ability to transform fibroblasts, indicating that this fragment is essential for fibroblast transformation. An A-MuLV mutant lacking the Bgl II 0.7 kbp fragment retained transforming activity for fibroblasts and lymphoid cells. Results of studies demonstrated that A-MuLV was capable of transforming fibroblasts in the absence of the 3' half of the A-MuLV cell-derived sequence (abl).

Project Description

Project 1: Molecular cloning of the transforming DNA sequences from human tumor cells.

Objectives:

- A. To clone transforming genes molecularly from human tumor cell DNA which transforms normal fibroblasts.
- B. 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.

Major Findings:

Distinct foci were induced by DNA from two out of 10 lung carcinoma cell lines. Human repetitive sequences (Alu) were detected in all of the transformed cells by Southern blot hybridization.

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:

- (1) Molecular cloning of the transforming DNA sequences and (2) comparison of the transforming sequences in the transformants with known retroviral oncogenes.

Project 2: Characterization of transforming genes and proteins of Abelson virus.

Objectives:

- A. To identify whether transforming proteins of Abelson virus responsible for fibroblast and lymphoid cell transformation are the same or different.
- B. To determine the transformation-specific regions of the Abelson virus genome.

Methods Employed:

1. Making deletion mutants by modifying the cloned Abelson virus DNA with restriction enzymes.
2. Transfection of deleted genomes of Abelson virus DNA on cultured fibroblasts.
3. Rescue of Abelson virus by superinfection with helper MuLV on the transformed cells.
4. In vitro and in vivo transformation assay of lymphoid cells by the rescued viruses.
5. Protein analysis by immunoprecipitation and protein kinase assay.
6. Southern blot hybridization analysis of the proviral genomes in the transformed cells.

Major Findings:

Abelson murine leukemia virus (A-MuLV) induces lymphomas in vivo and transforms fibroblasts and lymphoid cells in tissue culture. To analyze the A-MuLV transforming gene, we constructed deletion mutants of an infectious A-MuLV molecular clone. Two fragments, 1.6 kilobase pairs (kbp) at the center and 0.7 kbp at the 3' end of the A-MuLV cell-derived sequence (*abl*), are produced by Bgl II digestion. A-MuLV mutants, which deleted the 1.6 kbp fragment, lacked the ability to transform fibroblasts, indicating that this fragment is essential for fibroblast transformation. An A-MuLV mutant lacking the 0.7 kbp fragment retained transforming activity for fibroblasts and lymphoid cells. Such cells expressed a 90,000 dalton protein precipitable by anti-MuLV sera and demonstrated associated protein kinase activity. This result indicates that 0.7 kbp at the 3' end of the *abl* gene is not required for transformation of either fibroblasts or lymphoid cells. An independent DNA clone which contained the same 0.7 kbp deletion transformed fibroblasts less efficiently. One transformant containing this mutant expressed only a 70,000 MW protein with associated protein kinase activity. This protein was precipitable with anti-MuLV p15, p12 or p30 sera, indicating that it retained the 5' *gag* region of the A-MuLV transforming protein. These results demonstrate that A-MuLV can transform fibroblasts in the absence of the 3' half of *abl*.

Significance to Biomedical Research and the Program of the Institute:

The Abelson virus is the only known mammalian retrovirus which transforms both fibroblasts and lymphoid cells. It is very important to determine whether this virus has two different transforming proteins or only one responsible for both types of transformation. The Abelson virus is also very useful for analyzing the differentiation process of immunoglobulin-producing lymphocytes, since it specifically transforms pre-B-cells in vitro.

Proposed Course:

1. Determine the exact transforming regions of the Abelson virus genome responsible for fibroblast and lymphoid cell transformation by isolating many deletion mutants.
2. Analyze the proviral genomes in the transformed cells induced by the mutants.

Publications:

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, in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies Define Murine Mammary Tumor Viral Gene Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David Colcher	Microbiologist	LCMB	NCI
OTHERS:	Yoshio A. Teramoto	Sr. Staff Fellow	LCMB	NCI
	Jeffrey Schlom	Chief, Experimental Oncology Section	LCMB	NCI

COOPERATING UNITS (if any)

M. Potter, Laboratory of Cell Biology, DCBD, NCI, Bethesda, Maryland; S. DeNardo, Univ. of Calif., Davis, Calif.; A. Hackett, Peralta Cancer Research Institute, Oakland, Calif.

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies have been generated that are reactive with the major envelope and internal structural proteins of mouse mammary tumor viruses (MTVs). Different monoclonals were shown to be reactive with type-specific, group-specific, and interspecies determinants of MTVs. The monoclonals to type-specific determinants were used to demonstrate that differences exist between each of six MTVs derived from different strains of laboratory mice (M. musculus). MTVs grown in murine as well as feline cells were tested in order to rule out the possibility that the differences observed were due to host determinants. Antibodies to MTV interspecies determinants were obtained by immunizing mice with type B retroviruses obtained from M. cervicolor (MC-MTV) and M. cookii (MCo-MTV) and testing subsequent monoclonals for reactivity with MTVs of M. musculus. The monoclonals generated have been used to demonstrate the diversity of expression of individual antigenic determinants among mammary tumors and within a given tumor mass in primary mammary tumors of various Mus strains and species.

Project Description

Objectives:

To generate and characterize monoclonal antibodies to the major structural proteins of murine mammary tumor viruses (MMTVs) in order to (a) delineate type, group, and interspecies determinants among MMTVs derived from various strains of laboratory mice (M. musculus) and species of the genus Mus; (b) determine if viral coded antigenic determinants are expressed in naturally occurring neoplasms (emphasizing mammary neoplasias) of feral populations of species other than M. musculus; and (c) to determine the degree, if any, of antigenic heterogeneity that exists among naturally occurring mammary neoplasms of the genus Mus, between different mammary tumors of a given species and within a given tumor mass.

Methods Employed:

Hybridoma technology was employed to generate monoclonal antibodies to MTV gene products. These antibodies were screened by solid phase radioimmunoassays using purified MMTVs from various strains of mice, purified polypeptides from these viruses and membranes of tissue culture cells. The specificity of the monoclonal antibodies were further tested using immunoprecipitation of radiolabeled MTV viral polypeptides. Murine mammary tumors from various species of the genus Mus were tested for reactivity with the monoclonal antibodies using the immunoperoxidase technique.

Major Findings:

I. Specificity of monoclonal antibodies. Mice and rats were immunized with disrupted MMTVs from M. musculus (MMTV(C3H)), M. cervicolor (MC-MTV), and M. cookii (MCo-MTV). Spleen cells from immunized mice were fused with the NS-1 murine non-immunoglobulin secreting myeloma cell line and standard hybridoma technology was employed to obtain double cloned cultures synthesizing monoclonal antibodies. All the monoclonal antibodies bound selectively to disrupted MMTV. Reactivities were further examined for binding to membrane-enriched extracts of cells producing MMTV(C3H). All the monoclonal antibodies bound to membranes from both a C3H mammary tumor cell line and feline cells (CrFK) producing MMTV(C3H). No binding was observed, however, to membranes of C3H fibroblasts or to the uninfected feline cell line. These findings provide evidence that the antigenic determinants recognized by these antibodies are viral mediated and most probably viral coded.

The monoclonal antibodies that were generated against the type B retroviruses from M. cervicolor and M. cookii were tested for reactivity to retroviruses from a variety of species. The monoclonal antibodies M1.1 (prepared from animals immunized with MC-MTV) and M3.1 (prepared from animals immunized against MCo-MTV) both react to MTV's from M. cervicolor milk and from M. musculus produced by either murine or feline cells. None of the monoclonal antibodies tested bound to murine leukemia viruses from M. musculus, the M432 retrovirus from M. cervicolor, or a variety of retroviruses of different species.

The major MMTV(C3H) polypeptides, gp52, gp36, and p28, were purified using ion-exchange chromatography and molecular sieving. Monoclonal antibodies were

then tested in solid-phase RIA's against each purified polypeptide. Antibodies were identified in this manner as reactive with either the MMTV major external glycoproteins, gp52 or gp36, or with the major internal polypeptide p28. The polypeptide specificity of the monoclonal antibodies was further identified by immunoprecipitation of disrupted iodinated MMTV.

II. Diversity of MMTV gene products. The monoclonal antibodies were tested for their ability to distinguish various MMTVs from several strains of mice. Many of the viruses were also propagated in feline cells to ensure that the reactivities observed were directed against viral-coded determinants. All the MMTVs could be easily distinguished. In most cases, the source of the MMTV could be identified using only one monoclonal antibody. The only virus that required more than one monoclonal antibody to distinguish it from the others was the MMTV(BALB/c); that could be accomplished, however, using antibodies M1.1 and M2.1. Several monoclonal antibodies have also been generated that react to all the MMTVs tested.

III. Reactivity with mammary tumors of the genus Mus. The immunoperoxidase technique was utilized to determine if the monoclonal antibodies could be used to detect antigenic determinants in tissue sections of primary and transplanted murine mammary tumors. Two major types of staining patterns were observed: apical, in which the immune reaction was concentrated at the periphery of lumens of acini; and focal, in which the immune reaction was detected as discrete intracytoplasmic foci. Two phenomena became apparent when using monoclonal antibodies to monitor expression of a distinct antigenic determinant in tumor cells. The first was that a given antigenic determinant can be expressed in a different manner in two different mammary tumors. The second was the heterogeneity in staining within a given mammary tumor; i.e., most mammary tumors tested from a variety of M. musculus strains and Mus species presented some areas that were positive and some that were negative for expression of a given determinant.

The immunoperoxidase technique was also used to further define interspecies reactivities of certain monoclonal antibodies. Monoclonal antibody M3.1 reacted significantly with sections of primary mammary tumors of M. musculus (C3H), M. cookii, and M. cervicolor. This antibody did not, however, react with sections of lactating mammary glands of C57BL mice, a strain devoid of MTV antigen expression in its milk. The immunoperoxidase method has also proved to be extremely useful in the detection of tumor-associated antigens by monoclonal antibodies in segments of tumors too small to be used in other immunological assays.

Significance to Biomedical Research and the Program of the Institute:

These studies involve the generation and characterization of a repertoire of monoclonal antibodies to the major structural proteins of MMTVs. These antibodies have demonstrated the diversity of defined viral gene products in various mouse strains and provide defined immunologic reagents to investigate the most widely studied model of naturally occurring mammary neoplasia and preneoplasia. The use of the immunoperoxidase technique with monoclonal antibodies has revealed the heterogeneity of antigen expression in mammary tumors, i.e., that a single antigenic determinant may be expressed in a different manner not only among different mammary tumors but in different areas of the same mammary tumor. The monoclonal antibodies directed against various interspecies determinants of MTV

gene products now also make possible an evaluation of the association between the expression of specific viral gene products and mammary tumorigenesis in feral populations of species other than *M. musculus*. These monoclonal antibodies may prove quite useful in the study of anti-idiotypic responses to antibodies directed against tumor-associated antigens in the immunologic control of neoplasia. They may also serve as reagents in an excellent model for studies involving the radioimmunoassay and antibody-mediated therapy (either alone, or with conjugated drugs or isotopes) of naturally occurring mammary neoplasia, preneoplasia, and/or micrometastatic lesions.

Proposed Course:

These antibodies are being employed to help determine the degree of viral gene expression in naturally occurring neoplasias of feral populations of several species of the genus *Mus*. Several collaborations with other laboratories are currently in progress. Several of the anti-MMTV gp36 monoclonal antibodies will be injected into BALB/c mice to synthesize anti-idiotypic monoclonal antibodies, i.e., to determine what specific region of the anti-MMTV monoclonal is the anti-idiotypic directed. These antibodies are being used to further define reactivities of MMTV-related antigens in primary cultures of human breast tumors. These antibodies have been, and will continue to be, distributed to numerous other laboratories. We believe that this model system will eventually be extremely valuable in both diagnosis and therapy studies of a well-defined naturally occurring carcinoma and in the detection and therapy studies of both preneoplastic and micro-metastatic lesions.

Publications:

Colcher, D., Horan Hand, P., Teramoto, Y.A., Wunderlich, D., and Schlom, J.: Use of monoclonal antibodies to define the diversity of mammary tumor viral gene products in virions and mammary tumors of the genus *Mus*. Cancer Res. 41: 1451-1459, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies Reactive with Human Mammary Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David Colcher	Microbiologist	LCMB	NCI
OTHERS:	Marianna Nuti	Visiting Fellow	LCMB	NCI
	Jeffrey Schlom	Chief, Experimental Oncology Section	LCMB	NCI

COOPERATING UNITS (if any) Sidney Farber Cancer Institute, Boston, Massachusetts;
Department of Pathology, George Washington Univ., Washington, D.C.

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

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Experimental Oncology Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

2.4

PROFESSIONAL:

0.9

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice were immunized with membrane-enriched fractions of metastatic human mammary carcinoma lesions; splenic lymphocytes were fused with non-Ig secretor myeloma cells to generate subsequent hybridoma cultures synthesizing 13 monoclonal antibodies reactive with human mammary carcinoma cells, two which react with carcinoembryonic antigen. The monoclonals could be placed into six major groups based on (a) reactivity in solid phase RIAs with extracts of metastatic lesions of mammary carcinomas and (b) binding to the surface of live mammary tumor cells in culture using live cell RIAs and fluorescent-activated cell sorter analyses. Some of the monoclonals demonstrated a "pancarcinoma" activity, reacting with the surface of some non-breast carcinomas, but none of the 11 monoclonals reacted with the cell surface of melanomas, sarcomas, various hematopoietic malignancies and numerous apparently normal cell lines. The immunoperoxidase technique was used on fixed tissue sections to determine the extent of reactivity of the different monoclonals with various types of primary mammary tumors and with metastatic lesions in lymph nodes and at distal sites. These antibodies react with approximately 85 percent of human mammary tumors.

Project Description

Objectives:

To generate and characterize monoclonal antibodies that are reactive with human mammary tumor-associated antigens. These monoclonal antibodies may prove useful in the diagnosis, prognosis, monitoring of therapy, and eventual therapy of human carcinomas, as well as in the study of the biology of human carcinoma cell populations.

Methods Employed:

Hybridoma technology was used to generate monoclonal antibodies against human breast tumor metastases. The antibodies were screened in a solid phase radio-immunoassay against extracts of mammary tumor metastases to various organs and corresponding normal tissue. The reactivity of the antibodies to cell surfaces was examined using a live cell RIA and by fluorescent-activated cell sorting. The monoclonal antibodies were also assayed for cytotoxic activity. The reactivity of these antibodies to tissue sections of primary tumors and metastases to the lymph nodes and distal areas as well as normal tissue were examined using the immunoperoxidase technique.

Major Findings:

I. Generation and characterization of monoclonal antibodies. Mice were immunized with membrane-enriched fractions of human metastatic mammary carcinoma cells (designated Met 1 and Met 2) from either of two involved livers from two different patients. Spleen cells of immunized mice were fused with NS-1 mouse myeloma cells to generate 4,250 primary hybridoma cultures. Supernatant fluids from these cultures were screened in solid-phase RIAs for the presence of immunoglobulin reactive with extracts of metastatic mammary tumor cells from the involved livers and not reactive with similar extracts of apparently normal liver; 370 cultures synthesized immunoglobulin reactive only with the metastatic carcinoma cell extracts. After passage, double cloning of these cultures by endpoint dilution, and further testing, the monoclonal antibodies synthesized by 11 hybridoma cell lines were chosen for further study. The isotypes were first determined: 10 were IgGs of various subclasses and one was an IgM.

The primary screen for the monoclonal antibodies was a solid-phase RIA employing cell extracts of two breast tumor metastases (Met 1 and Met 2) and apparently normal human liver. The 11 monoclonal antibodies could immediately be divided into three major groups on the basis of their differential reactivity to Met 1 vs. Met 2. All 11 antibodies were negative when tested against similar extracts from normal human liver, the A204 rhabdomyosarcoma cell line, the HBL100 cell line derived from cultures of human milk, the Mm5mt/c₁ mouse mammary tumor cell line, the C3H10T_{1/2} mouse fibroblast cell line, the CrFK feline kidney cell line, disrupted mouse mammary tumor virus and mouse leukemia virus, purified carcino-embryonic antigen (CEA), and ferritin. Two monoclonal antibodies were used as controls in all these studies: (1) W6/32, an anti-human histocompatibility antigen and (2) B139, which was generated in these studies, and which showed reactivity to all human cells tested.

Binding to cell surface. To further define the reactivities of the 11 monoclonal antibodies, and to determine if they bind cell surface antigens, each antibody was tested for binding to live cells in culture. Test cells included three human mammary carcinoma cell lines (BT-20, MCF-7, and ZR-75-1). Nine monoclonals, grouped together on the basis of their binding to both metastatic cell extracts, could now be separated into three further groups on the basis of their differential binding to the surface of live cells in culture. Monoclonals B6.2, B14.2, B39.1, F64.5, F25.2, B84.1, and B38.1 all bound the three mammary carcinoma lines. Monoclonal B38.1 also bound to the A549 lung carcinoma line, the A431 vulva epidermoid carcinoma line, and the KB oral epidermoid carcinoma line. This antibody, however, did not bind to the sarcoma and melanoma cell lines tested. Monoclonals B50.4, B50.1, and B72.3 constituted a third grouping in that they reacted only with the surface of the MCF-7 cell line. None of the 11 monoclonal antibodies bound to the surface of any of the cell lines tested derived from apparently normal human tissues. Control monoclonal B139, however, did bind to all of these cells.

Fluorescent-activated cell sorter analyses. Monoclonal antibodies B6.2 and B38.1 were further analyzed for surface binding using fluorescent-activated cell sorter analyses to a panel of human cell lines. These studies were carried out in collaboration with Dr. D. Kufe, Sidney Farber Cancer Center. Antibody B6.2 was reactive with five of six breast carcinoma lines but was unreactive with most other carcinomas. Antibody B38.1, on the other hand, was strongly reactive with all breast carcinoma lines and was also reactive with many non-breast carcinoma lines. The breast tumor line HS0578T falls into a rare histologic grouping and at one point has been histologically designated a "carcinosarcoma"; it is unreactive with both antibody B6.2 and B38.1. Cell lines derived from melanomas, sarcomas and lymphoid tumors were uniformly unreactive with these antibodies. There was complete agreement in assay results when the same cell lines were tested in live cell RIA and by cell sorter analysis.

A variety of tissues obtained directly from biopsy were also evaluated for reactivity via fluorescence-activated cell sorter analyses. Breast carcinoma cells were examined from patients with malignant pleural effusions; three patterns of reactivity were noted. Pleural effusions 1 and 5 demonstrated moderate reactivity with both B6.2 and B38.1. Pleural effusion 4 demonstrated strong reactivity with antibody B6.2 but was unreactive with antibody B38.1; pleural effusion 2 showed the reciprocal reactivity. Single cell suspension derived from normal lymphoid tissue including bone marrow, lymph node, spleen and tonsil demonstrated no reactivity.

Cytotoxicity. Monoclonal antibodies B6.2 and B38.1 were tested for complement-mediated lysis (CDC), and antibody-dependent cell-mediated cytotoxicity (ADCC), using the MCF-7 and BT-20 mammary tumor cell lines as targets. All CDC assays, using various preparations of rabbit complement, were negative. In preliminary experiments, antibodies B6.2 and B38.1 both lysed MCF-7 mammary carcinoma cells in ADCC at various antibody dilutions, with antibody B38.1 activity being stronger at each dilution. Neither antibody B6.2 nor B38.1 lysed normal human lung, bone marrow, uterus, or skin. In contrast, control monoclonal antibody B139 lysed all of the above cells, and isotype-identical anti-MMTV monoclonals were negative. Rat and human lymphocytes were both positive as effector cells.

Binding to tumors. To further define the range of reactivity of each of the 11 monoclonal antibodies, the immunoperoxidase technique on tissue sections was employed. All the monoclonals reacted with primary mammary carcinoma cells (both infiltrating ductal and lobular), but the percentage of tumors that were reactive varied from approximately 50% to 85% for the different monoclonals. In many of the positive primary and metastatic mammary carcinomas, not all tumor cells stained. A high degree of selective reactivity with mammary tumor cells, and not with apparently normal mammary epithelium, stroma, blood vessels, or lymphocytes in the breast was observed with all 11 monoclonal antibodies. The staining patterns of mammary carcinoma cells varied among the different monoclonals. For example, monoclonal B50.4 was reactive with mammary carcinoma cells, displaying a dense focal staining. Monoclonal B6.2, on the other hand, reacts with alternate sections of the same mammary carcinoma, with a more diffuse cytoplasmic pattern. Frozen sections of primary mammary tumors were also tested with some of the monoclonals and were positive. Several of the monoclonals also showed reactivity to selected non-breast carcinomas, such as carcinoma of the colon, thus demonstrating a "pancarcinoma" reactivity.

A factor in the potential utility of any monoclonal antibody is its selective reactivity. The immunoperoxidase method of staining of fixed tissue sections with antibody has the advantage of screening large amounts of tissues in a relatively short period of time. Moreover, it permits the testing of antibody reactivity with tissues that would otherwise be inaccessible. For example, to date there are no cell lines available from *in situ* breast carcinoma. Monoclonal B38.1 has also been shown to be reactive with a variety of rapidly dividing epithelial cells using the immunoperoxidase technique, and may thus be reactive with a differentiation antigen. It is unreactive however, with the surface of all normal cells tested thus far. Since it is cell surface binding which is of clinical importance, one must distinguish between reactivity of an antibody with a cross-reactive internal antigen versus cell surface binding.

Experiments were carried out to determine if the 11 monoclonal antibodies could detect mammary carcinoma cell populations at distal sites, i.e., in metastases. Because the monoclonals were all generated by using metastatic mammary carcinoma cells as antigen, it was not unexpected that the monoclonals all reacted, but with different degrees, to various metastases. Several of the monoclonals reacted with metastatic mammary carcinoma cells in lymph nodes of 85 percent of patients, but did not react with uninvolved nodes. None of the monoclonals reacted with normal lymphocytes or stroma from any involved or uninvolved nodes. All 11 monoclonals were negative for reactivity with apparently normal tissues of the following organs: thyroid, intestine, lung, liver, bladder, tonsils, and prostate.

II. Differential binding to human mammary and non-mammary tumors of monoclonal antibodies reactive with carcinoembryonic antigen. The presence of high plasma levels of CEA has been reported to be an indicator of the possible presence of metastatic disease in patients with cancers of the digestive system, breast, lung, as well as other sites. Using assays based on antibodies to colonic CEA, elevated plasma levels of CEA have been reported in 38-79% of patients with mammary carcinomas. There have been several reports, however, indicating that "CEA" is a heterogeneous family of glycoproteins, some of which demonstrate cross-reactivity with each other, as well as with so-called "CEA-related" proteins. One issue that has not yet been clearly resolved is the possibility that a given tumor cell type

may produce, or maintain on the cell surface, a CEA that is only partially related to CEA's associated with other malignancies. Monoclonal antibodies should be a valuable reagent toward resolving this point. To date, monoclonal antibodies have been generated by several other groups using CEA from colon carcinomas as the immunogen. In the studies reported here, monoclonal antibodies were generated to membrane enriched fractions of human mammary carcinoma metastases and screened for reactivity with purified CEA. The differential binding properties of two of these antibodies (B1.1 and F5.5) to CEA as well as to breast and non-breast tumors was investigated. Monoclonal B1.1 is an IgG_{2a}, while F5.5 is an IgG₁.

Binding to cell surface. Monoclonals B1.1 and F5.5 were tested for binding to live cells in culture to define their range of reactivities and to ascertain if they bind to antigenic determinants that are present on the cell surface. Both monoclonals bound to the same three established human mammary carcinoma cell lines and to two colon carcinoma cell lines, but not to a lung carcinoma or vulva carcinoma cell line or to 13 normal human cell lines. The two monoclonals could be distinguished, however, by their differential reactivity to the surface of certain cell lines. B1.1 bound to three of four melanoma cell lines tested, while F5.5 did not bind to any of the four. Similar differential reactivity with B1.1 was also observed with late passages (greater than passage 80) of the A204 rhabdomyosarcoma cell line, while F5.5 did not bind A204 cells at any passage level.

Monoclonal antibodies B1.1 and F5.5 were compared for range of reactivity with two commercially available monoclonal antibodies prepared against colonic CEA. Monoclonal F5.5 could be distinguished from the three others in that it exhibited a greater binding to mammary cell lines compared to both purified colon carcinoma CEA and cell lines derived from colon carcinomas.

Binding to tumors. To further identify the range of reactivities of monoclonals B1.1 and F5.5 with human mammary carcinomas, the immunoperoxidase technique was used on formalin-fixed tumor sections. Both B1.1 and F5.5 reacted positively with malignant mammary tumor cells in primary lesions and with metastatic cells in lymph nodes and at distal sites. Positive staining was observed with three colon carcinomas and three lung carcinomas tested. Monoclonals F5.5 and B1.1 reacted positively with 55 and 66 percent, respectively, of the mammary carcinomas tested. The positive mammary tumors included infiltrating ductal, in situ, and medullary carcinomas.

III. Monoclonal antibody B72.3 defines the distribution of a novel tumor-associated antigen in human mammary carcinoma cell populations. Of the thirteen monoclonals described above, B72.3 (an IgG₁) displayed the most restricted range of reactivity for human mammary tumor versus normal cells. Monoclonal B72.3 was used at various concentrations in immunoperoxidase assays of tissue sections to determine the effect of antibody dose on the staining intensity and the percent of tumor cells stained. Since one cannot titrate antigen in the fixed tissue section, an antibody dilution experiment would give an indication of the relative titer of reactive antigen within a given tissue. A range of antibody concentrations, varying from 0.02 µg to 10 µg of purified immunoglobulin (per 200 µl) per tissue section, was used on each of four mammary carcinomas from different patients. The results demonstrate that: (a) different mammary tumors may vary in the amount of the antigen detected by B72.3, (b) a given mammary tumor may contain

tumor cell populations which vary in antigen density, and (c) some mammary tumors may score positive or negative depending on the dose of antibody employed.

To further characterize the range of reactivity of B72.3, the immuno-peroxidase technique was used to test a variety of malignant, benign and normal mammary tissues. Using 4 µg of monoclonal per slide, the percent of positive primary breast tumors was 46% (19/41); 62% (13/21) of the metastatic lesions scored positive. Several histologic types of primary mammary tumors scored positive: these were infiltrating duct, infiltrating lobular and comedo carcinomas. Many of the in situ elements present in the above lesions also stained. None of the six medullary carcinomas tested were positive. Approximately two thirds of the tumors that showed a positive reactivity demonstrated a cell-associated membrane and/or diffuse cytoplasmic staining, while approximately five percent showed discrete focal staining of the cytoplasm; approximately one-fourth of the reactive tumors showed an apical or marginal staining pattern. To date, not enough tumors have been tested to define any correlations between type of staining pattern and such parameters as tumor type or histologic grade. Metastatic breast carcinoma lesions that were positive were in axillary lymph nodes, and at the distal sites of skin, liver, lung, pleura and mesentery. Fifteen benign breast lesions were also tested; these included fibrocystic disease, fibroadenomas and sclerosing adenosis. Two specimens showed positive staining: one case of fibrocystic disease where a few cells in some ducts were faintly positive, and a case of intraductal papillomatosis and sclerosing adenosis with the majority of cells staining strongly. Other primary tumors were also tested for reactivity, including four colon carcinomas, two benign colon tumors, four lung carcinomas, two kidney carcinomas, one prostate carcinoma, one bladder carcinoma, one melanoma, two lymphomas and two sarcomas. Of the above, four colon carcinomas and two lung carcinomas were positive. Monoclonal B72.3 was also tested against normal breast tissue and normal lactating breast from non-cancer patients and showed no reactivity. Occasionally, the histiocytes or the polymorphonuclear leukocytes present in the stroma surrounding breast tumor cells showed positive cytoplasmic staining; this may be due to the reaction of B72.3 with antigen shed by tumor cells and phagocytized by the reactive cells. A variety of non-breast cells and tissues were tested and were negative; these included two uteruses, two livers, two spleens, three lungs, two bone marrows, five colons, one stomach, one salivary gland, five lymph nodes and one kidney.

Significance to Biomedical Research and the Program of the Institute:

These studies have led to the generation of a series of monoclonal antibodies that are reactive with approximately 85% of human mammary tumors examined. These antibodies may eventually prove useful in the diagnosis, prognosis, and treatment of human mammary neoplasias, as well as in the study of the basic biology of human mammary tumor cell populations.

Proposed Course:

We plan to further characterize the immunologic and biologic properties of the monoclonal antibodies described and to generate and characterize new monoclonal antibodies to mammary tumor-associated antigens. Specifically:

Definition of reactivities: To date, we have investigated the surface binding reactivities of each of the monoclonal antibodies described above to over 30 human cell lines; over 50 human mammary carcinomas and over a dozen normal organs have been tested using the immunoperoxidase technique and solid phase RIAs. However, there are several areas of investigation concerning the biological distribution of the antigens detected that will be thoroughly investigated. These include: (a) reactivity with "benign" mammary tumor lesions; (b) reactivity of non-breast tissues; and (c) reactivities to mammary biopsy specimens and primary cultures.

Cytotoxicity experiments. Initial efforts in our laboratory have indicated that several of the monoclonal antibodies described above are cytotoxic for human breast cancer cell lines in antibody-dependent cell mediated cytotoxicity experiments. We plan to work with several laboratories to investigate: (a) antibody-mediated cytotoxicity; (b) antibodies coupled to radioisotopes; and (c) antibodies coupled to toxins, such as ricin and diphtheria toxin.

Generation of new monoclonal antibodies: Not all primary and metastatic mammary tumor masses contain the antigens that are detected by the monoclonal antibodies we have generated to date. Furthermore, in some cases (such as with monoclonal B72.3) we have only one monoclonal, of a given isotype to a single determinant, to a novel tumor associated antigen. We plan to obtain new monoclonals to different determinants of this antigen, and of differing isotypes and affinities, to be utilized to further define the structure and function of this molecule, and for sandwich radioimmunoassays. We also plan to generate anti-idiotypic monoclonals against several of the monoclonals described for use in RIA.

Use as immunogen those primary and metastatic masses that do not react with any of the monoclonals characterized to date in an attempt to obtain a battery of monoclonal antibodies that are reactive with all primary and metastatic mammary tumor masses.

Publications:

Colcher, D., Horan Hand, P., Nuti, M., and Schlom, J.: Differential binding to human mammary and non-mammary tumors of monoclonal antibodies reactive with carcinoembryonic antigen. Cancer Investigation, in press.

Colcher, D., Horan Hand, P., Nuti, M., and Schlom, J.: A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc. Natl. Acad. Sci. USA 78: 3199-3203, 1981.

Nuti, M., Colcher, D., Horan Hand, P., Austin, F., and Schlom, J.: Generation and characterization of monoclonal antibodies reactive with human primary and metastatic mammary tumor cells. In Albertini, A. and Ekins, R. (Eds.): Monoclonal Antibodies and Developments in Immunoassay. New York, Elsevier/North-Holland, 1981, pp. 87-98.

Nuti, M., Teramoto, Y.A., Mariani-Costantini, R., Horan Hand, P., Colcher, D., and Schlom, J.: A monoclonal antibody (B72.3) defines patterns of distribution of a novel tumor associated antigen in human mammary carcinoma cell populations. Int. J. Cancer, in press.

Nuti, M., Teramoto, Y.A., Mariani-Costantini, R., Horan Hand, P., Colcher, D., and Schlom, J.: Reactivity of a monoclonal antibody (B72.3) with fixed sections of human mammary carcinomas. In Davis, W., Harrap, K., and Tanneberger, S. (Eds.): The Control of Tumour Growth and Its Biological Bases. Berlin-Buch, Germany, Academic Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05231-01 LCMB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Molecular Cloning and Characterization of Human Transforming Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. Santos	Visiting Fellow	LCMB	NCI
OTHERS:	M. Barbacid	Visiting Scientist	LCMB	NCI
	S. Pulciani	Visiting Fellow	LCMB	NCI
	V. Notario	Visiting Associate	LCMB	NCI
	S. Tronick	Microbiologist	LCMB	NCI
	K. Robbins	Expert	LCMB	NCI
	S.A. Aaronson	Chief	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: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies done during the course of this project include: (1) identification of dominant transforming genes present in human tumor cell lines and in naturally occurring human tumors; (2) molecular cloning of an oncogene present in T24 bladder carcinoma cells; (3) physical mapping of the T24 oncogene and characterization of the transforming region; (4) isolation of a normal gene homologous to the T24 oncogene from a library of human fetal liver DNA; (5) biochemical comparison of the normal and transforming alleles; (6) identification of the transcriptional and translational products of the T24 oncogene; (7) relationship of the T24 oncogene with other transforming genes including those present in acute transforming retroviruses.

Project Description

Objectives:

1. To determine the genetic differences between the T24 oncogene and its normal homologue form.
2. To determine whether the T24 oncogene is etiologically involved in naturally occurring human bladder tumors.
3. To study the processes by which the T24 oncogene induces malignant transformation.
4. To isolate by molecular cloning additional human oncogenes present in tumors other than bladder carcinomas.

Methods Employed:

Recombinant DNA technology includes the growth and use of different bacterial and phage strains, DNA and RNA isolation, restriction endonuclease analysis, Southern and northern blotting hybridization techniques and plasmid amplification and isolation.

Major Findings:

1. We have cloned the oncogene from T24 bladder carcinoma cells.
2. We have characterized the T24 oncogene, obtained its physical map and identified the sequences necessary for malignant transformation.
3. We have isolated the normal, nontransforming human sequences homologous to the T24 oncogene from a library of human fetal liver DNA.
4. By comparing the T24 oncogene with its normal counterpart, we have determined that no major rearrangements or modifications of the normal sequences have occurred to give rise to the T24 oncogene.
5. We have found that the T24 oncogene is different from oncogenes present in other human tumor cell lines as well as in naturally occurring tumors.
6. We have found that the T24 oncogene is highly related to the onc gene of BALB-MSV, an acute transforming retrovirus.
7. We have identified the transcriptional and translational products of the T24 oncogene.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of human transforming genes are of great importance to understanding the mechanisms involved in malignant transformation and to establish the etiology of human tumors.

Proposed Course:

1. To determine the genetic nature of the changes responsible for the acquisition of malignant properties by the T24 oncogene.
2. To study the involvement of the T24 oncogene in naturally occurring human bladder carcinomas.
3. To isolate, by molecular cloning, the oncogenes present in several naturally occurring human tumors obtained from different tissues.

Publications:

Pulciani, S., Santos, E., Lauver, A.V., Long, L.K., and Barbacid, M.: Transforming genes in human tumors. J. Cell. Biochem., in press.

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.

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., 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, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05232-01 LCMB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development of Murine Epithelial Cell Lines for In Vitro Transformation Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: B. Weissman Staff Fellow LCMB NCI
OTHER: S.A. Aaronson Chief LCMB NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A cell line derived from mouse epidermis has been established and characterized in our laboratory. The cell line is aneuploid, nontumorigenic, anchorage dependent and dependent on epidermal growth factor (EGF) for proliferation. It has been further designated as epithelial by morphology and biochemical markers such as keratin production. Infection of these cells with several different sarcoma viruses results in morphological change as well as a release from dependence on EGF. Detailed analyses of these virally transformed epithelial cells is currently in progress.

Project DescriptionObjectives:

1. To establish a murine epithelial cell line suitable for use as an assay for transformation.
2. To develop biochemical markers of differentiation in murine epithelial cells.
3. To examine the interaction of retroviruses with murine epithelial cells.

Methods Employed:

Murine epithelial cells are established from newborn mouse epidermis and developed into permanent cell lines. These cell lines are characterized for their biological features as well as biochemical markers of differentiation. The effect of different ion concentrations and growth factors in medium are also determined. Cloned retroviruses are used to infect these cells and potential changes in the growth properties of the infected cells are examined in vitro and in vivo.

Major Findings:

1. Permanent cell lines of murine epithelial keratinocytes have been established and characterized.
2. BALB-MSV, Kirsten sarcoma virus and Abelson MLV have been shown to transform these cell lines.
3. Transformation of murine keratinocytes by sarcoma viruses affects the pattern of keratin production in these cells.

Significance to Biomedical Research and the Program of the Institute:

Since the majority of human cancers are of epithelial cell origin, the study of virally transformed epithelial cells is of great interest. The effects of these viruses on a well-defined system of cellular differentiation, i.e., keratin production, is also equally important. The observation that BALB-MSV transforms epithelial cells in vitro is very relevant in light of the recent studies which show that a human oncogene isolated from a human tumor cell line is related to the *bas/ras* oncogene.

Proposed Course:

1. Extensively characterize the BALB-MSV and Abelson-transformed epithelial cell lines.
2. Determine whether other sarcoma viruses will transform murine epithelial cells.
3. Compare the similarities and/or differences between virally transformed fibroblasts and virally transformed epithelial cells.
4. Examine the effects of chemical carcinogens on murine epithelial cells.

Publications:

None.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Identification and Purification of Mammary Tumor Associated Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Daniela Stramignoni	Visiting Fellow	LCMB	NCI
OTHERS:	Jeffrey Schlom	Chief, Experimental Oncology Section	LCMB	NCI
	David Colcher	Microbiologist	LCMB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

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SECTION

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TOTAL MANYEARS:

1.3

PROFESSIONAL:

1.3

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies to human mammary tumor metastases were tested for reactivity to novel and known tumor-associated antigens. The monoclonals were used to immunoprecipitate antigens from a radiolabeled breast tumor metastasis extract. Monoclonal antibody B72.3 immunoprecipitated a high molecular weight polypeptide complex of approximately 220,000d. B6.2 and four other antibodies immunoprecipitated a 90,000d polypeptide. The four other antibodies cross-react in RIA for monoclonal B6.2 but differ in their ability to compete with the binding of B6.2. Two antibodies, B1.1 and F5.5, were shown to differentially react with carcinoembryonic antigen. The high molecular weight complex, identified by monoclonal B72.3, has been preparatively purified using molecular sieving and antibody affinity chromatography, without loss of immunoreactivity. Studies are in progress to develop radioimmunoassays with several of the monoclonals described.

Project Description

Objectives:

To identify and characterize tumor-associated antigens reactive with monoclonal antibodies. To determine if these monoclonal antibodies bind to antigens distinct from known tumor-associated antigens such as CEA, ferritin, T and M-N blood group antigens, and determine how many distinct tumor-associated antigens are detected by these monoclonal antibodies. To preparatively purify these tumor-associated antigens and develop radioimmunoassays (RIAs) for their detection in human tissues and biological fluids. The RIAs may eventually prove useful in the diagnosis, prognosis, detection of recurrence, and monitoring efficacy of treatment of human mammary cancer.

Methods Employed:

Presence of tumor-associated antigens, reactive with monoclonal antibodies, in primary tumor metastases, purified proteins and cell lines was determined by solid phase RIA. The molecular weight of these antigens was determined by immunoprecipitation and SDS polyacrylamide gel electrophoresis and western blotting technique. Purification of the antigens was performed by molecular sieving and antibody affinity column chromatography. A competitive binding assay was used to determine whether monoclonal antibodies reacting with the same antigen reacted also with the same binding site.

Major Findings:

Selection of an antigen source for the identification of tumor-associated antigens. As a first step to the identification of tumor-associated antigens reactive with the monoclonal antibodies described, the most immunoreactive antigen source for each antibody was determined. Monoclonal antibodies were first screened for reactivity with several previously well-characterized tumor-associated antigens, including carcinoembryonic antigen (CEA, derived from colon carcinoma), ferritin (derived from liver), acid placental ferritin, T antigen and M-N blood group antigens (obtained from George Springer, Northwestern University). Strong positive reactivity to CEA was observed only with monoclonal antibodies B1.1 and F5.5. No reactivity was observed to the other antigens with any of the monoclonal antibodies tested. As a next step in the identification of the best antigen source, monoclonal antibodies were screened by solid phase RIA for reactivity with a variety of mammary tumor extracts, including primary and metastatic tumors and established cell lines. Monoclonals B1.1 and B6.2 reacted similarly with tissue extracts and extracts of the cell lines. Antibody B72.3, however, showed very strong reactivity with some human tumor extracts, but reacted poorly with mammary tumor cell lines. Two breast tumor metastases to the liver were chosen as prime sources for antigen identification and purification on the basis of their broad immunoreactivity to all the monoclonal antibodies and the quantity of tumor tissue available.

Characterization of monoclonal antibodies to CEA. Antibodies B1.1 and F5.5 were shown to bind in a solid phase RIA to CEA purified from a colon carcinoma patient. Cross reactivities have been reported, however, between determinants on CEA and an

antigen expressed in normal spleen, termed normal cross-reacting antigen (NCA). Both monoclonals B1.1 and F5.5 were shown to react with determinants not shared by CEA and NCA. Purified immunoglobulin preparations of monoclonals B1.1 and F5.5 were then titered by serial dilution for binding to CEA preparations purified from five different patients with colon cancer. Monoclonal F5.5 reacted similarly with all five CEA preparations, whereas B1.1 bound differentially to the various preparations. The differences in binding of the antibodies to the different CEA preparations further demonstrate the heterogeneity of the CEA family of glycoproteins.

Immunoprecipitation of tumor associated antigens. Immunoprecipitation studies were initiated to determine the molecular weights of the tumor-associated antigens reactive with the monoclonal antibodies described. Purified CEA (obtained from H. Hansen, Hoffman La Roche) was iodinated and used as antigen source for the binding of B1.1 and F5.5. SDS-PAGE of the immunoprecipitates shows that the polypeptide precipitated by both monoclonal antibodies is a heterogeneous protein with an average molecular weight of 180,000. An extract of a breast tumor metastasis to the liver was used as the antigen source for the other monoclonal antibodies described. The reactivity of B1.1 and F5.5 to this breast tumor metastasis was also tested to determine if a polypeptide similar in molecular weight to CEA would be precipitated. Initial attempts to identify the various reactive antigens in radioiodinated extracts of the metastasis were unsuccessful. Experiments were undertaken to increase the relative antigen concentration by partial purification of the extract. The metastatic liver extract was detergent-disrupted and separated using molecular sieving on Ultrogel AcA34. The column fractions were assayed for reactivity with monoclonals B1.1, B6.2, and B72.3 by solid phase RIA. The appropriate immunoreactive fractions were then pooled and labeled with 125 I. SDS-PAGE analyses of the immunoprecipitates showed that B72.3 immunoprecipitated a complex of four bands with estimated molecular weights of approximately 220,000, 250,000, 285,000, and 340,000. B1.1 immunoprecipitated a heterogenous component with an average estimated molecular weight of 180,000. B6.2 immunoprecipitated a 90,000d component, as did several other monoclonal antibodies.

Analysis of cell extracts by "western" blotting. Extracts of a breast tumor metastasis to the liver, normal liver, and the MCF-7 breast tumor cell line were disrupted and run on an SDS-polyacrylamide gel. The polypeptides were electrophoretically transferred to nitrocellulose filters, and the filters were incubated with IgG from B1.1, B6.2 or B72.3. The filters were washed, and the remaining antibodies were detected with rabbit anti-murine IgG and 125 I-Protein A. B72.3 bound to a high-molecular weight complex of approximately 220,000d in the extract from the metastasis. B1.1 bound to a 180,000d polypeptide and B6.2 bound to a 90,000d polypeptide in extracts of both the breast tumor metastasis and the MCF-7 cell line. These data demonstrate that the immunoreactivity of the antigenic determinants are not destroyed by SDS and mercaptoethanol and that molecular weights of the polypeptides in the crude extracts are consistent with those obtained by the immunoprecipitations from semipurified extracts as described above.

Characterization of monoclonal antibodies to the 90,000d antigen. Five of the monoclonal antibodies, including B6.2, were reactive with an antigen of approximately 90,000d. To determine whether these antibodies reacted with the same

determinants, a competitive binding assay was established. Purified monoclonal antibody B6.2 was labeled with ^{125}I . Increasing amounts of unlabeled monoclonal antibodies were added to a breast tumor extract followed by the addition of ^{125}I -labeled B6.2 IgG. As little as 10 ng of B6.2 IgG was able to inhibit the binding of the labeled antibody by greater than 90%. Similar inhibition was observed with B84.1 IgG. Various degrees of competition were also observed with other antibodies (B39.1, B14.2, B50.4, F25.2, B84.1). The ability of some of the other monoclonal antibodies to compete for the binding of B6.2 to the breast tumor metastasis extract indicates that these antibodies react to the same antigen.

Purification of the 220,000d high molecular weight complex. Monoclonal antibody B72.3 has been shown to have highly selective reactivity to tumor versus normal tissues. We thus attempted to purify the antigen reactive with B72.3 first so that further immunological and biochemical characterization could be made. An extract of a breast tumor metastasis to the liver, which contained the highest immunoreactivity with B72.3, was used as the starting material for purification of the 220,000d high molecular weight complex. Following detergent disruption and high speed centrifugation, the supernatant was subjected to molecular sieving using Ultrogel ACA34. Immunoreactive fractions were then passed through a B72.3 antibody affinity column and eluted with 3 M KSCN. Radiolabeled aliquots from the various purification steps were analysed by SDS-PAGE. Only minimal radioactivity in the high molecular weight range was seen in gel patterns of the ACA34 pool, whereas the affinity column eluant demonstrated the four distinct bands of the 220,000d complex. ^{125}I -labeled B72.3 affinity-purified antigen was tested for immunoreactivity by solid phase RIA. Approximately 70% of the purified ^{125}I -labeled antigen was bound in B72.3 antibody excess. The identical method of purification was used with a normal human liver extract as the starting tissue. At no step within the purification scheme was any reactivity with B72.3 detected.

Significance to Biomedical Research and the Program of the Institute:

The identification and characterization of mammary tumor-associated antigens will enable us to compare these antigens with tumor-associated antigens from other sources. The purification of these antigens will also aid in the establishment of radioimmunoassays for their detection in human tissues and/or fluids. These assays may prove useful in the diagnosis and prognosis of patients with mammary cancer in the detection of recurrence of the disease and in monitoring the efficacy of various therapeutic regimens. Sequencing of the purified antigens and comparison to other polypeptides may aid in the determination of the biological activity and significance of the tumor-associated antigens. The sequence of the tumor-associated antigen will (a) enable the synthesis of portions of the peptide that may be useful in subsequent RIAs and (b) enable the synthesis of a cDNA copy that will be useful in studies to clone the genes coding for the peptide.

Proposed Course:

The antigens detected by antibodies B1.1 (180,000d, CEA), B6.2 (90,000d) and B72.3 (220,000d complex) will be purified from the most available source of antigen using gel filtration and antibody affinity chromatography. If these antigens lose immunoreactivity after elution from the affinity column, an alternative purification method will be employed. Ion-exchange chromatography will be

performed followed by separation based on the isoelectric point using either preparative isoelectrofocusing in a granular flat bed or chromatofocusing.

A collaboration has been established to sequence the polypeptides once sufficient quantities can be purified. The sequencing will be performed using either unlabeled antigens purified from breast tumor metastases or ³H-amino acid-labeled polypeptides isolated from cell lines. The sequencing studies will enable us to compare the tumor-associated antigens identified by the monoclonal antibodies to antigens that have been previously sequenced. Also, a synthetic peptide could be generated that may be useful in developing radioimmunoassays for the antigens.

Radioimmunoassays for the detection of tumor-associated antigens and the antibodies reactive with them will be established. The various RIAs described above will be used for the assay of blood and other biological fluids and for examination of the biological distribution of the tumor associated antigens in various tissues. Studies will be undertaken to examine sera of breast cancer patients obtained before and during their course of therapy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05234-01 LCMB
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Cloning and Characterization of Human Transforming Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. Notario	Visiting Associate	LCMB	NCI
OTHERS:	M. Barbacid	Visiting Scientist	LCMB	NCI
	S. Pulciani	Visiting Fellow	LCMB	NCI
	E. Santos	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: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A 14 kbp DNA fragment of an oncogene present in HT-1080 cells, a cell line derived from a human fibrosarcoma, has been cloned from DNA isolated from HT-1080-derived transformants utilizing lambda-1059 as the cloning vector. The human origin of the cloned DNA was demonstrated by the presence of human Alu repetitive sequences and by its poor hybridization to mouse cellular DNA. The cloned fragment was found to be present in all tested transfectants derived from HT-1080. The cloned oncogene fragment was found not to be related to either the T24 bladder carcinoma oncogene or to a number of retroviral onc genes.

Project DescriptionObjective:

To isolate and characterize human oncogenes.

Methods Employed:

A variety of human tumor cell lines, as well as naturally occurring human tumors, have been tested for the presence of oncogenes by DNA-mediated gene transfer experiments. Several tumors, including the fibrosarcoma HT-1080, were able to transmit the transformed phenotype to NIH/3T3 mouse cells. In each case, the transformed cells contained human sequences as determined by their ability to hybridize with probes specific for human Alu repetitive sequences. Human DNA sequences encompassing the transforming genes were identified by their cosegregation with the transformed phenotype.

After two or three cycles of transfection, these sequences were isolated by molecular cloning techniques. The human cellular DNA was partially digested with restriction endonucleases under conditions to avoid destroying their transforming ability and then ligated to vector DNA such as cosmid pHc 79, plasmid lambda-1059 or bacteriophage lambda-Charon 9. The ligated DNA was then packaged in vitro into phage particles which were used to infect the appropriate bacterial strains. Bacterial colonies or phage plaques were then screened for the presence of Alu sequences. Colonies or plaques giving positive hybridization to probes containing Alu sequences were isolated, grown and the DNA extracted from them used for transfection experiments to test the transforming ability of the cloned DNA.

Major Findings:

We have cloned a 14 kbp fragment of an oncogene present in HT-1080 cells, a human fibrosarcoma cell line. The cloned fragment has been found to be present in all tested HT-1080-derived transformants. The cloned DNA did not hybridize to a variety of probes from retroviral onc genes nor to an oncogene recently cloned from human bladder carcinoma cells. In transfection assays, the cloned oncogene fragment was unable to transform NIH/3T3 cells, indicating that the cloned DNA does not contain an intact HT-1080 oncogene.

Significance to Biomedical Research and the Program of the Institute:

The isolation of transforming genes from human tumors 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 the oncogenes present in transfectants of both HT-1080- and MNNG-HOS-derived cell lines. Since both oncogenes seem to have a big size (greater than 20 kbp), we are using cosmid as the cloning system and Mbo I partially cleaved cellular DNA isolated from third cycle transformants.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05235-01 LCMB
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PERIOD COVERED
January 12, 1982 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Molecular Cloning of the Unintegrated Circular DNA of Squirrel Monkey Retrovirus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	I-M. Chiu	Visiting Fellow	LCMB	NCI
OTHERS:	S. Tronick	Research Microbiologist	LCMB	NCI
	R. Callahan	Research Microbiologist	LCMB	NCI
	S. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cellular and Molecular Biology
SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.75	PROFESSIONAL: 0.75	OTHER: 0.0
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(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In efforts to detect retrovirus nucleotide sequences in human DNA, the squirrel monkey retrovirus (SMRV) was molecularly cloned in a bacteriophage vector. The cloned SMRV DNA was biologically active as demonstrated by DNA transfection assays. A detailed restriction enzyme map of the viral genome was constructed. Using this information, discrete fragments of SMRV DNA were prepared by sub-cloning in a plasmid vector. These subcloned DNA fragments, along with the genomic viral DNA, are being used as probes to detect homologous sequences in human DNA and also to study the relationship between SMRV and other retroviruses.

Project DescriptionObjectives:

1. Detect retroviral-related sequences in human DNA using molecularly cloned squirrel monkey retroviral probes.
2. Define the relationship of such sequences to human cancers.
3. Determine the relationship of the SMRV genome to other retroviruses.

Methods Employed:

Standard molecular biological techniques including gene enrichment, restriction enzyme analysis, cDNA synthesis, molecular cloning, gel electrophoresis, Southern transfer, filter hybridization, biological assays for retroviruses including DNA transfection, and library screening.

Major Findings:

1. The full length proviral DNA of SMRV has been cloned into λ gt WES- λ B.
2. Transfection of D-17 osteosarcoma cells with the cloned DNA resulted in the induction of reverse transcriptase activity, indicating that the clone was biologically active.
3. Four Hind III fragments composed of the full length of SMRV DNA and a 1.4 kb EcoRI-Pst I fragment have been subcloned into pBR322.
4. The cloned SMRV viral DNA has been used to detect the corresponding endogenous proviral sequences in genomic DNA from other primate species. Human DNA, when digested with Pvu I and Sal I, yielded one blurry band. Rhesus monkey DNA yielded a distinct band (~ 25 kb) when digested with Sal I and Pvu I. In contrast, there were 3-5 fragments with unequal intensity, ranging from 5-10 kbp, detected when rhesus monkey DNA was digested with Eco RI or Xba I. This implies that endogenous SMRV-related DNA may be present in multiple copies within certain Old World primates.

Significance to Biomedical Research and the Program of the Institute:

The availability of a molecularly cloned type D helper virus of primate origin makes possible a detailed analysis of the role retroviruses play in malignancies of primate species including humans. Furthermore, the relationship of this virus to other retroviruses can be more readily ascertained. The cloned SMRV genome may now make it possible to isolate specific retrovirus-related genes from the human genome.

Proposed Course:

1. To clone SMRV-related sequences from human DNA.
2. To determine the genetic relationship between SMRV and other retroviruses.
3. To continue to study in detail the physical structure and organization of the SMRV genome.
4. To study the evolutionary conservation of SMRV-related sequences in vertebrate DNAs.

Publications:

Chiu, I-M., Sittman, D.B., Pan, C., Cohn, R.H., Kedes, L.H., and Marzluff, W.F.: Isolation of two clusters of mouse histone genes. Proc. Natl. Acad. Sci. U.S.A. 78: 4078-4082, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Antigenic Heterogeneity of Human Mammary Tumor Cell Populations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marianna Nuti	Visiting Fellow	LCMB	NCI
OTHERS:	Jeffrey Schlom	Chief, Experimental Oncology Section	LCMB	NCI
	Patricia Horan Hand	Chemist	LCMB	NCI

COOPERATING UNITS (if any)

Sidney Farber Cancer Institute, Boston, Massachusetts

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.4

PROFESSIONAL:

0.7

OTHER:

0.7

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 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antigenic variation was observed in the expression of specific tumor-associated antigens within individual human mammary tumor masses using monoclonal antibodies. This variation was demonstrated by both the pattern and cellular localization of reactivity with a given antibody. This diversity was also observed in human mammary tumor cell lines grown in vivo and in vitro. Analyses of DNA content and cell surface binding of monoclonal antibodies during logarithmic growth phase, and at density-dependent arrest, demonstrated that the expression of some tumor-associated antigens is related to S-phase of the cell cycle. Membrane expression of the reactive antigens appeared to be stable despite prolonged exposure to antibody. Antigenic drift was observed with continued passage of mammary tumor cell lines; consistent with this finding, the "same" mammary tumor cell line obtained from different sources exhibited distinct antigenic phenotypes. Preliminary results indicate that exposure of mammary tumor cells to certain compounds may enhance the cell surface expression of some tumor-associated antigens.

Project Description

Objectives:

To define the antigenic heterogeneity that may exist within a given human mammary tumor mass. To determine parameters which mediate the expression of various antigenic phenotypes within mammary tumor cell populations. To develop methods of altering or controlling the expression of specific antigenic phenotypes.

Methods Employed:

The expression of human mammary tumor-associated antigens recognized by monoclonal antibodies is determined using (1) the immunoperoxidase method on sections of human mammary tumors and on mammary tumor cell lines; (2) fluorescent-activated cell sorter analyses of tumor and normal cell lines; and (3) the live cell radioimmunoassay which determines the binding of the antibody to the surface of cells in culture.

Major Findings:

I. Differential expression of tumor-associated antigens within a given mammary tumor mass. Antigenic variation was observed in the expression of tumor-associated antigens within a given mammary tumor. One pattern of antigenic reactivity observed repeatedly, using the immunoperoxidase method and tissue sections, was that one area of a mammary tumor contained tumor-associated antigens reactive with a particular monoclonal antibody, while another area of the same mammary tumor was not reactive with the identical antibody. Another type of antigenic heterogeneity was observed among cells in a given area of a tumor mass. This type of antigenic diversity, termed "patchwork," is demonstrated by the presence of tumor cells expressing a specific tumor-associated antigen directly adjacent to tumor cells negative for the same antigen. Patterns of reactivity with a specific monoclonal antibody were also observed to vary within a given mammary tumor mass. For example, monoclonal antibody B72.3 reacted with the 220,000d tumor-associated antigen present in the cytoplasm of cells in one part of the tumor mass and on the luminal edge of differentiated structures in a different part of the same tumor mass.

II. Heterogeneity of tumor-associated antigen expression within human mammary tumor cell lines. In an attempt to elucidate the phenomenon of heterogeneity of antigenic phenotypes within a human mammary tumor mass, model systems were examined. Human mammary tumor cell lines, transplanted in nude mice, also demonstrate antigenic heterogeneity. To determine if this phenomenon also exists in human mammary tumor cell lines grown in vitro, MCF-7 cells were tested for the presence of tumor-associated antigens using the cyto-spin/immunoperoxidase method. The MCF-7 cell line contained various subpopulations of cells as defined by variability in expression of the tumor-associated antigen reactive with monoclonal antibody B6.2. Positive MCF-7 cells were seen adjacent to cells which scored negative. In addition, variability was observed with respect to the quantity of the 90,000d tumor-associated antigen detected among the population of cells which were positive, as demonstrated by differences in the intensity of the stain. A similar heterogeneity, with fewer cells (less than 10%) scoring positive, was also observed using monoclonal B72.3.

III. Parameters affecting expression of tumor-associated antigens in vitro.

(a) Fluorescent-activated cell sorter (FACS) analyses. Monoclonal antibodies B6.2 and B38.1 were analyzed via FACS for surface binding to a panel of human cell lines. Both antibodies were reactive with breast carcinoma lines BT-20 and MCF-7. A non-reactive monoclonal antibody (anti-human IgM) of the identical IgG-1 isotype showed no binding. The fluorescence pattern with B38.1 was consistently more intense than that seen with B6.2. In contrast to the breast carcinoma cell lines, a cell line derived from prostate carcinoma (PC-3) showed strong reactivity with B38.1 but was unreactive with B6.2. Both antibodies were unreactive with WI-38 embryonic lung, CEM lymphoblasts, and normal bone marrow.

(b) Relationship of cellular growth patterns to expression of antigenic phenotypes. The cell surface binding of monoclonal antibody B6.2 was monitored on MCF-7 mammary carcinoma cells during logarithmic growth phase and at density-dependent arrest. Antibody binding was monitored each day for seven consecutive days after passage. One day after passage, B6.2 was strongly reactive with the MCF-7 cells. Over the following seven days, the reactivity of the monoclonal decreased progressively and was lowest at density-dependent arrest. Passage of these cells on day 7 resulted in the reappearance of the antigen within 24 hours. One explanation for these results would be a cell cycle related expression of the antigen. Similar results were observed with monoclonal B38.1.

To investigate the relationship of antigen expression to cell cycle phase, day 7 cells at density-dependent arrest were passed and monitored 24 hours later for both DNA content and cell surface antigen expression via FACS. DNA staining by Hoechst dye demonstrated that the majority of cells were in the G_0/G_1 phase. The remainder of cells were distributed evenly throughout S-phase and G_2/M phase. G_0/G_1 cells express background fluorescence. In contrast, monoclonal antibody B38.1 was most reactive with cells in S-phase. This S-phase differential binding was also observed using monoclonal antibody B6.2.

c) Effect of prolonged exposure of MCF-7 cells to antibody on expression of cell surface tumor-associated antigens.

In light of the phenomena of "capping," "internalization," and "shedding" of lymphocyte surface antigens after exposure to monoclonal antibodies, studies were undertaken to determine the effect of prolonged exposure of MCF-7 cells to monoclonal antibodies B6.2 and B38.1 on cell surface expression of the reactive antigens. MCF-7 cells were incubated in the presence of monoclonals B6.2 or B38.1 for two hours or 24 hours. The fluorescence patterns after exposure to G/M (goat anti-mouse) FITC were similar for each exposure. MCF-7 cells were also incubated for 2 hours, washed extensively and then refed with growth medium. Cells were then incubated for an additional 22 hours and analyzed following the addition of G/M FITC. The fluorescence profile was again similar to that achieved during a 2-hour or 24-hour antibody incubation. The membrane expression of the reactive antigens therefore appears to be stable, despite continued exposure to antibody.

IV. Antigenic drift of mammary tumor cell populations. Studies were undertaken to determine if any drift in antigenic phenotypes could be observed in human mammary tumor cell lines over an extended period of time. The BT-20 cell line, obtained at passage 288 from the Breast Cancer Task Force, was serially passaged

and assayed at each passage level during S-phase of the growth cycle. A cell surface HLA antigen detected by monoclonal antibody W6/32, was present at all passage levels, as was the tumor-associated antigen detected by monoclonal antibody B38.1. The 90,000d antigen detected by monoclonal antibody B6.2 was expressed on the BT-20 cell surface up to passage 319, but was not evident after this passage level. Similarly, monoclonal B14.2 reacted with BT-20 cells only up to passage 317. This phenomenon was observed in three separate experiments at approximately the same passage levels, using cells thawed from a BT-20 culture viably frozen at passage level 302. Antigenic drift was also observed with the MCF-7 cell line as manifested by the differential binding of several monoclonal antibodies to the cell surface. MCF-7 cell lines were obtained from four sources and were examined for surface expression of several tumor-associated antigens. Karyotype profiles of the four cell lines were examined (W. Peterson, Child Research Center, Detroit) and were identical and characteristic of the MCF-7 cell line. A single LDH band, characteristic of only a few breast tumor cell lines including MCF-7, was also supportive evidence that these cell lines were indeed MCF-7. Using a live cell solid phase RIA, which detects the reactivity of antigens at the cell surface, immunologic profiles of the four MCF-7 cell lines were determined. Monoclonal antibody B139, which recognizes an antigen on the surface of all human cells reacted with all four cell lines. However, when other monoclonal antibodies were tested, several different antigenic phenotypes emerge. The MCF-7 cell line obtained from the Breast Cancer Task Force contained all the tumor-associated antigens assayed for, while the MCF-7 (GC) cell line expressed none. Similar results were also observed with BT-20 cell lines obtained from different sources.

Significance to Biomedical Research and the Program of the Institute:

Heterogeneity of cells within a given tumor mass, including antigenicity, response to drugs, and presence of cell surface receptors has been observed. The differences among the cell populations present a problem in the development and optimization of immunodiagnostic and therapeutic procedures for breast cancer. Knowledge about the nature of this antigenic heterogeneity may help to predict or control the expression of a specific antigenic phenotype. The studies described here have enabled us to demonstrate this antigenic heterogeneity within human mammary tumor populations both in vivo and in vitro. An examination of the effect of cell cycle, cellular growth patterns, and continued cell passage has enabled us to determine some of the parameters associated with a change in phenotype. Moreover, preliminary studies relating the effect of retinoic acid or progesterone on the expression of specific antigens indicate that the potential exists to regulate or amplify the tumor antigen level on the surface of cells. These studies may therefore ultimately aid in both the detection and therapy of breast cancer.

Proposed Course:

Future studies will involve the development and use of cloned mammary tumor cell populations for the delineation of the nature of antigenic diversity. Several parameters of antigenic heterogeneity will be examined with cloned cell populations exhibiting distinct antigenic phenotypes, including the relationship between the expression of a specific antigenic phenotype and such parameters as morphology, tumorigenicity, drug susceptibility, estrogen receptors, and growth

rate. Studies will also be conducted to determine the ability of cell surface tumor-associated antigens to modulate following exposure to a variety of agents including hormones, vitamin A derivatives, and interferon. If success is obtained in the in vitro system in enhancing the expression of specific tumor-associated antigens, experiments will be undertaken to see if the same effects can be observed in vivo, employing human tumors transplanted in athymic mice.

Publications:

Colcher, D., Horan Hand, P., Nuti, M., and Schlom, J.: Differential binding to human mammary and non-mammary tumors of monoclonal antibodies reactive with carcinoembryonic antigen. Cancer Investigation, in press.

Colcher, D., Horan Hand, P., Nuti, M., and Schlom, J.: A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc. Natl. Acad. Sci. USA 78: 3199-3203, 1981.

Nuti, M., Colcher, D., Horan Hand, P., Austin, F., and Schlom, J.: Generation and characterization of monoclonal antibodies reactive with human primary and metastatic mammary tumor cells. In Albertini, A. and Ekins, R. (Eds.): Monoclonal Antibodies and Developments in Immunoassay. New York, Elsevier/North-Holland, 1981, pp. 87-98.

Nuti, M., Teramoto, Y. A., Mariani-Costantini, R., Horan Hand, P., Colcher, D., and Schlom, J.: A monoclonal antibody (B72.3) defines patterns of distribution of a novel tumor associated antigen in human mammary carcinoma cell populations. Int. J. Cancer, in press.

Nuti, M., Teramoto, Y.A., Mariani-Costantini, R., Horan Hand, P., Colcher, D., and Schlom, J.: Reactivity of a monoclonal antibody (B72.3) with fixed sections of human mammary carcinomas. In Davis, W., Harrap, K., and Tanneberger, S. (Eds.): The Control of Tumour Growth and Its Biological Bases. Berlin-Buch, Germany, Academic Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05244-01 LCMB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Localization of Human Mammary Tumors Using Radiolabeled Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: David Colcher Microbiologist LCMB NCI
OTHER: Jeffrey Schlom Chief, Experimental LCMB NCI
Oncology Section

COOPERATING UNITS (if any)

O. Gansow, Laboratory of Chemical Physics, NIADD, NIH;
M. Zalutsky and W. Kaplan, Sidney Farber Cancer Institute, Boston,
Massachusetts

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Experimental Oncology Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

1.7

PROFESSIONAL:

0.7

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Purified IgG, F(ab')₂ fragments, and Fab' fragments of monoclonal B6.2 have been generated. The IgG and its fragments were radiolabeled with I-125 without loss of immunoreactivity and were injected into athymic mice bearing human mammary tumor transplants. The radiolabeled antibody localized in the tumor within 24 hours with tumor to tissue ratios rising over a 96-hour period. The F(ab')₂ was better than the IgG and gave tumor to liver and spleen ratios of 15 to 20:1, and tumor to muscle and brain ratios of 50 to 110:1. No localization was observed in mice bearing human melanomas or with radiolabeled normal murine IgG in human mammary tumor bearing mice. The ability of the radiolabeled antibody to localize in mammary tumors was sufficient to give high quality gamma scans of tumor bearing mice. Several monoclonal antibodies are now being labeled with other radioactive isotopes that may be more appropriate for clinical studies.

Project Description

Objectives:

To radiolabel monoclonal antibodies reactive with human mammary tumor-associated antigens without loss of immunoreactivity and use them to localize human tumors in athymic mice. To determine what form of the antibody should be used for the radiolocalization studies, and if successful in model systems, to localize tumors in lymph nodes (via lymphangiography) and at distal sites in breast cancer patients.

Methods Employed:

Monoclonal antibodies were purified from ascitic fluids of mice by ion exchange chromatography and molecular sieving. Pepsin was then used to generate $F(ab')_2$ and Fab' fragments. The IgG and its fragments were radiolabeled with ^{125}I and assayed for immunoreactivity in solid phase radioimmunoassays. The radiolabeled antibodies were injected IV into nude mice bearing human tumors and in vivo distribution of the label was determined by analyzing the tissues for radioactivity and by gamma scanning of the mice.

Major Findings:

Labeling of antibody B6.2 IgG and fragments. Monoclonal B6.2 was purified by salt precipitation ion exchange chromatography. Some of the purified IgG was used to generate $F(ab')_2$ and Fab' fragments by pepsin digestion. The fragments were purified by molecular sieving and retained all their immunoreactivity when compared on a molar basis to the intact IgG. The IgG and its fragments were labeled with ^{125}I using a variety of techniques, including lactoperoxidase, chloramine T and iodogen. The iodogen method gave the highest yield of labeled antibody without loss of immunoreactivity. The binding of the IgG, $F(ab')_2$, and Fab' was better to the extracts from a breast tumor metastasis than to extracts of the MCF-7 breast tumor cell line. No binding was observed to extracts from normal human liver, lymphoid cells, or a rhabdomyosarcoma. The labeled antibody was shown to bind to the surface of MCF-7 cells and retained the same specificity as the unlabeled antibody. More than 70% of the antibody remained immunoreactive after labeling.

Tumor distribution studies. Nude mice were treated with pieces of a transplantable human mammary tumor (Clouser). After approximately 10-20 days, the tumors grew to detectable nodules. The growth rate of the tumors varied as did the final size obtained (0.5 to 2.5 cm diameter). Nude mice were also injected subcutaneously with cells from a human breast tumor cell line (MCF-7) and a human melanoma cell line (A375). The mice injected with MCF-7 cells required subcutaneous implants of estrogen for the tumors to progress.

Mice were injected intravenously in the tail vein with approximately 0.1 μg of ^{125}I labeled IgG or fragments, sacrificed at various times and the tissues assayed. The number of counts per mg of each tissue was determined and compared to that observed in the tumor of the same mouse.

Nude mice bearing the Clouser human mammary tumor were first injected with B6.2 IgG labeled with ^{125}I to a specific activity of approximately 15 μCi per μg . The ratio of cpm/mg in the tumor versus various tissues rose over a 4-day period and then fell at 7 days. The tumor to tissue ratio was greater than 10:1 in the liver, spleen and kidney at day 4. Ratios of the counts in the tumor to that found in the brain and muscle were greater than 50:1 and as high as 110:1. Lower tumor to tissue ratios were obtained with blood and lungs (with its large blood pool). The rate of clearance of the labeled IgG from the blood was slightly faster than that published by other investigators, possibly because of the difference in isotopes.

When the Clouser mice were injected with ^{125}I -F(ab')₂ fragments of B6.2, higher tumor to tissue ratios were obtained. The highest tumor to tissue ratios of the organs tested were obtained with liver and spleen with ratios of 15 to 20:1 at 96 hours. The tumor to tissue ratios were somewhat lower with blood and lungs, but were higher than those obtained using IgG. This is probably due to the faster clearance of the F(ab')₂ fragments as compared to the IgG. The tumor to kidney ratio was relatively low and was due to the clearance of Fab' fragments which are either generated from the F(ab')₂ in vivo by the breakage of the cross linking disulfide bonds or are present as minor contaminants (less than 1%) of the starting material. Nude mice bearing Clouser mammary tumors were also injected with ^{125}I -labeled B6.2 Fab'. The clearance rate of the Fab' fragment was considerably faster than the larger F(ab')₂ fragment and the intact IgG. Good tumor to tissue ratios were obtained within 8 hours, but the fast clearance rate resulted in a large amount of the labeled Fab' being found in the kidney and bladder.

Nude mice bearing human melanomas were used as controls for nonspecific binding of the labeled antibody. No preferential localization of the monoclonal antibody was observed in the tumor; in fact, counts per mg in the melanoma were lower than that found in many organs. Similar negative results were found when either normal murine IgG, F(ab')₂ or IgG₁ (the same isotype as B6.2) from a murine myeloma were inoculated into nude mice with Clouser mammary tumor or melanoma transplants.

There was a variability in the tumor to blood ratio observed between different nude mice bearing the Clouser mammary tumor. At 96 hours the ratios observed varied between 4 and 14:1. The variation may be due to either differences observed in tumor growth rate or antigenic differences among tumors (even though they arose from the same inoculum). The variability in tumor to blood ratios between mice may also be due to differences in blood supply to the tumors and thus in the ability of the labeled antibody to bind to the tumor. The mice bearing tumors derived from MCF-7 cells and BT-20 cells gave positive localization of the label, but at lower levels than that obtained with Clouser tumors. This result was not unexpected in that monoclonal B6.2 has consistently bound better to human tumor biopsy material than to tissue culture cells.

Scanning of nude mice bearing human tumors. Studies were undertaken with the Diagnostic Radiology Departments of the NCJ and Sidney Farber Cancer Institute to determine whether the localization of the ^{125}I -labeled antibody in the tumors was sufficient to detect using a gamma camera. Nude mice bearing the Clouser mammary tumor or the A375 melanoma were injected IV with approximately 30 μCi of B6.2 IgG. The mice were scanned and then sacrificed at 24 hour intervals. The B6.2 IgG was

easily detected in the Clouser tumor at 24 hours with a small amount of activity visible in the blood pool. The tumor remained strongly positive over the 4 day period with the background activity decreasing to the point where it was barely detectable at 96 hours. No localization was observed in the mice bearing the human melanoma which is unreactive with B6.2. Mice were also injected with ^{125}I -B6.2 F(ab')₂. The mice cleared the fragments faster than the intact IgG and a significant amount of activity can be observed in the kidneys and bladder at 24 hours, but the tumor is clearly positive for localization of the ^{125}I -B6.2 F(ab')₂ fragments. The activity is cleared from the kidney and bladder by 48 hours and the tumor to background ratio increased over the 4-day period of scanning with little background activity observed at 96 hours. No localization of activity was observed in the nude mice bearing the A375 melanoma.

Significance to Biomedical Research and the Program of the Institute:

Radiolabeled polyclonal antibodies have previously been used to detect metastases. The use of monoclonal antibodies will give to this technique an added specificity and thus less computer manipulation and general applicability. The ability to assess internal mammary chain involvement may be of benefit in the staging of patients. The ability of the antibody to localize metastatic lesions may also prove useful as an adjunct in monitoring efficacy of therapy.

Proposed Course:

F(ab')₂ fragments of monoclonal antibody B6.2 labeled with ^{125}I will be used to determine the minimum size tumor that can be detected by gamma camera scanning. The distribution of the antibody in the tumor will be examined by autoradiography of tumor sections of scanned mice. The efficacy of other radiolabeled monoclonal antibodies made against human breast tumor metastases for radioimmunolocalization will be studied.

While ^{125}I -labeled monoclonal antibodies work well in the murine model systems, ^{125}I is not applicable in human studies because of its low energy. Other radioactive iodines will be used to determine their effect on the antibody specific activity, obtainable and efficiency of localization in mice. Other isotopes such as $^{99\text{m}}\text{Tc}$ and ^{111}In are currently being used in nuclear medicine. The feasibility of binding these isotopes to monoclonal antibodies, i.e., maintaining the stability of the label-antibody bond and immunoreactivity, will be examined.

We plan to use radioactively labeled monoclonal antibody B6.2 to localize human mammary tumors in patients with advanced disease. Iodinated antibody will be used in initial studies. The human experiments to detect distal metastases and lymph node lesions via lymphangiography will be performed in collaboration with the Diagnostic Radiology Departments of the Sidney Farber Cancer Institute and the NCI.

Publications:

Colcher, D., Horan Hand, P., Nuti, M., and Schlom, J.: Differential binding to human mammary and non-mammary tumors of monoclonal antibodies reactive with carcinoembryonic antigen. Cancer Investigation, in press.

Colcher, D., Horan Hand, P., Nuti, M., and Schlom, J.: A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc. Natl. Acad. of Sci. USA 78: 3199-3203, 1981.

Colcher, D., Horan Hand, P., Teramoto, Y.A., Wunderlich, D., and Schlom, J.: Use of monoclonal antibodies define diversity of mammary tumor viral gene products in virions and mammary tumors of the genus Mus. Cancer Res. 41: 1451-1459, 1981.

Nuti, M., Colcher, D., Horan Hand, P., Austin, F., and Schlom, J.: Generation and characterization of monoclonal antibodies reactive with human primary and metastatic mammary tumor cells. In Albertini, A. and Ekins, R. (Eds.): Monoclonal Antibodies and Developments in Immunoassay. New York, Elsevier/North-Holland, 1981, pp. 87-98.

CONTRACT NARRATIVE
LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1982

HAZLETON LABORATORIES AMERICA, INC. (N01-CP-01017)

Title: Support Services for the Laboratory of Cellular and Molecular Biology

Contractor's Project Director: Mr. James Gargus

Project Officer (NCI): Dr. Keith C. Robbins

Objectives: The purpose of the contract is to provide support services for research conducted by the LCMB.

Major Findings: Since the purpose of this contract is to provide support services for research conducted by LCMB, a discussion of major findings is inappropriate.

Methods Employed and Performance: The contractor performs the following tasks: (a) purification of retrovirus proteins, (b) performance of radio-immunoassays, (c) analysis of translational products synthesized by virus-infected and uninfected cells, (d) isolation of cellular and viral nucleic acids, (e) preparation of viral nucleic acid probes, (f) performance of molecular hybridization assays, (g) restriction endonuclease digestion of viral and cellular DNAs, and agarose gel electrophoresis, (h) biological assays for RNA tumor viruses, (i) growth of cells and viruses, (j) maintenance of animals, and (k) preparation and quality control of tissue culture medium.

Significance to Biomedical Research and the Program of the Institute: This program provides essential support services for research of the LCMB aimed at determining the etiology of naturally occurring cancers, elucidation of mechanisms of transformation, and the development of approaches capable of prevention of spontaneous and virus-induced tumors.

Proposed Course: To continue to provide timely, high quality support services for the LCMB.

Date Contract Initiated: June 16, 1980

Current Annual Level: \$705,000

CONTRACT NARRATIVE
LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
DIVISION OF CANCER CAUSE AND PREVENTION
FISCAL YEAR 1982

LITTON BIONETICS, INC. (N01-C0-75380; Subcontract FOD-0400L2196)

Title: Feral Mouse Breeding Colony

Contractor's Project Director: Marty McGowan

Project Officer (NIH): Dr. Robert Callahan

Objectives: This project provides the capability of maintaining pedigreed breeding colonies of feral mice from various geographical areas of the world, to test the effect of various biological (hormones and mouse mammary tumor virus) and chemical carcinogens on the incidence of mammary tumors, and provide a source of tumor tissue to test for the expression of tumor associated genes and retroviral genes.

Methods Employed: The contractor breeds and maintains various pedigreed breeding colonies of feral mice (1000) which are derived from a number of different geographical locations (United States, Europe, Asia, and North Africa). In addition the contractor performs the following tasks: a) maintenance of pedigree records, b) observation of mice for tumor development, c) surgery on live animals, d) dissection of sacrificed animals, d) injections, e) preparation of tissues for electron microscopy and histology, and f) provide blood and other animal products.

Major Findings: We have identified a pedigreed breeding colony of feral Mus musculus musculus which lack the genetically transmitted MMTV proviral genome in their germline. Several other colonies of feral M. musculus contain individuals which carry either one or two MMTV genomes in their germline or lack the viral genome. Thus, approximately 50% of the M. m. brevisrostris colony contains a single MMTV genome in their cellular DNA, the remainder lack the viral genome. The results of our study leads us to conclude that natural demes of mice are heterozygous for a limited number of genetically transmitted genomes and that mice which lack them arose as a result of random chromosomal segregation. We have also identified a feral M. m. domesticus breeding colony which contains only the long terminal repeat (LTR) portion of a MMTV proviral genome in its germline. Our studies suggest that the structural genes of the MMTV provirus were lost during an aberrant integration event or a reciprocal recombination event.

Significance to Biomedical Research and the Program of the Institute: The development of pedigreed breeding colonies from an evolutionary diverse group of species from the genus Mus provides a rich source of new genetic material as well as model systems for studying tumorigenesis. The breeding colonies of MMTV-negative mice will allow us to dissect the genetic and molecular interaction between genetically transmitted MMTV proviral genome and exogenous carcinogens.

Proposed Course: These services and materials are of essential importance to this research program. Mice from these colonies are currently being utilized in the following experiments: (1) evaluation of the effect of exogenous biological (hormones and MMTV) and chemical carcinogens (administered separately and in concert) on the etiology of mammary gland neoplasias in MMTV-negative mice; (2) introduction, by selective breeding, of single endogenous MMTV proviral genomes into the genetic background of the MMTV-negative mice. These mice will be used to determine the extent to which endogenous MMTV genomes contribute to spontaneous and carcinogen-induced mammary tumors; (3) evaluation of the extent to which the novel endogenous retroviral genes are involved in the development of mammary gland tumors; (4) determine whether genes corresponding to tumor-associated antigens identified in human mammary neoplasias have a genetic and biologic counterpart in murine mammary tumors; (5) study the genetic organization and evolution of endogenous retroviral genes in the genus Mus, as well as organizational rearrangements which are associated with tumor development.

The procurement for this colony was originally at the Frederick Cancer Research Facility. An administrative decision by DCCP resulted in the issuance of a subcontract by Litton Bionetics, Inc., Frederick Cancer Research Facility, Frederick, Maryland, to Litton Bionetics, Kensington, Maryland, for the maintenance and breeding of these feral mouse colonies. We have proposed a noncompetitive contract for the continuance of this program for three years. This period of time would insure that our breeding program would have an adequate time for success.

Date Contract Initiated: November 16, 1981

Current Annual Level: \$78,000

CONTRACT NARRATIVE
LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
DIVISION OF CANCER CAUSE AND PREVENTION
FISCAL YEAR 1982

MELOY LABORATORIES (N01-CP-01018)

Title: Support Services for the Experimental Oncology Section, Laboratory of Cellular and Molecular Biology

Contractor's Project Director: Mr. Fred Rogusky

Project Officer (NCI): Dr. Jeffrey Schlom

Objective: The purpose of this contract is to supply routine support services for the Experimental Oncology Section of the Laboratory of Cellular and Molecular Biology.

Methods Employed: The contractor provides proper facilities and technical support to carry out the following routine protocols designated by the NCI Project Officer and his staff: (a) standard and routine hybridoma techniques such as cell fusion, passage of cultures, and cell cloning; (b) preparation of mouse ascites fluids containing monoclonal antibodies; (c) concentration and purification of immunoglobulins from ascites fluids; (d) preparation of cell extracts; (e) routine solid phase RIAs for the detection of murine and primate monoclonal antibodies; (f) cutting of tissue sections from paraffin embedded blocks; (g) routine immunoperoxidase assays; (h) routine passage and maintenance of established cell lines in culture; (i) routine extraction of DNA and RNA from tissues; (j) routine molecular hybridization (Southern blotting) assays; (k) monitoring of lab safety for isotope use and disposal, and for controlled substances (chemicals); (l) glassware washing and sterilization.

Significance to Biomedical Research and the Program of the Institute: To continue support services for the Experimental Oncology Section, Laboratory of Cellular and Molecular Biology. The Experimental Oncology Section has made significant progress in areas that may be applicable in the diagnosis, prognosis and treatment of several human neoplasms.

Proposed Course: To continue the routine tasks and assays described in Methods Employed.

Date Contract Initiated: November 20, 1980

Current Annual Level: \$480,055

ANNUAL REPORT OF THE LABORATORY OF CHEMOPREVENTION

NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

The problem of the isolation and characterization of transforming polypeptide growth factors (TGFs) is occupying 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, which 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 already been developed in our laboratory during the past year.

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 and its ability to induce colony formation in soft agar is not enhanced by EGF. TGF β does not compete for binding to EGF receptors, but it requires the presence of a polypeptide that can bind to the EGF receptor for expression of its biological activity. Using TGF α and TGF β isolated from the acid-ethanol extract of murine-sarcoma virus-transformed mouse cells, it has been shown that either TGF α or EGF can function to potentiate the ability of TGF β to form large colonies in soft agar; neither TGF α or EGF by themselves have significant ability to induce formation of large colonies. Experiments using chemically modified analogues of EGF show that the ability of these analogues to potentiate the colony-forming activity of TGF β can also be correlated with their ability to compete with native EGF for receptor binding. These results can be generalized to all TGF β 's isolated thus far from both neoplastic and non-neoplastic tissues of murine, bovine, or human genomes. Similarly, TGF from human melanoma cells has the same properties described here for TGF α from virally transformed mouse cells.

TGF β represents the major TGF of tissues when assayed in the presence of EGF. It is currently being purified from virally transformed mouse cells grown in culture, from mouse kidney, and from bovine salivary gland and kidney. Use of slaughterhouse tissue (bovine) as starting material for the isolation of TGF has allowed the economical scale-up of procedures to the extraction of 1-50 Kg of tissue. The average yield of TGF β is approximately 10-100 μ g per Kg tissue extracted. Thus far the TGF β 's from the transformed mouse cells and from the bovine tissues have been purified over 1000-fold using a sequence of purification steps involving acid-ethanol extraction, ethanol-ether precipitation, chromatography on sizing gels, ion-exchange chromatography, and two steps using high pressure liquid chromatography. At this stage of purification, the various TGF β 's appear to be similar to each other and distinctly different from either TGF or EGF. They migrate as a single band on SDS-PAGE at molecular weight 13,000. Activity in the soft-agar colony forming assay is optimal at 1-2 ng/ml purified TGF β , assayed in the presence of EGF. Work has begun on the chemical characterization of these TGF β 's in terms of their amino acid composition and sequence.

Since TGF β has been found in all tissues examined thus far, including blood platelets, it can be assumed that these TGFs have a normal physiological role, perhaps in tissue repair. Experiments designed to test the effects of bovine TGF β on wound healing have shown that TGF β treatment results in increased accumulation of protein, DNA, and collagen in stainless steel wire mesh wound healing chambers implanted subcutaneously in the backs of rats. Control chambers were treated with equal quantities of bovine serum albumin. These experiments represent the first demonstration of *in vivo* activity of a TGF.

Human TGFs and possible controlling factors are being purified from normal human placenta and from two human tumor cell lines, HeLa and a rhabdomyosarcoma cell line (A673). The TGF from human placenta has been purified by chromatography of the residue obtained from an acid-ethanol extract of the tissue on a Bio-Gel P-30 gel filtration column followed by chromatography on a conventional cation-exchange column, a reverse-phase HPLC column and finally a cation-exchange HPLC column. This last column has separated the human TGF into 3 regions of transforming activity. One region appears to be homogeneous as determined by SDS-polyacrylamide gel electrophoresis. This peak of transforming activity is now undergoing further characterization using amino acid analysis and sequencing techniques. In addition, an inhibitor of cell proliferation has been detected in the acid-ethanol extract of normal human placenta and A673 cells. This factor appears to be non-protein in nature and has an apparent molecular weight of 2000 on gel permeation columns. The inhibitory factor is currently being purified for characterization by mass spectroscopy and nuclear magnetic resonance techniques.

TGF activity has also been purified from human blood platelets. Clinically outdated human platelets were subjected to acid-ethanol extraction and the soluble peptides were precipitated with ether. The extract induced NRK-fibroblasts to undergo anchorage-independent growth at a concentration of 100 μ g/ml. Addition of EGF (2 ng/ml) to the incubation mixture increased the potency of the extract 1000-fold; EGF alone had little biological activity. These findings demonstrate the presence of a TGF β in platelets. Gel filtration of the extract on Bio-Gel P-60 revealed a single peak of biological activity (molecular weight = 20,000). The elution position of this peptide was distinct from that of platelet-derived growth factor (molecular weight = 33,000). Further purification of platelet-derived TGF β by HPLC and a preparative gel electrophoresis is currently in progress.

The Laboratory of Chemoprevention continues to investigate the role of retinoids in prevention of cancer in experimental animal models. A large number of retinoic acid-amide derivatives have been found to be effective agents for prevention of bladder cancer in a mouse model that closely resembles the human disease. Studies on prevention of breast cancer in animal models has concentrated on the use of combination chemoprevention, or ancillary treatments, such as ovariectomy, in addition to treatment with retinoids. We are also interested in studying possible interactions between transforming growth factors and retinoids. Accordingly, we have established a cell line derived from a primary culture of bovine arterial smooth muscle cells; excessive proliferation of this cell type *in vivo* can result in atherosclerosis. Notably, the growth of these cells requires platelet-derived mitogens; they are quiescent when cultured with plasma-derived serum and mitotic when cultured with whole blood-derived serum. The differential effects of these sera should permit an analysis of the mechanisms whereby these cells respond to platelet-derived mitogens and, in particular, platelet-derived TGF β . In addition,

preliminary results indicate that retinoic acid at a concentration of 1 micromolar inhibits the stimulatory effect of whole blood serum on the growth of these cultured cells. Thus, this cell line can serve as a physiologically appropriate model to examine the roles of TGF β and retinoids in the genesis and prevention of atherosclerosis, respectively. These experiments also provide an excellent model for studying mechanistic interactions of retinoids and TGFs, as related to the ability of retinoids to inhibit the development of cancer.

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 chemopreventive and chemotherapeutic agents. Recent reports from laboratories other than ours 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 in our own laboratory 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 of a reality.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Isolation of Polypeptide Transforming Factors from Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Anita B. Roberts	Staff Scientist	LC	NCI
OTHER: Mario A. Anzano	Visiting Fellow	LC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

4.0

PROFESSIONAL:

2.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 murine and bovine kidney. These TGF betas of neoplastic and non-neoplastic derivation and different genomes will then be compared both with regard to biological activity and to chemical composition.

Project Description

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. Once the proteins are purified, it is our intention to investigate the mechanism of action of these TGFs in the carcinogenic transformation of cells as well as the mechanism of their inhibition by potential synthetic polypeptide antagonists or the retinoids, which are known to have anti-promoter activity.

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 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 and its ability to induce colony formation in soft agar is not enhanced by EGF. TGF β does not compete for binding to EGF receptors, but it requires the presence of a polypeptide that can bind to the EGF receptor for expression of its biological activity.

Using TGF α and TGF β isolated from murine-sarcoma virus-transformed mouse cells, it has been shown that either TGF α or EGF can function to potentiate the ability of TGF β to form large colonies in soft agar; neither TGF α or EGF by themselves have significant ability to induce formation of large colonies. Experiments using chemically modified analogues of EGF show that the ability of these analogues to potentiate the colony-forming activity of TGF β can also be correlated with their ability to compete with native EGF for receptor binding. These results can be generalized to all TGF β 's isolated thus far, from both neoplastic and non-neoplastic tissues of murine, bovine, or human genomes. Similarly, TGF α from human melanoma cells has the same properties described here for TGF α from virally-transformed mouse cells.

TGF β represents the major TGF of tissues, when assayed in the presence of EGF. It is currently being purified from virally transformed mouse cells

grown in culture, from mouse kidney, and from bovine salivary gland and kidney. Use of slaughterhouse tissue (bovine) as starting material for the isolation of TGF β has allowed the economical scale-up of procedures for the extraction of 1-50 kg of tissue. The average yield of TGF β is approximately 10-100 μ g per Kg tissue extracted. Thus far the TGF β 's from the transformed mouse cells and from the bovine tissues have been purified over 1000-fold using a sequence of purification steps involving acid-ethanol extraction, ethanol-ether precipitation, chromatography on sizing gels, ion-exchange chromatography, and two steps using high pressure liquid chromatography. At this stage of purification, the various TGF β 's appear to be similar to each other and distinctly different from either TGF α or EGF. They migrate as a single band on SDS-PAGE at molecular weight 13,000. Activity in the soft-agar colony forming assay is optimal at 1-2 ng/ml purified TGF β , assayed in the presence of EGF. Work has begun on the chemical characterization of these TGF β 's in terms of their amino acid composition and sequence.

Since TGF β has been found in all tissues examined thus far, including blood platelets, it can be assumed that these TGFs have a normal physiological role, perhaps in tissue repair. Experiments designed to test the effects of bovine TGF β on wound healing have shown that TGF β treatment results in increased accumulation of protein, DNA, and collagen in stainless steel wire mesh wound healing chambers implanted subcutaneously in the backs of rats. Control chambers were treated with equal quantities of bovine serum albumin. These experiments represent the first demonstration of in vivo activity of a TGF.

Significance to Biomedical Research and the Program of the Institute: The discovery in all tissues, both neoplastic and non-neoplastic, of polypeptides which can induce the expression of the transformed phenotype in normal cells 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.

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. (In press).

Roberts, A.B., Anzano, C.A., Frolik, C.A., and Sporn, M.B.: Transforming growth factors - characterization of two classes of factors from neoplastic and non-neoplastic tissues. Cold Spring Harbor Conferences on Cell Proliferation, Volume 9. Cold Spring Harbor Symposium. (In press).

Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., Frolik, C.A., Marquardt, H., Todaro, G.J., and Sporn, M.B.: Isolation from murine sarcoma cells of novel transforming growth factors potentiated by EGF. Nature 295: 417-419, 1982.

Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., and Sporn, M.B.: New class of transforming growth factors potentiated by epidermal growth factor: Isolation from non-neoplastic tissues. Proc. Natl. Acad. Sci. USA 78: 5339-5343, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Characterization of Transforming Growth Factors from Human Tissue and Cell Lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles A. Frolik Chemist LC NCI

OTHERS: Chester A. Meyers Expert LC NCI
S. Russ Lehrman 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:

3.7

PROFESSIONAL:

1.2

OTHER:

2.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Acid-stable polypeptides have been isolated 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 will concentrate on the purification and characterization of these polypeptides from normal human placenta as well as from HeLa and A673 (rhabdomyosarcoma) tumor cell lines 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 will be investigated in order to gain a greater understanding of the process of carcinogenesis.

Project Description

Objectives: The purpose of this study is to explore new methods that may be useful in the chemoprevention of cancer. Special emphasis will initially be 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 by gel filtration and ion-exchange chromatography followed by reverse-phase high pressure liquid chromatography (HPLC) and finally cation-exchange 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. After purification of the factors to homogeneity, they will be subjected to amino acid analysis and to sequence determination.

Major Findings: 1) ISOLATION AND PURIFICATION OF TGFs. TGFs have been detected in all human tissues and cell lines investigated to date. Several cell lines which display the highest activity have been selected for further purification of these TGFs. These include a cervical carcinoma cell line (HeLa) and a rhabdomyosarcoma (A673) cell line. 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 has been purified by chromatography of the residue obtained from an acid-ethanol extract of the tissue on a Bio-Gel P-30 gel filtration column followed by chromatography on a conventional cation-exchange column, a reverse-phase HPLC column and finally a cation-exchange HPLC column. This last column separates human TGF β into 3 regions of activity. One region appears to be homogeneous with a molecular weight of approximately 6000 as determined by SDS-polyacrylamide gel electrophoresis. This peak of transforming activity is now undergoing further characterization using amino acid analysis and sequencing techniques. In addition, other TGFs are being isolated from the HeLa and A673 cell lines using an analogous purification scheme.

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 is currently being purified for characterization by mass spectroscopy and nuclear magnetic resonance techniques.

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 may be involved in the control of the TGFs will be further investigated. It is also anticipated that initial investigation can begin into the mode of action of TGF in causing a reversible morphological transformation of normal cells.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Synthetic and Biological Studies on Epidermal and Transforming Growth Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Chester A. Meyers	Expert	LC	NCI
OTHERS:	Akira Komoriya	Staff Fellow	LC	NCI
	Nancy Acton Roth	Expert	LC	NCI
	S. Russ Lehrman	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:

3.5

PROFESSIONAL:

1.75

OTHER:

1.75

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A group of transforming growth factors (TGFs) 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 apparently peptides closely associated with epidermal growth factor (EGF). EGF enhances the activity of some TGFs, 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. Cyclic fragments of all 3 cyclic regions of EGF have been synthesized by solid phase methods, as well as complete overlapping linear and cyclic sequences. These have been tested in a variety of assays for receptor binding and biological activity. Chemically modified native EGF peptides have also been examined. A computer model of the tertiary structure of EGF has been generated and is consistent with the data obtained from the above peptides. The model will be used for designing new analogs. Efforts to prepare still larger synthetic fragments using fragment condensation techniques are planned.

Project Description

Objectives: The initial objective of the project is to determine the regions of the EGF molecule responsible for receptor binding and biological activity. These structure-function relationships, once determined by a combination of testing synthetic peptides and computer modeling techniques, will provide the basis for the rational design of synthetic peptide analogs which would be effective antagonists to the native peptide. These analogs would be used to investigate the manner in which TGFs cause cellular transformation; they may also have potential as therapeutic agents in the prevention or treatment of cancer and other proliferative diseases. As the TGFs themselves near purification to homogeneity, chemical characterization studies and synthetic work similar to that described for EGF can also begin.

Methods Employed and Major Findings: The project embodies a broad program of chemical synthesis, modification, sequencing, analysis, chromatographic separations and biological testing. As a part of preparative scale isolation of TGFs by A. Roberts, 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 14-31 and possibly, 20-31). This view is consistent with a computer model of EGF tertiary structure derived from completely different information. As the TGFs from several sources approach purification to near homogeneity, chemical characterization studies are now in progress, including quantitative amino acid composition analysis at submicrogram levels (10-25 picomoles) and amino acid sequence analysis.

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 analogs, have potential as clinical drugs in the treatment or prevention of such diseases.

Proposed Course: To continue as described above.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05210-02 LC

PERIOD COVERED October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Synthesis and Characterization of Some Pseudodipeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Nancy Acton Roth Expert LC NCI

OTHER: Akira Komoriya Sr. Staff Fellow LC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A pseudodipeptide is a dipeptide in which the amide linkage has been substituted with a thioether linkage. We have made several of these compounds in order to determine to what extent the molecular structure of these thioethers mimics that of a normal peptide. We are incorporating one of these derivatives into a tetrapeptide, and this tetrapeptide (AlaPhe-pseudo-PheAla) will be submitted for x-ray analysis. In addition, the same pseudodipeptide [S,S(-)-Phe-pseudo-Phe] has been incorporated into a pentapeptide which is related to the C-terminus of EGF (epidermal growth factor). This pentapeptide will then be incorporated into a larger EGF fragment either chemically or enzymatically, and the biological activity of the resulting material will be assayed.

Project Description

Objectives: Synthesis of polypeptides combining the pseudodipeptide (thioether) linkage for possible elimination of all protease substrate sites, thereby lengthening the biological stability and lifetime.

Methods Employed: The pseudodipeptides are being incorporated into other peptides via their active (p-nitrophenyl) ester derivatives.

Major Findings: A pentapeptide containing S,S(-)-Phe-pseudo-Phe, i.e. BOC-Phe-pseudo-Phe Glu (OB₂)Leu Arg(NO₂)OCH₃, has been prepared and characterized.

Significance to Biomedical Research and the Program of the Institute: Several polypeptides in the group of growth factors are thought to play a role in malignancy. By studying chemically altered growth factors, e.g. by replacing amino acid residues with pseudodipeptides, it may be possible to develop a material with growth factor inhibitory activity since the thioether linkage is expected to be more hydrophobic than the peptide amide linkage. Furthermore, substitution of a protease substrate residue by a corresponding pseudo-amino acid will eliminate, completely, any degradation of peptide by protease thereby increasing the overall potency of the peptide.

Proposed Course: Synthesis of a pseudo polypeptide suitable for x-ray analysis and synthesis of EGF fragment(s) containing the pseudo linkage followed by structural studies with NMR and biological assay of these analogs would complete this project.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05266-01 LC
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Synthesis and Biological Activity of EGF Fragments and Analogs		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="text-align: center;"> PI: Akira Komoroya Sr. Staff Fellow LC NCI OTHER: Nancy Acton Roth Expert LC NCI </div>		
COOPERATING UNITS (if any) Dr. Thomas Blundell, Birkbeck College, Univ. of London, U.K.; Dr. Alex Wlodawer, National Measurements Laboratory, National Bureau of Standards; Dr. Gary Gilliland, Laboratory of Biophysics, NIADDK, NIH		
LAB/BRANCH Laboratory of Chemoprevention		
SECTION		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.25	PROFESSIONAL: 1.00	OTHER: 0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) EGF (<u>epidermal growth factor</u>) has been chemically modified to give nitrated, acetylated, and methylated derivatives. It has also been cleaved, both chemically (CNBr) and enzymatically to give <u>EGF analogs</u> , all of which have been tested for EGF <u>receptor</u> competition and for <u>mitogenic</u> activity. We have synthesized a series of <u>overlapping</u> fragments of mouse EGF. Biological assays of these fragments suggest that the 14-31 region of EGF is most important for binding. <u>Enzymatic resynthesis</u> 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 degradation of native EGF. In collaboration with the units listed above, we are undertaking x-ray structural studies of EGF. Using <u>computer graphics</u> , a <u>modeled EGF tertiary structure</u> was derived.		

Project Description:

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 developing 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 14-31 or the 20-31 region of EGF is probably 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.

Significance to Biomedical Research and the Program of the Institute: Several growth factors have been implicated as being involved in cell transformation. An understanding of the regulation of these polypeptides may be important for the understanding of malignancy. Development of EGF inhibitors may lead to a procedure for chemical control of the hormone activity.

Proposed Course: The molecular structure of native EGF will be studied in detail by x-ray diffraction. The EGF fragments and analogs will be more completely characterized by enzyme mapping and their structures analyzed by circular dichroism. Intrinsic mitogenic activity of synthetic EGF fragments will be determined to identify, if possible, its biological signaling residues. New derivatives will be designed based on the modeled EGF tertiary structure (see project #Z01 CP 05209-02 LC).

Publications:

Chaiken, I.M., Komoriya, A., Ohno, M., and Widmer, F.: Use of enzymes in peptide synthesis. Applied Biochemistry and Biotechnology. 1982. (In press)

PERIOD COVERED October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Identification of Platelet-derived Transforming Growth Factor-beta

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: 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:

.75

PROFESSIONAL:

.75

OTHER:

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clinically outdated human platelets were extracted with acid-ethanol and precipitated with ether. The extract induced NRK-fibroblasts to undergo anchorage-independent growth. Addition of EGF to the incubation mixture increased the potency of the extract by 1000-fold; EGF alone had little biological activity. Gel filtration of the extract on Bio-Gel P-60 revealed a single peak of biological activity having an apparent molecular weight of 20,000. The elution position of this peptide was distinct from that of radioiodinated PDGF. These findings demonstrate the presence of TGF-beta in platelets.

Project Description

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 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: TGF has been detected in human platelets and serum; the beta form (EGF-activated) predominates. The biological activity elutes as one major component during gel filtration on Bio-Gel P-60 ($M_r = 20,000$), high pressure liquid chromatography, and pH 4.5 polyacrylamide gel electrophoresis.

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: Platelet-derived TGF-beta will be purified further and its effect on gene expression and protein synthesis will be examined in cultures of fibroblasts and smooth muscle cells.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Chemical Studies of Transforming Growth Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. Russ Lehrman Guest Worker LC NCI

OTHER: 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:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Polypeptides capable of transforming normal cells in vitro have been isolated from neoplastic and non-neoplastic 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 nonroutine 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 salivary gland, transformed murine MSV cells and human placenta. Sequence analysis of TGF isolated from human placenta has produced tentative assignments for 24 of 30 residues. These results are consistent with the amino acid analysis performed on this compound. The immediate goal is to determine the primary sequence of several intracellular TGFs. This information will be used in the synthesis of TGF antagonists and by others in the laboratory studying the molecular biology of the TGF gene.

Project Description

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 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 N-terminal sequence of human placental TGF is presently being studied using a Beckman 890C automated sequencer. The PTH-amino acids formed from aqueous acid treatment of thiazolinone amino acids are identified using HPLC on Zorbax ODS and CN columns and mass spectral analysis. Further structural information requires denaturation, chemical derivatization and enzymatic digestion of this transforming growth factor. The first steps are accomplished by treatment of native TGF with iodoacetic acid in 6 M urea. Since the initial amino acid analysis indicates the presence of one arginine residue, it may be sufficient to treat the altered protein with clostripain or submaxillaris protease, two enzymes known to cleave proteins on the C-terminal end of arginine. The peptide fragments generated could then be separated by standard HPLC methodology and sequenced as above.

Major Findings: To date, initial amino acid analyses have been obtained on human placental, murine, bovine kidney, and salivary gland TGF. Sequence analysis of the N-terminal region of human placenta TGF (MW 7000) has produced tentative identification for 24 out of 30 residues. The later study is, at this point, consistent with amino acid analysis obtained with submicrogram amounts of material.

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 determine 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-TGF receptor interaction and is useful for the design of TGF antagonists.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05269-01 LC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Transforming Growth Factor mRNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mark E. Smith Sr. Staff Fellow LC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Production of mRNA's coding for transforming growth factors by selected cell lines was investigated. VSV-transformed mouse fibroblast line 3B11-1C, human rhabdomyosarcoma A673 and human embryonic lung HEL299 have been used as source for whole cell RNA isolation. Conditions for extraction of translatable mRNA from these cell lines have been optimized for maximum translation of radiolabeled protein in rabbit reticulocyte lysate protein synthesis systems. Two approaches for monitoring TGF mRNA translation have been initiated. The production of TGFs in the in vitro protein synthesis will be of such low levels that the soft agar colony formation assay currently in use will not be sufficiently sensitive. A micro semi-quantitative assay has been developed which is capable of detecting less than 25 picograms of partially purified beta TGF. This detection level is well within the range of potential in vitro mRNS-directed TGF synthesis. Also, antibodies to beta TGF have been raised in rats which will be used to follow beta TGF synthesis by specific immunoprecipitation of the radiolabeled proteins. Either or both of these methods should allow sufficient detection and quantitation of beta TGF mRNS for further characterization and purification.

Project Description

Objectives: The primary objectives of this project are 1) isolation of RNA coding for human and mouse β -transforming growth factors (β TGF); 2) preparation of a c-DNA library and selection of clones containing β TGF genes; 3) characterization and sequencing of these β TGF genes.

Methods Employed and Major Findings: Initially, messenger RNA production is being studied using standard isolation and translation procedures. A micro semiquantitative soft agar assay was developed for use in screening and quantitating the efficiency of TGF messenger RNA translation. Immuno-precipitation of radiolabeled translation products also has been used as a means of quantitating messenger RNA-directed β TGF synthesis. Messenger RNA from selected cell lines has been isolated, purified and used to direct protein translation in reticulocyte lysate and wheat germ translation system. Polyclonal antibodies to human TGF have been produced in rats.

Significance to Biomedical Research and the Program of the Institute: The isolation and characterization of transforming growth factor genes is of primary importance in developing probes to investigate the role of TGF in normal growth and wound repair and in neoplastic transformation.

Proposed Course: When the messenger RNA for β TGF is isolated and partially purified, a cDNA library will be made and screened by either hybridization selected translation, or a synthetic oligo nucleotide probe made from amino acid sequence of β TGF. The positive clones from these screens will be isolated and the DNA sequenced. This will allow location and primary structure of β TGF genes to be determined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05285-01 LC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Interaction of Retinoids and Peptide Growth Factors in Smooth Muscle Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael B. Sporn Chief LC NCI
OTHER: Richard K. Assoian Guest Researcher LC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to define a cell culture system in which the antiproliferative effects of retinoids can be studied from a mechanistic point of view. This will involve study of the potential antagonism between retinoids and the peptide growth factors of serum using vascular smooth muscle cell cultures as the test system. Particular attention will be paid to transforming growth factors which have been isolated recently from blood platelets.

Project Description

Objectives: The ultimate goal of this project is to study the ability of retinoids to antagonize the proliferative effects of several peptide growth factors in a defined cell culture system. These studies should further our understanding of the mechanism of action of retinoids in suppressing transformation of cells, a problem which has significant application to chemoprevention of cancer.

Methods Employed: Culture of bovine arterial smooth muscle cells in monolayer culture is a well-defined cell culture system. Cells remain quiescent when grown in the presence of plasma and then undergo a striking mitogenic response when peptide growth factors derived from blood platelets are applied. Platelet factors may be provided by whole serum or by purified extracts from platelets. The latter include the well-known platelet-derived growth factor, as well as a newly discovered transforming growth factor (TGF) obtained by acid-ethanol extraction and gel-filtration of platelets.

Major Findings: This is a new project and efforts thus far have been directed largely at defining new test systems. However, it is already clear that all-trans-retinoic acid exerts a potent antiproliferative effect on the mitogenic response of smooth muscle cells elicited by exposure of these cells to serum. Other retinoic acid analogs are currently being tested, and purified platelet growth factors are currently being studied as the mitogenic agents.

Significance to Biomedical Research and the Program of the Institute: Understanding of the antiproliferative effects of retinoids is of obvious importance for further development of these agents for cancer chemoprevention.

Proposed Course: This project should offer further opportunity to study mechanistic aspects of the control of cell growth and transformation by retinoids. The experimental work that will be done in the next year will concentrate on the use of defined peptides as mitogens and measurement of biochemical effects of retinoids on peptide action.

CONTRACT NARRATIVE
LABORATORY OF CHEMOPREVENTION
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1982

IIT RESEARCH INSTITUTE (N01-CP-15742)

Title: Resource for Long-Term Animal Experiments to Study Prevention of Cancer by Retinoids and Related Materials.

Contractor's Project Director: Dr. Richard C. Moon

Project Officer (NCI): Dr. Michael B. Sporn

Objectives: The major objective of this contract is to provide the Laboratory of Chemoprevention, NCI, with an animal resource for evaluation of the chemopreventive activity of retinoids, natural and synthetic derivatives of vitamin A, and other related chemopreventive compounds in experimental models for cellular differentiation and tumorigenesis of the breast, lung, sebaceous glands and vascular system.

Methods Employed: Standard animal carcinogenesis bioassays have been used in most experiments.

Major Findings: Mammary Cancer. A single intravenous administration of N-methyl-N-nitrosourea (MNU) has been shown to induce mammary cancers in female Sprague-Dawley rats in a dose-related manner with no acute toxicity. MNU-induced mammary cancers invade locally and metastasize to distant sites. A synergistic interaction was found between N-(4-hydroxyphenyl)retinamide (HPR) and bilateral ovariectomy in the inhibition of mammary carcinogenesis induced by MNU or 7,12-dimethylbenz(a)anthracene (DMBA). By contrast, although both agents inhibited MNU-induced mammary carcinogenesis, no synergism in cancer inhibition was noted between HPR and MVE-2, an inducer of interferon synthesis. In addition, a study is in progress to determine the anticarcinogenic activity of 6-methyl-dimethylstilbenic acid in the MNU mammary cancer model.

Urinary Bladder Cancer. Dose-response parameters have been defined for the induction of transitional cell carcinomas in male BDF mice by intragastric administration of the carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (OH-BBN). The retinoids N-(2-hydroxyethyl)retinamide, 13-cis-(2-hydroxyethyl)-retinamide, and N-(5-tetrazolyl)retinamide were found to have approximately equal activity in bladder cancer prevention, while 13-cis-(5-tetrazolyl)-retinamide was inactive. Animal studies completed in the present contract period found that N-butyl-retinamide, 13-cis-butyl-retinamide, N-(2-hydroxybutyl) retinamide, and 13-cis-(2-hydroxybutyl)retinamide had no anticarcinogenic activity in the OH-BBN bladder cancer model, while HPR was an effective inhibitor of urinary bladder carcinogenesis. A study to test for interactions between HPR and MVE-2 in the inhibition of urinary bladder carcinogenesis noted that, while HPR and MVE-2 both were effective inhibitors when administered alone, no additive or synergistic anticancer activity was achieved with combined HPR and MVE-2 administration.

Vascular Proliferative Lesions. The temporal relationship between cholesterol accumulation, cellular proliferation, and gross lesion appearance in the aorta and brachiocephalic arteries of male S.E.A. Japanese quail has been determined.

Pilot studies have been conducted to determine the tolerance of Japanese quail to retinoid-supplemented diets, and dose-response and time-response parameters of cholesterol-induced lesion development have been defined. A pilot study has indicated that 13-cis-retinoic acid may possess arterial antiproliferative activity in quail fed an atherogenic diet. Studies currently are in progress to determine the influence of 13-cis-retinoic acid and HPR on cell proliferation, cholesterol deposition, and atherosclerotic lesion development in quail fed an atherogenic diet supplemented with 0.25% cholesterol and 0.125% cholic acid. In addition, studies to develop an organ culture model for proliferative lesions in the quail aorta and brachiocephalic arteries are in progress.

Sebaceous Gland Differentiation. Studies to determine the effect of 13-cis-retinoic acid, N-(4-hydroxyphenyl)retinamide and the trimethylmethoxyphenyl analog of retinoic acid ethyl ester (RO 10-9359) on cellular differentiation of a modified sebaceous gland of the hamster (flank organ) have been completed. Administration of 13-cis-retinoic acid resulted in a significant reduction in gland size and altered patterns of differentiation in treated animals. RO 10-9359 was less active than 13-cis retinoic acid, while HPR had no effect on gland size or differentiation. Animal studies to determine the effect of several stilbenoids on the flank organ have been completed and histological processing of tissues is in progress.

Significance to Biomedical Research and the Program of the Institute: Studies performed under this contract have led to the development of new models for the safe but rapid induction of highly proliferative lesions and cancers in animals. These models have not only provided suitable systems for the evaluation of chemopreventive activity of retinoids but also provided techniques by which other retinoids and chemopreventive compounds can be evaluated at the cellular level. The data obtained from the evaluation of retinoids in various epithelial cancer models will hopefully lead to the clinical application of these compounds for prevention of cancer in groups at high risk.

Proposed Course: Newly synthesized retinoids and other chemopreventive agents will be evaluated for prevention of respiratory, breast, urinary bladder, and esophageal cancer as well as determining the antiproliferative effect of these compounds in the above animal models.

Date Contract Initiated: January 18, 1981

Current Annual Level: \$704,000

Man Years: 11

ANNUAL REPORT OF THE LABORATORY OF COMPARATIVE CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

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.

Establishment. 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. The Laboratory was established in July, 1981, in the course of reorganization of the Carcinogenesis Intramural Program, by reassignment of existing sections from several laboratories. The Perinatal Carcinogenesis Section (PCS), previously part of the Laboratory of Experimental Pathology, and the Nutrition and Metabolism Section (NMS), previously part of the Laboratory of Carcinogen Metabolism, moved from Bethesda to Frederick in May, 1981, and began renovation of laboratory space before the formal creation of the Laboratory. The Ultrastructural Studies Section (USS), previously part of the Laboratory of Viral Carcinogenesis, transferred to the new Laboratory in July, 1981, at which time the Tumor Pathology and Pathogenesis Section (TPPS) was created around a nucleus of veterinary pathologists from the Tumor Pathology Branch of the former NCI Carcinogenesis Testing Program, now part of the National Toxicology Program.

During the course of this fiscal year, laboratory renovations have been completed and a practical means of carrying out long-term carcinogenesis studies in rodents has been established at the Frederick Cancer Research Facility. The research programs of the PCS and NMS have been successfully relocated and are continuing along previously established lines. The research program of the USS has made progress in transferring its focus from viral to chemical carcinogenesis, and the new research program of the TPPS has been effectively initiated.

Summary Report: 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. 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 program has been established in this laboratory to identify previously unsuspected promoting agents; to rigorously establish the limits of cellular specificity for tumor promotion by specific agents, and to compare dose/effect relationships from one species to another, including both rodent and nonhuman primate species. The TPPS has undertaken to investigate whether substances such as phthalates, which have a capacity to induce tumors in rodents that is remarkably limited to a specific cell type, are in fact promoters. This has led to a careful and comprehensive program to define, both biologically and histologically, the naturally occurring proliferative lesions of experimental animals commonly used in carcinogenesis research since certain of these lesions, the neoplastic nature of which is uncertain, are among those "promoted" by chemical agents. The PCS and the Office of the Chief have undertaken studies on the barbiturates which appear in rodents to selectively promote neoplasia in certain epithelia (including lining epithelia, from which most human cancers originate) depending on the pattern of molecular substitution at the 5 position of the pyrimidine ring, while the NMS, in collaboration with the USS, has investigated nutritional deficiencies which promote development of neoplasia. Efforts are in progress to develop mechanistic hypothesis from the data obtained in vivo.

In addition to this major coordinated program, studies continue on age-dependence of susceptibility to chemical carcinogens and on carcinogenesis by salts of metals such as Ni, Cd, and Pb. Details are given in the following summary reports of each research group.

Conferences: Laboratory staff have been instrumental in organizing two major research conferences and in editing the proceedings for publication in book form.

During March 2-4, 1981, a joint NCI-EPA Symposium on Organ and Species Specificity in Chemical Carcinogenesis was held in Raleigh, North Carolina. The proceedings of the Symposium, which comprised 40 invited papers, are now in press and will appear as a photo-offset volume edited by R. Langenbach, S. Nesnow, and J. M. Rice, and published by Plenum Press.

On December 8, 1981, a "Symposium on the Use of Small Fish Species in Carcinogenicity Testing" was convened in Bethesda at the Lister Hill Center. The organizing committee was chaired by Dr. Karen Hoover of the Laboratory of Comparative Carcinogenesis. This three-day workshop, supported by DCCP, examined the potential of more than 10 fish species as bioassay and/or environmental

indicator animals. Biological and biochemical responsiveness of fish species to carcinogens such as aflatoxin, nitrosamines, and polycyclic aromatic hydrocarbons, and the feasibility of increasing laboratory utilization of fish were also discussed. Thirty-eight papers were presented by thirty-two participants in five sessions including 1) proposed species for carcinogenicity testing *in vivo*; 2) alternative testing systems such as embryo exposure and *in vitro* techniques; 3) laboratory maintenance of fish; 4) special problems related to fish (such as different organ systems and histology); and 5) the metabolic handling of xenobiotics by fish. The papers are to be published by the Journal of the National Cancer Institute as Monograph 64 or 65 in its Monograph series.

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.

Programs involving nonhuman primates have been consolidated in the Office of the Chief. These include the program on transplacental carcinogenesis in patas monkeys, previously part of the program of the Perinatal Carcinogenesis Section, and a new project on tumor promotion in cynomolgus monkeys. The Laboratory now has adequate veterinary medical expertise in-house to carry out these projects independent of any collaborating unit.

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.

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 until the carcinogen has ceased; and that phenobarbital may be a promoter of hepatocellular neoplasia in this species as it is in rats and mice.

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 B₁₂ 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. Further, chronic administration of the liver tumor promoters, phenobarbital and DDT, decreased the hepatic levels of S-adenosylmethionine in rats. Such decreases could be completely prevented by the simultaneous feeding of methionine and could be partially inhibited by the dietary administration of choline. These results are consistent with the hypothesis that in the rat liver, at least, methyl insufficiency is a promoter of liver carcinogenesis. Further evidence for a contributing role of methyl deprivation in hepatocarcinogenesis was provided by recent results with the methionine antagonist, ethionine. Ethionine is now exhibiting carcinogenic activity in C3H and Swiss female mice. In addition, both ethionine and S-adenosylethionine were shown to transform rat liver epithelial cells maintained in methionine-deficient, homocysteine-supplemented medium. Following implantation of the treated hepatocytes into syngeneic hosts, tumors arose faster in the animals receiving the S-adenosylethionine-treated cells than in those receiving the ethionine-treated cells; this observation provides evidence that ethionine exerts its carcinogenic activity through its metabolism to S-adenosylethionine.

The production of lung adenomas in strain A mice by nickel acetate and lead subacetate was inhibited by the simultaneous administration of calcium and magnesium acetates. Similarly, the simultaneous injection of magnesium completely prevented the formation of injection site sarcomas and delayed the onset of testicular tumors in rats treated with cadmium.

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.

The Perinatal Carcinogenesis Section's research program has been reorganized in the course of transfer of the Section from the Laboratory of Experimental Pathology to the Laboratory of Comparative Carcinogenesis. Tumor pathology is now performed in collaboration with the Tumor Pathology and Pathogenesis Section, and transplacental carcinogenesis studies in nonhuman primates have been consolidated with related projects in the Office of the Chief. The current program of the Section is focused on mechanisms of organ specificity and species differences in transplacental carcinogenesis and has three components: (1) in vivo studies with special emphasis on transplacental carcinogenesis

followed by postnatal exposure to tumor promoters; (2) in vitro studies; and (3) biochemical processes that determine organ specificity.

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 have been initiated. 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.

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 biology and pathology of naturally occurring and experimentally induced preneoplastic and neoplastic lesions of rodents, monkeys and humans are characterized and compared. Through the work of the section, criteria for differentiation of natural and induced tumors are being identified. Characteristics of tumors induced by specific chemicals occasionally allow us to identify the chemical cause of a specific tumor by the morphology and biology of the tumors that develop in exposed animals. Other markers such as tumor antigens, enzymes and transplantability are identified and characterized. Tumor promoters would,

perhaps, increase the incidence of certain naturally occurring (spontaneous) tumors when given alone, or may increase the incidence of types of tumors induced by a carcinogen given as an initiator. The naturally occurring tumors of the F344 rat are being systematically characterized by transplantation. Latency period after transplantation was related to tumor malignancy and tumor type. Histologically and clinically benign tumors were readily transplanted, a phenomenon not previously appreciated.

The major cause of death of aging F344 rats was identified as a large granular lymphocyte (LGL) leukemia with morphologic, immunologic and functional similarities to the normal LGL. Transplantable LGL tumors with high natural killer cell activity were characterized. These transplantable tumors are being developed to serve as a model for the study of NK activity and LGL lineage. Most studies on these LGL tumors was done in collaboration with Dr. C. Reynolds of the Biological Response Modifiers Program, Division of Cancer Treatment.

The comparative pathology of initiation and promotion as potential mechanisms in the genesis of cancer are being studied in rats, mice and monkeys. The role of initiation and promotion in the induction of tumors by weak genotoxic and nongenotoxic carcinogens are being studied utilizing formaldehyde, methapyrilene and a phthalate plasticizer as models.

Preliminary evidence indicates that formaldehyde is a so-called "weak" initiator, poor promoter and "weak" carcinogen for mouse skin, that is "weak" or "poor" in comparison to DMBA and TPA. Gamma-glutamyl transpeptidase (GGT) has been used as a marker in rat liver for preneoplasia and neoplasia. In our studies of naturally occurring potential preneoplastic basophilic hepatic foci and tumors in F344 rats, we found that these foci and tumors did not contain the enzyme. In old rats exposed to phenobarbital, enzyme-positive foci appeared and sometimes progressed to tumors. In patas and rhesus monkeys, the enzyme is present on normal hepatocyte cell membranes in monkeys and not on bile duct epithelium while in adult rats it is present only on bile duct epithelium. Monkey liver cell tumors had the enzyme pattern of normal liver. Thus, GGT may be a marker for preneoplasia and neoplasia only in some species and only under certain conditions. The results in old F344 rats suggests that natural and induced tumors may be differentiated by the presence of GGT in induced tumors.

The TPPS has utilized the ACI rat as an animal model of naturally occurring prostatic cancer to study factors which prevent the disease and prolong life. A study with 3 retinoids is in progress. One of the retinoids appears to have prolonged the life span of the rats. The histology of this phenomenon is being reviewed.

The TPPS provides guidance in pathology to other scientists at NCI and U.S. federal agencies. Aid has been provided to Drs. Sporn (DCCP), Tarone (DCCP), Rapp (DCCP), Reynolds (DCT), Lijinsky (FCRF) and others at NCI and FCRF. Requested consultations in pathology and data evaluation with members of FDA, OSHA, NIOSH, and EPA occur on a regular basis.

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.

Establishment, in Building 538 (NCI/FCRF), of new laboratories for the section was achieved by the end of 1981. The section now occupies one laboratory for electron microscopic preparative work and two rooms equipped with a transmission and a scanning microscope, respectively. Ancillary instruments for TEM and SEM have been installed and a dark room has been constructed. The section is now fully operative. The duties of the section are of dual nature and include establishment of a research program in the framework of the Laboratory and, on a collaborative basis, provision of support to members of the Laboratory and other institutions with regard to various ultrastructural 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 major themes: 1) characterization of chemically transformed liver cell lines by morphological means and 2) study of morphological and functional changes in the mouse thymus during Moloney virus-induced lymphoma development.

Ethionine, the ethyl analog of methionine, is a known liver carcinogen in the rat. 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. Short-term ultrastructural studies on the action of ethionine have shown well-defined morphological changes in the nucleus and especially in the nucleolus that parallel changes in the cell metabolism due to ATP deficiency and/or incomplete rRNA methylation.

In the Nutrition and Metabolism Section of the Laboratory several liver cell lines, 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 a program to characterize the chemically transformed cell lines by the following aspects:

- 1) The role of the cytoskeleton in transformation.
- 2) Characterization of cell-to-cell and cell-to-substrate interactions during carcinogenesis.
- 3) Elucidation of the cell surface architecture and its alterations accompanying carcinogenesis.

The methods used are transmission and scanning electron microscopy in combination with immunomicroscopy of nontransformed and ethionine-transformed cells grown in vitro.

In preliminary experiments, an SEM study revealed an altered surface architecture in transformed cells which affects both the distribution of microvilli and the expression of the primary cilium. These changes from the normal may reflect deviations in membrane fluidity in cytoskeletal architecture, as well as alterations in certain phases of the cell cycle. TEM revealed the following alterations in the nuclei of transformed cells: formation of membranous inclusions, decrease of heterochromatin, accumulation of perichromatin granules, and hypertrophy of the nucleolus with enlargement of the pars granulosa. In the cytoplasm, alterations in the mitochondrial architecture and changes in the size of cell junctions are outstanding.

The Section Head has been invited to join a research project at the University of Cologne, Germany. She has spent part of a sabbatical as guest of Prof. Krueger in the Institute of Pathology of that University. The research project encompasses studies on morphological and functional changes in the mouse thymus during Moloney virus-induced lymphoma development. In short, in previous experiments carried out in Cologne it has been shown that during the early phase of M-MuLV-induced lymphomagenesis in mice prethymic stem cells of the T-lineage derived from blood-forming tissue accumulate in the thymus where they encounter a differentiation block. Subsequently, an uncontrolled progressive proliferation of prethymic stem cells will lead to generalized malignant lymphoma of this cell type. In the present study, a number of parameters will be correlated to further elucidate the mechanism of the differentiation block in the thymus. This collaborative effort concentrates on the characterization of ultrastructural changes in thymic tissue and isolated thymic cells with special emphasis on thymic epithelial cell and microfilament/microtubule distribution.

In collaborative studies the Section is supporting investigations on chemical carcinogenesis in primates. Transmission electron microscopy is being used to provide ultrastructural characterization of gestational choriocarcinoma and of mesenchymomas, both characteristic neoplasms in patas monkeys given ethylnitrosomea, respectively, during pregnancy or transplacentally. Transmission electron microscopy is also being used to compare the responses of rodent target tissues to tumor promoters with corresponding tissues of nonhuman primates exposed to the same agents.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Role of Lipotropes in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lionel A. Poirier	Chief, Nutrition & Metabolism Section	LCC NCI
OTHER:	Mary J. Wilson	Chemist	LCC NCI
	Narayan Shivapurkar	Visiting Fellow	LCC NCI
	Karen Hoover	Staff Fellow	LCC NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Nutrition and Metabolism Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

5.5

PROFESSIONAL:

3.2

OTHER:

2.3

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 levels of S-adenosylmethionine in the livers of animals treated with hepatocarcinogens and liver tumor promoters are being determined. The effects of methylase inhibitors on the methylation of macromolecules, on cell transformation in vitro and on carcinogenesis in vivo are determined.

Projection Description

Objectives: The dietary lipotropes methionine, choline, and vitamin B₁₂ significantly modify the production of tumors by certain chemical carcinogens. The extent of and the mechanisms behind these effects are being investigated. In addition, the mutual metabolic interactions of the lipotropes and the chemical carcinogens are to be determined.

Methods Employed: The carcinogenic activities of standard carcinogens are compared in rodents fed chemically defined and chow diets. S-adenosylmethionine, -ethionine, and -homocysteine are determined using appropriate combinations of HPLC, thin layer chromatography, spectrophotometry and radioisotopes. Fibroblasts are transformed in vitro by chemical carcinogens, and their growth constants in nutritionally deprived medium are determined.

Major Findings: The chronic administration of amino acid-defined methyl-deficient diets to diethylnitrosamine-initiated rats led to the markedly increased formation of hepatocellular carcinomas. Such effects were most marked in animals receiving a diet devoid of both methionine and choline but could also be noted in rats fed diets with a single deficiency of either methionine or choline. The hepatic levels of ornithine decarboxylase, a marker of tumor promotion, was found to be inversely proportional to the liver levels of S-adenosylmethionine in rats fed methionine- and choline-deficient diets or ethionine-supplemented diets. The chronic administration of the liver tumor promoters phenobarbital and DDT led to decreased hepatic contents of S-adenosylmethionine; these decreases were reversed by the simultaneous administration of methionine and choline. The livers of methionine-deficient rats, like those of diethylnitrosamine-treated rats, contained elevated levels of methylcobalamin which are required for methionine biosynthesis. Such results provide evidence that liver tumor promotion may occur via a methyl insufficiency. Chronic ethionine feeding to mice produced hepatocellular carcinomas. As in the rat, the chronic administration of ethionine to mice led to decreased hepatic levels of S-adenosylmethionine and to very high levels of S-adenosylethionine.

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 carcinogens induce tumors. The aim of these studies is to determine whether methyl insufficiency is a promoter of liver tumor formation.

Proposed Course: Carcinogenesis by standard carcinogens in animals fed chemically defined diets will be determined. The carcinogenicity of ethionine in different species will be completed. The effects of methionine deficiency on tumor promotion will be determined. The inhibition by methionine of liver tumor promotion will be attempted and methyl insufficiency as a promoter of carcinogenesis will be tested in other systems.

Publications:

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2. Mikol, Y.B. and Poirier, L.A.: An inverse correlation between hepatic ornithine decarboxylase and S-adenosylmethionine. Cancer Letters 13: 195-201, 1981.
3. Nawata, H., Yamamoto, R.S. and Poirier, L.A.: Elevated levels of ornithine decarboxylase and polyamines in the kidneys of estradiol-treated male hamsters. Carcinogenesis 2: 1207-1211, 1981.
4. Nawata, H., Yamamoto, R.S. and Poirier, L.A.: An inverse correlation between uterine and ovarian levels of ornithine decarboxylase and S-adenosylmethionine decarboxylase in the rat. Proc. Soc. Exp. Biol. Med. 167: 563-566, 1981.
5. Nawata, H., Yamamoto, R.S. and Poirier, L.A.: Tissue distribution of ornithine decarboxylase and S-adenosylmethionine decarboxylase in male and female rats. Biomedical Research 2: 659-663, 1981.
6. 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 Borchardt, R.T., Creveling, C.R. and Usdin, E.D. (Eds.): The Chemistry and Pharmacology of S-adenosylmethionine. London, MacMillan Co., LTD., 1982. (In press)
7. Raj, H.G. and Poirier, L.A.: Stimulation of choline and methionine incorporation into liver phospholipids of rats following chronic treatment with diethylnitrosamine. ICRS Med. Sci. 9: 899-900, 1981.
8. Shivapurkar, N. and Poirier, L.A.: Decreased levels of S-adenosylmethionine in the livers of rats fed phenobarbital and DDT. Carcinogenesis. 1982 (In press)
9. Wilson, M.J., Hatfield, D.L. and Poirier, L.A.: Aminoacylation of ethionine to rat liver tRNA^{Met} and its incorporation into protein. FEBS Letters 128: 157-160, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04582-07 LCC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure Activity Correlations in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Lionel A. Poirier Chief, Nutrition & Metabolism Section LCC NCI
OTHER: Kazimierz Kasprzak Visiting Scientist LCC NCI
Karen Hoover Staff Fellow LCC NCI

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LAB/BRANCH

Laboratory of Comparative Carcinogenesis

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TOTAL MANYEARS:

0.8

PROFESSIONAL:

0.8

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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. Magnesium and calcium completely prevented lung adenoma production in strain A mice by nickel and lead acetates. Magnesium, but not calcium, prevented the formation of injection site sarcomas and delayed the development of testicular carcinomas in rats receiving cadmium.

Project Description

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 tumorigenic activities of nickel acetate and lead subacetate towards the lungs of strain A mice were completely prevented by the simultaneous administration of calcium acetate and of magnesium acetate. Magnesium injection completely prevented the formation of injection site sarcomas and delayed the time of onset of testicular carcinomas in rats receiving a single injection of cadmium.

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 metal carcinogens can be diminished. The evidence accumulated to date indicates that an antagonism to the divalent cations, calcium or magnesium, may be part of the mechanism by which the divalent metal carcinogens exert their activity.

Proposed Course: The carcinogenicity of inorganic carcinogens will continue to be examined. These include oxidized arsenic and chromium compounds. Attempts will be made to extend the observed antagonism between carcinogenic divalent metals and calcium and magnesium.

Publications:

Hoover, K.L.: Carcinogenicity of selenium sulfide in Fischer 344 rats and B6C3F1 mice. J. Environ. Pathol. Toxicol. Oncol. 1982 (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04680-12 LCC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development and Application of In Vitro Systems Involving Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mary J. Wilson Chemist

LCC NCI

COOPERATING UNITS (if any)

Dr. Ursula I. Heine, Ultrastructural Studies Section, LCC, NCI

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Nutrition and Metabolism Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Epithelial cells derived from livers of 10-day old Fischer 344 rats are utilized in transformation and metabolism studies. The importance of nutrients in the culture medium to the transformation process are being assessed following exposure of the cells to chemical carcinogens.

Project Description

Objectives: Successful culture of epithelial-like 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 chemical carcinogens were discussed in previous reports. Since neoplasia in man and animals involves mainly epithelial cells, our principal aim in current investigations has been to determine further the potential of in vitro epithelial systems for use in rapid bioassay procedures for chemical carcinogens and in examining the biochemical mechanisms of carcinogenesis.

Methods Employed: Methods for culturing and cloning rat liver cells for culture continue to be developed in this laboratory. In addition, the reliability of short-term markers of cell transformation is also being examined.

Major Findings: Irreversible morphological alterations were produced in cultures of liver cells treated for three months with 5.0 mM or 7.5 mM DL-ethionine. Growth in soft agar was observed in both treatment groups after 5 additional months in culture. Only those cells treated with 7.5 mM DL-ethionine produced tumors when injected subcutaneously into syngeneic hosts.

Cells treated with L-S-adenosylethionine (SAE) (0.2 mM and 0.065 mM) and L-ethionine (0.2 mM and 0.375 mM), in medium in which methionine was replaced by homocysteine, were able to grow in soft agar after 3 months of treatment and an additional 5-7 months in culture. When syngeneic hosts were injected with cells treated with 0.2 mM SAE and 0.375 mM ethionine for 3 months and cultured an additional 2 months tumors were produced. Three months of treatment with 0.2 mM ethionine and 0.065 mM SAE plus an additional 5 months in culture were required for tumor production. Thus, at equimolar levels SAE resulted in cell transformation, as indicated by tumor production, at a faster rate than ethionine.

Two problems encountered in past investigations were the relatively long time required to develop cultures which were proliferating adequately to provide enough cells for biochemical investigations and the fact that some sublines of control cells underwent spontaneous malignant transformation as demonstrated by tumor formation following injection of cells into syngeneic hosts. Unfortunately, no distinctive morphological changes were apparent in the cultured cells which induced tumors. In addition, the spontaneous transformation was not correlated with a specific time in culture nor with the number of times a line had undergone subculturing. Therefore, we have emphasized the development of methods for producing a greater number of cultures with similar characteristics so that studies can be repeated as required in more replicable systems. Investigation of pertinent biochemical properties of untreated or chemically exposed cultured cells in regard to the effects of various cultural conditions and in comparison to the characteristics of liver tissue from whole animals should help assess the value of this in vitro system.

Significance to Biomedical Research and the Program of the Institute: An assay system which can be designed to give results more rapidly, specifically, and reliably than the customary long-term tests for carcinogenicity is highly desirable with the dual purpose of studying fundamental phenomena and the detection

of harmful agents in our environment. Full exploitation of workable in vitro systems has not been achieved. It is our goal to utilize the in vitro transformation system to study the mechanism of cell transformation by ethionine and the effect on transformation exerted by nutritional components of the culture medium.

Proposed Course: Experiments recently initiated, which will be continued, include a two-stage study of cell transformation using ethionine as the initiating agent and methionine-deficient medium as the promoting agent. Electron microscopic examination of cells treated with ethionine and SAE are currently being conducted to gain information relating to the mechanism of ethionine's carcinogenicity. DNA from cells undergoing treatment with ethionine will be examined to determine the effect of ethionine on methylcytosine content.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 04812-14 LCC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Morphological and Functional Changes During Transformation of Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ursula Heine Research Microbiologist LCC NCI
OTHER: M. Wilson Chemist LCC NCI

COOPERATING UNITS (if any)

G. Krueger, Pathology Institute, University of Cologne, West Germany;
Litton Bionetics, Inc., FCRF, Frederick, MD

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

ultrastructural Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

2

PROFESSIONAL:

1

OTHER:

1

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to determine and to compare the role of cyto-skeletal proteins in epithelial cells during cell transformation by chemical and biological carcinogens. The effects of ethionine, a liver cell carcinogen, are evaluated in vitro and initial studies suggest deviations in cytoskeletal architecture concomitant with altered membrane fluidity in the course of transformation. In vivo experiments, using the Moloney virus-induced lymphoma as a model, are utilized to elucidate the mechanism of the differentiation block in the thymus resulting in generalized malignant lymphoma. Studies concentrate on thymic tissue and isolated thymic cells with special emphasis on the thymic epithelial cell and its microfilament/microtubule distribution.

Project Description

Objectives: To identify the precise nature of morphological and functional changes in epithelial cells during transformation with special emphasis on the transformed cell per se and on nontransformed epithelial cells providing a microenvironment favorable to tumor development.

Methods Employed: Tissue culture techniques, in vivo tumor production, light, transmission, and scanning electron microscopy, antibody-mediated labeling, fluorescence microscopy and immunomicroscopy at the level of the electron microscope.

Major Findings: Recent studies have stressed the importance of the cytoskeleton (microtubules, actin cables, intermediate filaments) for the preservation and regulation of cellular configuration in interphase cells and during mitosis. Changes in phenotype are used as one criterion for cell transformation. Several liver cell lines, untransformed and ethionine-transformed, provide a model for the study of different parameters in the course of transformation. Ultrastructural studies on the action of ethionine in such liver cells have shown well-defined morphological changes in the nucleus and especially in the nucleolus which parallel changes in the cell metabolism due to ATP deficiency and/or incomplete rRNA methylation. These changes include decrease in heterochromatin, accumulation of perichromatin granules, and hypertrophy of the nucleolus with enlargement of the pars granulosa. In the cytoplasm, alterations in the mitochondrial architecture and changes in the size of cell junctions are outstanding. Moreover, in preliminary experiments, SEM studies revealed an altered surface architecture in transformed cells, which affects both the distribution of microvilli and the expression of the primary cilium, possibly reflecting changes in cytoskeletal architecture, alterations of the cell cycle and deviations in membrane fluidity.

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 the non-lymphoid, epithelial cells to be able to differentiate. It is understood that the thymic epithelial cells provide a specific microenvironment capable of directing proliferation and maturation. It has been shown previously that during the early phase of M-MuLV-induced lymphomagenesis in mice 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 leads to generalized lymphoma of the stem cells. A study has been initiated to evaluate the differentiation block by characterizing changes in thymic tissue and isolated thymic cells with special emphasis on thymic epithelial cell and microfilament/microtubule distribution.

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 the mesoderm. In our studies we utilize two epithelial cell models: the liver epithelial cell is used to investigate functional changes during transformation in the epithelium per se, whereas the thymus model serves for studies of the role of nontransformed epithelial cells in malignancy.

Proposed Course: The qualitative results obtained thus far will be quantitated. The thymus study will be extended to include early changes in lymphomagenesis.

Publications:

Heine, U.I., Keski-Oja, J., and Wetzel, B.: Rapid membrane changes in mouse epithelial cells after exposure to epidermal growth factor. J. Ultrastructural Research 77: 335-343, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05092-04 LCC
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Transplacental Carcinogenesis and Tumor Promotion in Nonhuman Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jerry M. Rice	Laboratory Chief	LCC	NCI
OTHER:	Amos E. Palmer	Research Veterinarian	LCC	NCI
	Jerrold M. Ward	Head, TPPS	LCC	NCI
	William T. London	Section Head	NINCDS	NIH

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, MD 21701

TOTAL MANYEARS: 2.4	PROFESSIONAL: 1.4	OTHER: 1.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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, and in some cases subsequently to agents that promote development of specific neoplasms in rodents. Pathologic changes, including neoplasms, are evaluated by histopathology and ultrastructural methods. Mechanisms of organ and species differences in the effects of chemical carcinogens and tumor promoters in rodent and nonhuman primate species are investigated.

Project Description

Objectives: To explore and define the varying susceptibilities of different organ systems in rodents and primates to direct-acting and metabolism dependent carcinogens during prenatal and postnatal development; and precisely to characterize neoplastic and selected non-neoplastic conditions by light and electron microscopy, histochemistry, transplantation, explantation to cell and organ culture, and by other procedures as required. To establish the phenomenon of tumor promotion in nonhuman primates and to determine whether cell and tissue specificities of tumor-promoting agents that are known solely from their effects in rodents will be the same or different.

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. Carcinogens in precise dosages are administered to nonpregnant or exactly timed-pregnant rodent (rat, mouse) or nonhuman primate (patas, rhesus or cynomolgus monkey) subjects and the treated animals and their offspring are followed carefully for tumor development. Agents that previously have been adequately characterized as tumor promoters in rodents are similarly purchased and purified, or synthesized de novo and administered to primates after completion of an "initiating" regimen of exposure to organ-specific carcinogens.

Surgical procedures are devised as necessary to remove single operable growths from primates in order to prolong life and allow further observation for additional tumor development. Selected neoplasms and fetal tissues are explanted to cell culture and to athymic (Nu/Nu) mice for further study of their properties. Tumors are carefully classified histogenetically by light and electron microscopy and by histochemistry.

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.

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 α -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 to target organ and target cell in both rodents and this primate species.

Significance to Biomedical Research and the Program of the Institute:

Research of 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 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 vs. 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. Pharmacodynamic studies will be continued to further study maternal-fetal distribution and tissue/organ localization of ENU-¹⁴C in this species in comparison with rats and mice.

Publications:

Palmer, A. E., London, W. T., Brown, R. L., and Rice, J. M. Color changes in the haircoat of patas monkeys (*Erythrocebus patas*). Amer. J. Primatol. 1: 371-378, 1981.

Rice, J. M., Williams, G. M., Palmer, A. E., London, W. T., and Sly, D. L. Pathology of gestational choriocarcinoma induced in patas monkeys by ethylnitrosourea given during pregnancy. Placenta (Suppl. 3): 223-230, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05093-04 LCC
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) In Vitro Studies on Organ Specificity in Transplacental Carcinogenesis		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Jerry M. Rice Chief, LCC and Head, PCS LCC NCI OTHER: Paul Donovan Chemist LCC NCI Alan Perantoni Microbiologist 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: 3.2	PROFESSIONAL: 2.2	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The roles of <u>morphogenetic differentiation</u> in controlling the phenotypic expression of <u>neoplastic transformation</u> , the degree of malignancy of <u>tumors</u> , and the susceptibility of developing organs to <u>carcinogenesis</u> are studied using <u>organ culture</u> and <u>tissue transplantation</u> techniques, with current emphasis on the <u>kidney</u> . The presence of <u>genotoxic damage</u> in target and non-target tissues exposed transplacentally to chemical carcinogens during embryonal and fetal development is assayed by detection of mutations at 3 loci in cells cultured from embryos exposed in utero and is correlated with organ specificity for chemical carcinogenesis by the same agents.		

Project Description

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 determine mutation frequencies in cells isolated from selected organs from different species after transplacental treatment with carcinogens at comparable stages in gestation. To determine the sensitivity to transplacentally induced mutation induction as a function of different stages of gestation. To determine transplacentally induced mutation dose curves with selected known carcinogens and noncarcinogens.

Methods Employed: 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.

Mutation frequencies are determined for cells isolated from embryos treated transplacentally with various carcinogens and non-carcinogens. Gravid animals are injected with different doses of the agents at precise periods of gestation. Primaries are made from either whole carcass or selected organs of different species. At experimentally determined optimum expression times, cells are treated in vitro with the selective agents for several markers. These include resistance to 6-thioguanine, resistance to ouabain or resistance to diphtheria toxin.

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 organ rudiments in organ culture and their effects on differentiation of normal and neoplastic tissues 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: Previous reports have described our development of procedures for culturing fetal organ rudiments and for producing primary and established monolayer cultures of fetal and adult epithelial organs.

A continuing effort to manipulate morphology and differentiation of normal and neoplastic renal tissue through media additives is under way. Mouse kidneys develop purely epithelial, well-differentiated tumors of adult type in response to transplacental carcinogens, while rat kidneys generate adult epithelial, nephroblastic, and undifferentiated (blastemal cell) tumors; the ability of rat and mouse renal tumor cells to respond to differentiation signals and the influence of the latter on their morphology, growth, and biologic behavior are being investigated. Work has continued on maintaining kidney rudiments in organ culture. Enzymatic dissociation of fetal rat and mouse metanephric rudiments has been achieved, with quantitative separation of mesenchyma from inductive ureteric bud epithelium, and experiments are underway to determine the relative sensitivity of response to homo- and heterotypic inducers of morphogenetic differentiation in normal metanephric rudiments from these two species and in tumors derived from them.

Dose curves of selected transplacental carcinogens have been determined in cells cultured from fetuses of Syrian hamsters injected on day 12 of gestation. 7,12-Dimethylbenz[a]anthracene has been found to be the most active transplacental mutagen, followed by ethylnitrosourea, and then at much lower levels, benzo[a]pyrene, diethylnitrosamine, and ethylcarbamate.

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 "blastema-cell" tumors will continue to be studied in transplantation and in cell and organ culture to determine whether the morphologically undifferentiated tumors 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.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemical Mechanisms of Organ Specificity in Chemical Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jerry M. Rice	Chief, LCC, and Head, PCS	LCC	NCI
OTHER: Beatrice Chen	Research Chemist	LCC	NCI
Alan Perantoni	microbiologist	LCC	NCI

COOPERATING UNITS (if any)

Dr. David Kaufman, University of North Carolina, Chapel Hill, North Carolina
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:

2.2

PROFESSIONAL:

1.7

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 post-natal 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, 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. Factors anticipated for future study include the induction and genetics of enzymes metabolizing carcinogens.

Project Description

Objectives: To define the roles of biochemical processes 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 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 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 that for a number of cell lines sensitivity to toxicity by the carcinogen L-azaserine correlates positively with intracellular levels of GGT. Studies of possible mechanisms for

the observed correlation between gamma-glutamyltranspeptidase (GGT) activity and sensitivity to azaserine-induced toxicity have continued. GGT does not contribute to active transport of azaserine into cells. Neither is GGT capable of metabolizing azaserine to a reactive intermediate. However, GGT has been shown by others to influence glutathione availability by degrading the tripeptide. Although GGT-deficient cell strains of TuWi, a cell line established from a human nephroblastoma, contain intracellular glutathione levels comparable to that of the parent line, they release significantly more glutathione to the culture medium. This suggests that these GGT-deficient cells are producing more glutathione as a result of suppression of the degradative activity of GGT. Azaserine is reactive with thiol moieties in enzymes and with glutathione. The nature of this reaction remains to be determined; however, a glutathione conjugate, which contains the diazo moiety and the acetyl group from azaserine, can be separated from reaction mixtures by HPLC. Several observations support the hypothesis that glutathione not only reacts with azaserine but also participates in its detoxification. Reduced glutathione and cysteine, but not oxidized glutathione, protect TuWi cells from azaserine toxicity. In addition, serine and borate complex to inhibit GGT activity and also to reduce azaserine toxicity. Finally, maleic acid, which compromises the ability of glutathione to function in detoxification, enhances cellular susceptibility to azaserine. These results are consistent with the hypothesis that cells high in GGT activity are more sensitive to azaserine damage than cells deficient in GGT because of their reduced production of glutathione, which is necessary for azaserine detoxification.

A final mechanism under consideration postulates a role for GGT in the preferential cellular retention of GGT substrates. GGT can add multiple glutamyl residues to its amino acid substrates. This process might reduce diffusion of the substrate azaserine out of target cells without impairing its ability to damage the cell. This situation has been observed for the folate analog methotrexate. Polyglutamyl methotrexate molecules predominate intracellularly even after methotrexate has been removed from the environment of the cell; yet the polyglutamyl addition does not impair the ability of this drug to compete with folic acid. We have found that GGT-deficient cells have a reduced capacity to retain isotope following removal of labeled azaserine from culture media. Purified GGT and homogenates of TuWi are capable of adding glutamyl residues to azaserine; however, analyses of HPLC separations of intracellular forms of azaserine suggest that the azaserine is primarily in a free unmodified state or it is part of a glutathione conjugate. No short-chain glutamyl containing azaserine species have been isolated from intact cells following exposure to azaserine.

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 β -galactosidase (β G) to function in an in vitro DNA-dependent system in which β G is synthesized. The DNA λ h80d1acp^S was made apurinic (AP) by treatment with acid (pH 4.0) and low heat (37°). 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 indicate 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. The conclusions are especially convincing since the onset of S could be delayed by as much as 10 hours by administering the hormone hydrocortisone by intraperitoneal injection at intervals after partial hepatectomy, resulting not only in delay in onset of S, but improved synchrony once decay of the block allowed the cell cycle to proceed in the regenerating liver. Delay of 10 hours in the onset of S delayed the peak tumor incidence in carcinogen-treated rats by the same interval. 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 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-¹⁴C is essentially constant, irrespective of the stage of the cell cycle in regenerating liver when the labeled carcinogen was given.

Significance to Biomedical Research and the Program of the Institute: 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.

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 role of target cell constituents, such as GGT, as possible determinants of organ specific carcinogenesis is a step in this direction.

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 of DNA in liver, brain, and other tissues will continue to be studied by assays for activity of repair enzymes in a continuing effort to titrate physical repair of single-strand breaks at dosages of simple monofunctional methylating and ethylating agents below cytotoxic levels, as judged from histological sections of organs of interest. This procedure should allow for greater resolution than the relatively crude alkaline sucrose gradients initially employed, will eliminate artifacts due to radioactive decay of tritium labels and the severe selection process of short-term in vitro cultivation for purposes of labeling, and should 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 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:

Kaufmann, W. K., Kaufman, D. G., Rice, J. M., and Wenk, M. L. Reversible inhibition of rat hepatocyte proliferation by hydrocortisone and its effect on cell cycle-dependent hepatocarcinogenesis by N-methyl-N-nitrosourea. Cancer Res. 41: 4653-4660, 1981.

Ohmori, T., Rice, J. M., and Williams, G. M. Histochemical characteristics of spontaneous and chemically induced hepatocellular neoplasms in mice and the development of neoplasms with -glutamyl transpeptidase activity during phenobarbital exposure. Histochem. J. 13: 85-99, 1981.

Rice, J. M. Prenatal susceptibility to carcinogenesis by xenobiotic substances including vinyl chloride. Environmental Health Perspectives 41: 179-188, 1981.

Rice, J. M. Foreword: Current concepts in transplacental chemical carcinogenesis. Int. J. Biol. Res. Pregnancy 2: 149, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05299-01 LCC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Interspecies Differences in Transplacental Carcinogenesis and Tumor Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bhalchandra Diwan	Expert	LCC	NCI
OTHER:	Jerry M. Rice	Chief, LCC and Head, PCS	LCC	NCI
	Amos Palmer	Research Veterinarian	LCC	NCI
	Jerrold M. Ward	Head, TPPS	LCC	NCI

COOPERATING UNITS (if any)

Microbiological Associates, Inc., Bethesda, MD
Litton Bionetics, Inc., FCRF, Frederick, MD

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

TOTAL MANYEARS:

2.2

PROFESSIONAL:

1.6

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tumor promotion is systematically explored in various rodent species in conjunction with transplacental carcinogenesis. Organ specificity and interspecies correlations in tumor promotion are established and used in investigations of mechanisms of promotion phenomena.

Project Description

Objectives: To characterize and define the limits of organ specificity exhibited by nongenotoxic tumor-promoting agents, and to utilize these agents to demonstrate the presence of latent, potentially neoplastic cells in organs and tissues which appear unaffected by transient transplacental or systemic exposure to genotoxic carcinogens alone.

Methods Employed: Precisely timed-pregnant mice, rats, and Syrian hamsters are exposed to selected chemical carcinogens at defined periods during gestation. Offspring, and animals exposed to carcinogens by conventional routes during postnatal life, are subsequently given sustained exposure to nongenotoxic agents known or suspected to promote tumorigenesis in one or more organ systems. Tumors and preneoplastic proliferative processes resulting from such treatments are classified by histological, histochemical, and ultrastructural parameters.

Major Findings: This is a new project and results are not yet available. Systematic studies on organ-specific effects of barbiturates, which in rats display apparent organ specificity for hepatocytes, intestinal mucosa, and bladder urothelium, are in progress in rats, mice and Syrian hamsters.

Significance to Biomedical Research and the Program of the Institute: The existence of tumor-promoting activity at therapeutic dosage levels as a characteristic of widely used therapeutic agents is of obvious significance to public health if such promoting action is not limited to rodent species. Prediction of the effects of such agents in man is supportable if organ specificity and dosage requirements prove to be consistent in many nonhuman species. Rigorous empirical definition of organ specificity in tumor promotion by barbiturates may provide a route towards demonstration of mechanisms of promoting action of these agents.

Proposed Course: Studies in at least 3 rodent species--mouse, rat, and Syrian hamster--should be extended to rigorous determination of the range of cell types effectively promoted by different barbiturates and structure/activity correlations established. Dose/effect relationships for each tissue in each species should be determined. If organ-specific activity varies markedly with structure in rodents, these studies should be extended to nonhuman primates.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05301-01 LCC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Biology of Natural and Induced Tumors in Rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jerrold M. Ward Head, TPPS LCC NCI

COOPERATING UNITS (if any)

Biological Response Modifiers Program, Division of Cancer Treatment, NCI
Litton Bionetics, Inc., Frederick, MD

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The biology and pathology of natural and induced tumors are characterized. Natural tumors are characteristic for each strain of rat, while induced tumors may be different biologically and morphologically from natural tumors. Natural tumors from approximately 150 F344 rats were transplanted into the mammary fat pad of weanling F344 rats. The tumor latent period, growth rate, invasiveness and metastatic rate were directly related to the histologic appearance of the tumor and tumor cell type. Both malignant and benign tumors were transplanted although only the former were invasive or metastatic in syngeneic hosts. The experiment provides evidence that characteristics of tumor transplantation can be indicative of the degree of malignancy of a primary tumor, but not whether it is benign. Leukemia in the F344 rat was identified as a large granular lymphocyte leukemia, the major effective cell for natural killer activity in the rat. Its role in natural tumor immunity is being studied.

Project Description

Objectives: To characterize the biology and pathology of natural and induced tumors of rats; to identify differences between natural and induced tumors.

Methods Employed: Induced and natural tumors of rats are routinely characterized by histologic methods and by immunologic and ultrastructural procedures. Selected lesions are transplanted to correlate biology with histological features.

Major Findings: Histologic diagnosis of benign and malignant tumors is subjective in nature and is based on one's education, experience and review of the literature (other pathologists' views and experience). Some investigators believe that tumor transplantation is prima facie evidence of malignancy and that small nodules in tissues are not neoplastic, but rather hyperplastic. We transplanted 400 naturally occurring tumors of various types and sizes from 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 but none were invasive or metastasized in tumor recipients. This experiment provides support to the concept that tumor transplantation, latency, and morphology is related to degree of malignancy but not to whether or not the tumor is malignant.

The large granular lymphocyte leukemia, the most common cause of death of aged F344 rats, was identified and characterized by immunologic procedures in vitro, 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 are characterized as to NK activity. Transplantable tumors are heterogeneous in nature and represent the spectrum of tumors from those with morphologic and immunologic characteristics identical to normal LGL-NK cells to those poorly differentiated tumors of possible stem cell origin.

Induced tumors of liver, lung, and kidney from several studies have been shown to differ biologically, morphologically and histochemically from those of control rats.

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 transplant study and characterization of LGL tumor.

Publications:

1. Goodman, D. G., Bates, R., Ward, J. M., Frith, C. H., Sauer, R. M., Jones, S. R., Strandberg, J. E., Squire, R. A., Montali, R. J. and Parker, G. A. Common lesions in aged B6C3F1 (C57B1/6NXC3H/HeN)F1 and Balb/c St Cr/fcC3H/Nctr mice. Armed Forces Institute of Pathology, Washington, D.C., 1982, 44 pp.
2. Reynolds, C. W., Ward, J. M., Denn III, A. C. and Bere Jr., E. W. Identification and characterization of large granular lymphocyte (LGL) leukemias in F344 rats. In R. B. Herberman (Ed.): Natural Immunity, Volume 2, New York, Academic Press, 1982. (In press)
3. Reznik, G. and Ward, J. M. Refinements of Rodent Pathology Protocol and the Pathologist's Contribution to Better Evaluation of Carcinogenesis. Prog. Exp. Tumor Res. 26, 1982. (In press)
4. Ward, J. M. Background Data and Variations in Tumor Incidence of Control Animals. Prog. Exp. Tumor Res. 26, 1982. (In press)
5. Ward, J. M. Background Variations of Tumor Incidence in Rodent Populations. In F. Homburger (Ed.): Proc. Int. Conference on Safety Evaluation and Regulation of Chemicals. Basel, S. Karger, 1982. (In press)
6. Ward, J. M. Pathology of Toxic, Preneoplastic and Neoplastic Lesions in Rodents. In Douglas, J. F. (Ed.): Carcinogenesis-Mutagenesis. New York, Humana Press, 1982. (In press)
7. Ward, J. M. and Rice, J. M. Naturally occurring and chemically induced brain tumors of rats and mice in carcinogenesis bioassays. Annals NY Acad. Sci., 1982. (In press)
8. Wenk, M., Ward, J. M., Reznik, G. and Dean, R. J. Effects of three retinoids on colon adenocarcinomas, sarcomas and hyperplastic polyps induced by intra-rectal N-methyl-N-nitrosourea administered in male F344 rats. Carcinogenesis 2: 1161-1166, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Animal Models for Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jerrold M. Ward	Head, TPSS	LCC	NCI
OTHER:	Michael Sporn	Head, LCP	LCC	NCI
	Craig W. Reynolds	Staff Fellow	DCT	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

0.5

PROFESSIONAL:

0.2

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Natural occurring and induced tumors of rodents may be used to study the possible causes, nature and prevention of human cancer. We have been developing the ACI rat prostate cancer model and have characterized the histogenesis of the prostate preneoplastic and neoplastic lesions. In a long-term lifetime experiment we are studying the effects of 3 retinoids on survival, naturally occurring tumors, and prostate lesions. Interim results suggest that one retinoid prolonged survival of rats. The large granular lymphocyte leukemia in F344 rats is in development as a model for the study of the natural killer cell and its role in tumor immunity. Transplantable LGL tumors with high or low NK activity are utilized to study the potential lineage and activity of LGLs. Experiments are planned to study the role of NK-LGL cells in induced and natural tumor immunity.

Project Description

Objectives: To develop and utilize animal models of human tumors or carcinogenesis processes to help characterize the etiology, pathogenesis and methods of prevention of human cancer.

Methods Employed: a) We are using the ACI rat prostate cancer model which involves these rats with a high incidence of age-related preneoplastic, precancerous and neoplastic lesions. The high incidence of latent lesions is similar to the human disease. In one large experiment, ACI rats, 24 months of age, were placed on one of 3 diets with one of 3 synthetic retinoids or on control diets. Each rat will be allowed to live out its life span. Cause of death and incidences of neoplastic and nonneoplastic lesions will be analyzed. The differences between groups will be statistically evaluated. b) The large granular lymphocyte (LGL) leukemia of F344 rats is being studied to characterize the role of natural killer cells in tumor immunity and pathogenesis. The rat LGL is similar to human LGLs. Tissue distribution of LGLs in normal and neoplastic tissues is being characterized by immunoperoxidase techniques for localization of lymphocyte cell surface antigens. Similar studies will be performed using parallel human surface antigens in human tissues.

Major Findings: The ACI rat prostate tumor model experiment is still in progress. Thus far, one retinoid, trans-N (4-pivaloyloxyphenyl) retinamide, has prolonged the life span of aging ACI rats. The incidence of certain tumors appears reduced.

Primary and transplantable LGL leukemia in the F344 has been characterized as a heterogeneous lymphocytic leukemia. Some cases have high natural killer cell activity. Localization of lymphocyte cell surface antigens has allowed us to localize LGL (and natural killer cells) in various tissues of the rat including epithelium, lymphoid organs and parenchymal organs.

Significance to Biomedical Research and the Program of the Institute: Animal models of human tumors or neoplastic process provide a laboratory study for better understanding the cause, nature, pathogenesis and methods of prevention of human cancer. The prostate cancer model provides a unique opportunity to study so-called "latent" prostate lesions and the sequential development to clinical cancer. Chemoprevention, a possible method for reducing human cancer risk, offers promise for prolonging lives. Animal studies can identify potential chemopreventive agents.

Natural tumor immunity is an emerging area of cancer research. The role of natural immunity, particularly LGLs and NK cells, in neoplastic development, is not yet fully known. Characterization of this animal model system would add greatly to our understanding of the NK cell and tumor immunity.

Proposed Course: a) Completion of the ACI rat prostate cancer model experiment, b) further development of the rat LGL-NK model and its comparison with human LGL-NK cells, and c) development of additional new animal models.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Natural and Induced Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jerrold M. Ward	Head, TPPS	LCC	NCI
OTHER:	Amos Palmer	Research Veterinarian	LCC	NCI
	Mary J. Wilson	Chemist	LCC	NCI

COOPERATING UNITS (if any)

Bioassay Research, FCRF, Litton Bionetics, Inc., Frederick, MD
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:

0.5

PROFESSIONAL:

0.3

OTHER:

0.2

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 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The sequence of development of selected tumors from the initial event to overt cancer is being studied by the biopsy and serial sacrifice of animals, initiation-promotion bioassays using skin and liver models and serial karyotyping of target cells. Liver cancer histogenesis and histochemistry in monkeys, rats and mice are being compared. Significant differences were found as well as similarities. Karyotyping and initiation-promotion bioassays are being used to characterize possible mechanisms of cancer development for chemicals not demonstrated to be genotoxic, including ethionine, phthalates and formaldehyde.

Project Description

Objectives: To characterize the sequential steps (pathogenesis) in the development of cancer from the pathologist's viewpoint.

Methods Employed: The role of initiation and/or promotion in the development of tumors is being studied using formaldehyde and dioctyl phthalate as models. Skin painting and liver initiation-promotion assays are compared with results of two in vitro initiation-promotion systems. The common endpoint in rodent carcinogenesis bioassays is the mouse liver. The mouse liver is being utilized for defining the sequence of cancer development and the role of initiators and/or promoters on liver tumor development. Mice are injected once with diethylnitrosamine and later placed on diets containing the potential promoter, phthalate. Control groups include those given the phthalate once at a dose 1/2 of the LD₁₀, followed by phenobarbital in the diet. Thus, the initiating and/or promoting activity of the phthalate in this system can be tested. Skin and in vitro results will be compared.

So-called "nongenotoxic" carcinogens are being studied for their possible pathogenetic mechanisms. Dioctyl phthalate is being studied, as above, for its possible promotional role in carcinogenesis. Methapyrilene is also in similar studies. Ethionine, a carcinogen which appears to act as an antimetabolite or competitive inhibitor, is being studied in vitro (rat liver cells) as to possible induction of karyotypic changes in vitro during the process of in vitro cell transformation.

Major Findings: Dioctyl phthalate, a so-called nongenotoxic carcinogen, has been shown to have promotional activities in two in vitro tumor promotion systems and in a mouse liver tumor system. Initiating activity has not been demonstrated as yet. The experiments are in progress. Formaldehyde, however, a weakly genotoxic chemical, with purported irritational and possible promoting activities in nasal carcinogenesis studies, has been shown in SENCAR mouse skin painting studies to lack promoting activity after DMBA application, but to have "weak" initiating activity when given prior to a phorbol ester. Chromosomal aberrations have been found in rat liver cells transformed by ethionine. Methapyrilene lacked initiating activity after 30 weeks of feeding phenobarbital to rats but had significant cocarcinogenic and promoting activity with phenobarbital.

Significance to Biomedical Research and the Program of the Institute: The mechanisms of chemical carcinogenesis are not precisely known, i.e., genotoxic, nongenotoxic, or other. For each chemical, whether demonstrated to be genotoxic or not, in vitro/in vivo studies of the sequence of tumor development and role of initiation and promotional aspects must be studied to understand the possible significance of in vitro and in vivo genotoxicity results. Human cancer risk to chemical exposure may depend, in part, on the mechanism of tumor formation and events that occur after the initial event of carcinogen-cell interaction. These rodent studies with formaldehyde, phthalates and methapyrilene, all important chemicals, should lead toward understanding the mechanisms of cancer development.

Proposed Course: Continue studies with dioctyl phthalate to define mechanism of tumor induction by this chemical, complete formaldehyde skin painting study, and ethionine liver cell karyotyping studies.

Publications:

Ward, J. M.: Morphology of Potential Preneoplastic Hepatocyte Lesions and Liver Tumors in Mice and A Comparison with Other Species. In Popp, J.A. (Ed.): Proceedings of the CIIT Conference on the Mouse Liver Neoplasia. New York, Hemisphere Press, Inc., 1982. (In press)

CONTRACT INDEX

LABORATORY OF COMPARATIVE CARCINOGENESIS
 DIVISION OF CANCER CAUSE AND PREVENTION

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CONTRACT NARRATIVES
LABORATORY OF COMPARATIVE CARCINOGENESIS
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1982

LITTON-BIONETICS, INC. (N01-CP-01039)

Title: Holding Facility for Small Laboratory Animals

Contractor's Project Director: Dr. Allen Manus

Project Officer (NCI): Dr. Lionel A. Poirier

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.

Methods Employed: Standard carcinogenesis feeding and injection studies with metals and organic compounds are conducted. Initiation and promotion studies in rat liver are done by administering short pulses of the hepatocarcinogens 2-acetylaminofluorene and N-nitrosodiethylamine. Promotion is performed by feeding methionine antagonists. Typically, carcinogenesis studies are performed for 1.5 to 2 years. Preliminary toxicity studies are conducted to determine the appropriate carcinogen levels and dietary regimens to be employed. Other services include the routine weighing, termination, and necropsies of carcinogen-treated animals.

Major Findings: Ethionine has been shown to be a hepatocarcinogen in female C3H and Swiss mice.

Proposed Course: The animal holding facilities will continue to be used during the administration of chemical carcinogens. The studies with metals will be expanded to include other inorganic carcinogens whose mode of activity is being examined. The effects of methylase inhibitors on chemical carcinogenesis will be determined. Over the next few years this effort will be gradually transferred to NCI intramural laboratories at FCRF.

Current Annual Level: \$198,000.

Man Years: 2.5

MELOY LABORATORIES, INC. (N01-CP-15766)

Title: Resources for Transplacental Carcinogenesis in Primates

Contractor's Project Director: Dr. John Cicmanec

Project Officer(s) (NCI): Jerry M. Rice, Ph.D.

Objectives: This project is designed to demonstrate and characterize transplacental carcinogenesis in nonhuman primates, especially the Old-World monkey *Erythrocebus patas*, by direct-acting and metabolism-dependent chemical carcinogens. In addition, related phenomena are studied including increased risk of carcinogenesis in adult females during pregnancy, tumor promotion, mechanisms of cell and organ specificity and of species differences in the effects of both chemical carcinogens and tumor promoters.

Methods Employed: A closed colony of patas monkeys is maintained by a breeding program that includes production of time-mated animals for experimental purposes. Chemical carcinogens are administered intravenously, while tumor promoters are given in diet or drinking water. Induced neoplasms are surgically removed whenever possible. Effects of chemicals in primate tissues are evaluated by histo- and ultrastructural pathology.

Major Findings: Through collaboration with investigators at NINCDS, it has been found that a direct-acting alkylating agent, ethylnitrosourea (ENU) is a potent transplacental carcinogen in a second species of nonhuman primate, the rhesus monkey, at similar dosage levels. In this species, as in the patas, the fetus is more susceptible than the adult and susceptibility is pronounced during the first and second trimesters, rather than near term as in rodents. However, the kinds of tumor seen in the rhesus are generally different from those that occur in patas in response to transplacental ENU. One neoplasm (a myelomonocytic leukemia) developed at the age of 69 months in a patas monkey exposed only transplacentally to ENU, which corresponds to young adulthood in this species which becomes sexually mature at 3-4 years. While no tumors have been seen in either mothers or offspring following exposure to diethylnitrosamine (DEN) during pregnancy, even after 5 years of observation, multiple hepatocellular neoplasms have developed in one transplacentally exposed patas given human therapeutic levels of phenobarbital beginning at 4 years of age. This suggests that phenobarbital may promote hepatocellular carcinogenesis in this species as it does in rats and mice; that DEN is also a transplacental carcinogen for primates; and that potentially neoplastic cells may persist for prolonged periods in nonhuman primates, as in rodents, and may be stimulated to proliferate to form tumors by exposure to tumor promoters.

Significance to Biomedical Research and the Program of the Institute:

With recognition of the association between exposure to diethylstilbestrol in utero and development of vaginal adenocarcinoma during the second decade of life, the possible significance to human health of transplacental chemical carcinogens has become increasingly a matter of concern. Experimental studies in transplacental chemical carcinogenesis have been limited to rodent species, which appear to differ significantly from man in ways likely to be of importance for understanding the different consequences of exposure to chemical carcinogens during fetal versus adult life. Among these differences are the more rapid rates of fetal and neonatal development and maturation in rodents. The short latencies for tumor appearance and the type of tumors observed to date in the patas monkey provide experimental support for the hypothesis that at least some tumors of infancy and childhood, including certain congenital tumors, may result from prenatal exposure to carcinogens. The demonstration that agents which promote tumor development in rodents behave similarly in nonhuman primates emphasizes the importance of such agents for man.

Proposed Course: Animals previously exposed transplacentally to carcinogens will be monitored for tumor development and tumor type as they progress in age. In addition, a variety of studies is being initiated to explore the cocarcinogenic effects of several tumor promoting agents. Both phenobarbital and asbestos have been shown to promote tumorigenesis in rodent studies. The question of whether these types of agents, which are present in the human environment, also intensify or accelerate tumors in primates and in man is extremely important. These studies will determine if limited prenatal exposure to carcinogens previously shown to produce sarcomas in monkeys will, with subsequent exposure to a tumor-promoting agent, give rise to carcinomas of lining epithelia, which are the major forms of cancer in man.

Date Contract Initiated: March 23, 1981.

Current Annual Level: \$229,993

Man Years:

MICROBIOLOGICAL ASSOCIATES (N01-CP-15744)

Title: Laboratory Rodent and Rabbit Facility As A Resource to the Laboratory of Comparative Carcinogenesis

Contractor's Project Director: Martin L. Wenk, Ph.D.

Project Officer (NCI): Jerry M. Rice, Ph.D.

Objectives: The purpose of this contract is to provide support services for the Laboratory of Comparative Carcinogenesis (LCC) and the Laboratory of Cellular Carcinogenesis and Tumor Promotion (LCCTP) within the Carcinogenesis Intramural Program of NCI in carrying out long-term treatment, holding, and observation of animals in carcinogenesis investigations emphasizing life-time tumor induction in rodents and related activities.

Methods Employed: 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. These resources are employed to carry out protocols developed in collaboration with intramural NCI investigators and approved by the 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 as protocolled, or experimental diets as required; storage of labile animal diets, reagents, tissues, or other materials under conditions of temperature

regulation (40°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, radioautography, biochemistry, or histochemistry at NCI.

Major Findings: During the course of the last year, support has been made available to 28 NCI investigators in conducting 36 individual experimental protocols. These protocols involved pathogenesis of chemically induced neoplasms and evaluation of the modifying influence on chemical carcinogenesis of various factors including age, sex, genetic determinants, target organ cell cycle kinetics, natural or synthetic dietary additives, route of exposure, and exposure level. In addition, the contract resources have been devoted to the supply of immunological reagents, breeding or holding of animals not routinely available through other sources, and maintenance of an athymic nude mouse colony.

Significance to Biomedical Research and the Program of the Institute:

This contract offers facilities, expertise, and manpower to support the ongoing and developing scientific programs of the Carcinogenesis Intramural Program that require long-term or lifetime studies in experimental rodents. The ability to conduct rapid tests of new approaches in experimental pathology, to conduct both short- or long-term in vivo experimentation involving large numbers of animals, and to rapidly initiate studies that are relevant to the overall objectives of the Carcinogenesis Intramural Program are critical to advancement of the Institute's scientific program.

Proposed Course: To continue to offer a diversified facility for the support of ongoing scientific programs within the Carcinogenesis Intramural Program.

Date Contract Initiated: December 30, 1980

Current Annual Level: \$611,171

Man Years:

ANNUAL REPORT OF THE LABORATORY OF EXPERIMENTAL PATHOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

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 level.

Relocation and Renovations: The Laboratory of Experimental Pathology (LEP) was reorganized during Fiscal Year 1981. New Laboratories were established from previous Sections in LEP, namely the Laboratory of Cellular Carcinogenesis and Tumor Promotion (Chief, S. H. Yuspa, M.D.) including programs of the In Vitro Pathogenesis Section and of the Differentiation Control Section in the former LEP; the Laboratory of Comparative Carcinogenesis (Chief, Jerry M. Rice, Ph.D.) including programs of the Perinatal Carcinogenesis Section of the former LEP; and the Laboratory of Human Carcinogenesis (Chief, C.C. Harris, M.D.) including programs of the Human Tissues Studies Section of the LEP. The new program of LEP and the newly established Laboratory of Comparative Carcinogenesis were moved to Frederick during Fiscal Year 1981, while the two new laboratories of Cellular Carcinogenesis and Tumor Promotion and of Human Carcinogenesis remained in Building 37 in Bethesda. Each of the new laboratories thus acquired more space and resources and formed a new point of departure for further progress in different but related areas in the growing field of experimental pathology research in chemical and physical carcinogenesis.

The reorganized LEP, in its new facility at Frederick, has maintained previous programs in the Office of the Chief, adding new research activities, and developed two new sections--one on respiratory carcinogenesis and one on tissue culture. Relocation of the LEP to Building 560 at the Frederick Cancer Research Facility started in May 1981 with the relocation of the Office of the Chief in temporary office and laboratory space. Plans were drawn up for new laboratory areas and renovations were implemented in stages. The tissue culture area was first renovated in the summer of 1981 and further renovations were completed in the spring of 1982; the pathology laboratory area and office area were renovated with a completely new space design, completed in January 1981. The biochemistry and histochemistry areas were renovated by May 1982. The LEP now occupies 3,235 sq. feet of contiguous space in Building 560. In addition, an animal facility of 553 sq. feet in Building 539, floor 2, was made available to LEP in April 1982. During Fiscal Year 1982 these new facilities were equipped and organized to become effectively operational and appropriate research instruments were selected, installed and calibrated.

Organization and Recruitment: The LEP now includes the following components:

Office of the Chief (U. Saffiotti, M.D., Chief)
Respiratory Carcinogenesis Section (U. Saffiotti, M.D., Chief)
Tissue Culture Section (M.E. Kaighn, Ph.D., Acting Chief)

The Laboratory's program is developing as an integrated whole, with the Respiratory Carcinogenesis Section providing a focus on animal and tissue pathology, the Tissue Culture Section providing a focus on tissue and cell culture studies, and the Office of the Chief providing a focus on underlying biochemical and molecular mechanisms for both in vivo and in vitro systems.

The initial staff nucleus that moved to FCRF during Fiscal Year 1981 included: U. Saffiotti, M.D., S. Stinson, Ph.D. (respiratory pathology), E. Cortesi, M.D., (Visiting Fellow, tissue culture) and J. Whitlock (Management Assistant). Additional recruitment has included: T. Gindhart, M.D. (Expert, Board certified pathologist, cell biology), M. Copley, D.V.M. (Staff Fellow, cell biology), C. Callahan (Secretary) and a biochemist (recruitment pending) in the Office of the Chief; F. Bertolero, M.D. (Visiting Fellow, metal toxicology) and J. Keller (Histotechnologist) in the Respiratory Carcinogenesis Section; M. E. Kaighn, Ph.D. (Expert, cell biology), M. Bignami, Ph.D. (Guest Researcher, genetics and cell biology), C. Ficorella, M.D. (Visiting Fellow, cell biology) and a pathologist experienced in cell biology (recruitment pending). In addition, three technical positions were provided by the FCRF/LBI contract support.

General Research Objectives: The main program of investigations in this laboratory continues to be focused on two correlated problems: (a) the comparative pathogenesis of chemically induced neoplastic disease at all levels of biological organization, ranging from human tissues and animal models to organ and cell cultures and to molecular interactions, with particular emphasis on studies on chemical carcinogenesis in lining epithelia which are the tissues of origin of most human cancers; and (b) the interactions resulting from concurrent exposures to different carcinogens with emphasis on synergism and 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 resulting in 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 different levels of observation. An approach that is considered particularly promising in this respect 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 linking together the response mechanisms from the subcellular to the organismic levels. A suitable sequence of biological targets for correlative studies on the response to carcinogens must represent stepwise connections linking macromolecular targets in controlled microenvironments, organized cell systems and 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 new LEP program represents a logical sequence to these advances.

A systematic investigative approach has become possible 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 will be further extended in its new 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; (c) organ explant culture systems for target epithelia with the possible outgrowth of epithelial cell lines from animal and human sources; (d) mammalian epithelial cell systems for neoplastic transformation (e.g. epidermal, respiratory and secretory epithelia); (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 and their expression in mammalian cell transformation.

Emphasis on epithelial systems: In the past decade the development of culture systems for human epithelial tissues and cells received major emphasis in the Human Tissues Section of LEP. LEP is now engaged in further methodological cell culture developments, not only for some human cell systems, but primarily 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. 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 and subsequent work in several laboratories has recently led to the development of preliminary respiratory epithelial cell culture systems. Methods for chemically induced neoplastic transformation of epithelial cells in culture have started to develop in the last decade. In recent years efforts in several laboratories were directed to the establishment of quantitative methods for the evaluation of chemically induced neoplastic transformation in epithelial cell systems, but these methods need to be further developed and more rigorously defined. As new 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 sub-cellular and molecular levels.

Development of optimal culture conditions: Several cell culture systems are used for studies on neoplastic transformation in this Laboratory. Quantitative dose-response experiments with single and combined carcinogens have been carried out in the mouse BALB 3T3 clone A31-1-1 cell line. Although this system is one of

the few established models for chemically induced transformation assays, there were difficulties in obtaining accurate and reproducible quantitative data. First, the number of induced transformed foci per dish is not a direct function of inoculum size. The literature offers conflicting data and interpretations concerning this phenomenon, which could be due to feeder effects between affected and unaffected cells or to some undefined "probability" function. Methods are needed to permit determinations of cytotoxic, mutational and transformational events in aliquots of the same cell population, treated at equal cell density. Another disturbing factor is the lot-to-lot variation in serum. The standard transformation assays employ a relatively high serum level (10%). Its variability with the consequent need for cumbersome controls and its high cost are reasons for developing adequate serum-free culture conditions for quantitative transformation studies. Recently (Shipley and Ham, 1981) a new medium (MCDB402) was developed that supported clonal growth of 3T3 cells at serum protein levels as low as 125 ug/ml (ca 0.3%). This medium is now being tested in the BALB 3T3 transformation system. Even this low serum requirement could be eliminated by adding several growth factors. If effective, such a defined medium would facilitate standardization of the assay and either eliminate or significantly reduce the need for serum with its variable effects.

Development of chemically defined media is also planned for at least two other cell systems, including normal Syrian golden hamster bronchial epithelial cell cultures. It is now apparent that the development of epithelial cell lines in defined media is possible from several tissue sources. A general requirement appears to be suppression of terminal differentiation by various medium modifications such as low Ca^{++} levels, addition of cholera toxin, alteration of the basal nutrient composition and, most importantly, elimination of serum.

Other factors will be also considered in optimizing culture standards, such as the surface of the culture vessel and the use of mild methods to disperse cells for replating, allowing transformation assays to be performed on aliquots of the same cell population used for cytotoxicity and mutagenesis assays.

Mechanisms of combined effects of different carcinogens: 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 limited to cumbersome in vivo animal studies; among the relatively few studies in the literature, usually limited to two carcinogens at a time, several examples of marked synergism were reported.

The development of the above mentioned sequence of interrelated biological models has made it possible to study combined effects of carcinogens in relevant in vitro and short-term systems for mutagenesis and cell transformation and to extend these studies to molecular mechanisms, particularly by DNA transfection.

Combinations of carcinogenic factors or co-factors 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), on their metabolic activation and detoxication, and on the cellular expression of neoplastic transformation. Such combinations may derive from the same source of exposure

(e.g., inhalation) or from different sources including exogenous and endogenous factors.

Selected combinations of factors, particularly if found to be synergistic, are investigated in a series of biological systems and for appropriate mechanisms of interaction. These studies include, at the present time, a comparison of the molecular effects of phorbol ester promoting agents with the effects of full carcinogens, singly and in combination; these studies investigate whether the mechanisms of "promotion" are present, in part if at all, in the mechanisms of full carcinogens acting alone or in combinations.

Metabolism of several carcinogens, both organic and inorganic, is studied to determine their pathways of activation, deactivation and binding, in vivo and in vitro in the different biological models, with emphasis on their possible interactions following combined exposures. Studies are under way on arsenic metabolism and others are being planned for aflatoxin, polycyclic aromatic hydrocarbons, aromatic amines and N-nitroso compounds.

In summary, the Laboratory of Experimental Pathology is developing its new program around three complementary lines of approach: (1) the development and definition of biological models for chemical carcinogenesis in epithelial tissues in vivo and in vitro, with emphasis on carcinogenesis models relevant to major forms of human cancer such as carcinogenesis in respiratory, epidermal and secretory epithelia; (2) the development and definition of cell culture models for chemically induced neoplastic transformation, particularly for epithelial cells derived from the systems mentioned above, with emphasis on developing chemically defined media and optimal conditions for growth, characterization and transformation of cells in culture; and (3) the study of mechanisms of chemical carcinogenesis, connecting the different levels of biological organization in vivo and in vitro, with emphasis on the combined effects of different carcinogens and on the multifactorial origin of cancer, at the organismic, tissue, cellular, genetic, and molecular levels.

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 reeseach on carcinogenesis 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.

This Office coordinated the reorganization of LEP at FCRF, the renovation of its facilities and the selection and installation of its equipment.

The scientific activities of the staff assigned to this Office include research programs involving the LEP at large and are focused on molecular mechanisms of carcinogenesis. This work is also integrated with projects in the Respiratory Carcinogenesis Section and in the Tissue Culture Section.

The continuously developing state of knowledge on mechanisms of chemical carcinogenesis, derived from studies in this and other laboratories, is 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 assessment are also evaluated.

A theoretical model, based on the recognition of the multifactorial origin of cancer, was further developed to account for the overlapping role of several concurrent causative factors. This model, initially developed from human cancer risk estimates for different categories of factors, is also studied in relation to experimental models for combined exposures to different carcinogens and their quantitative evaluation.

A systematic investigative approach has been outlined for a program of studies aimed at correlating molecular mechanisms of carcinogenesis by single and combined factors, through a series of sequentially connected biological models *in vitro* and *in vivo*, with emphasis on epithelial target tissues. The systems are designed to connect observations made in human tissues in culture with studies on corresponding animal models for epithelial carcinogenesis, with corresponding organ cultures and cell cultures, and to extend the comparisons to selected systems for cell transformation, mutagenesis, cellular and molecular toxicity, analysis of molecular targets, and identification of genes and gene products involved in the process of neoplastic transformation and its expression.

Quantitative studies on concurrent exposures to numerous carcinogens *in vitro* were continued and resulted in new evidence of synergistic combinations not only in mutagenesis systems but also in the BALB 3T3 system for cytotoxicity and for neoplastic transformation. Facilities were installed and plans made to initiate biochemical studies on the metabolism, DNA binding and mutual interactions of several carcinogens.

A new project is directed to identify specific DNA sequences affected by chemical carcinogens in the process of neoplastic transformation, and to investigate the biochemical events which determine or derive from the interactions between chemical carcinogens and the specific DNA sequences relevant to transformation. Effects of chemical carcinogens on transforming DNA sequences are being studied. Transforming genes, activated by chemical carcinogens interacting differently with DNA, are transfected into NIH 3T3 cells and compared according to their patterns of sensitivity to restriction endonucleases.

Initial studies are comparing transfectable transforming DNA sequences activated by a variety of agents in BALB 3T3 cells to determine whether different agents activated a single target-cell-specific DNA sequence or different agent-specific DNA sequences. The role of indicator-cell phenotype in the measurement of transfectable transforming genes will be evaluated by comparing the restriction endonuclease patterns of transforming DNA's expressed constitutively in NIH 3T3 cells and in a tumor promoter inducible fashion in JB6-Cl30 mouse epidermal cells. A transfection assay for genes which suppress the transformed phenotype will be developed to determine if and how chemical carcinogens inactivate such suppressor genes in malignant transformation.

Molecular mechanisms in multistage carcinogenesis are the object of other studies. These studies analyze basic molecular mechanisms underlying specific stage transitions in multistage epithelial carcinogenesis using three complementary approaches.

Specific receptors for tumor promoting phorbol esters are being isolated from human platelet membranes to elucidate directly the molecular mechanism of tumor promoter-mediated late-stage preneoplastic progression.

The roles of hexose transport and of receptors for epidermal growth factor (EGF) are studied in relation to the mitogenic and transforming effects of tumor promoters on mouse epidermal cell lines. Phorbol ester induced mitogenesis was found to require both enhancement of hexose transport and the presence of EGF receptors while promotion of transformation requires neither.

Alterations in protein kinase activities during tumor promoter-dependent induction of transformation and subsequent promoter-independent maintenance of transformation are studied in a battery of clonal sublines of the JB6 mouse epidermal cell line.

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 carcinogens 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.

In order to use syngeneic animals in studies requiring reimplantation in vivo of organ and cell cultures, 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 non-inbred clean breeding colony, Syrian/CG.FOD, was also established. Normal control studies of cellular characterization in the different segments of the respiratory tract were started, together with colony control studies on spontaneous pathology and tumor occurrence. Intratracheal instillation studies with particulate carriers were undertaken. Studies on different inorganic particulates and different carcinogens are planned in this system, with emphasis on combined effects. Histologic, histochemical, immunochemical and autoradiographic techniques are planned for the characterization of the segmental response of the respiratory epithelium to different carcinogens and their combinations. Biochemical studies of carcinogen metabolism, binding and interactions are also planned. In preparation for this new research project, extensive literature reviews were conducted on carcinogenesis in different segments of the respiratory tract.

Two long-term studies on respiratory carcinogenesis were completed and their pathology evaluated. An unusual finding was the induction by N-methyl-N-nitrosourea, applied topically to hamster tracheas in buffered solution at pH 4.5, of spindle cell carcinomas, previously unreported in respiratory carcinogenesis, which were characterized by electron microscopy.

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 hamster more effectively than by the rat; 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; 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.

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 focused on the response to carcinogens at the cellular level. In cooperation with other components of LEP, a major goal is to investigate the action of carcinogens at all levels of biological organization from the molecular, cellular and tissue levels to the intact animal *in vivo*. The emphasis of this Section will be on carcinogenesis of epithelial cells from important organ sites of human cancer (respiratory tract, prostate, bladder, skin). Both rodent and human culture systems will be employed.

In addition to the development of models and their use in evaluating the activity and mechanism of carcinogens, another function of the Section is to make available to other investigators in the LEP resources, expertise and collaboration in cell culture biology.

Work of the Section was started in partially renovated facilities in September 1981 and in the last several months further renovations were made.

Initial cell culture studies conducted in the Section and in collaboration with other units of LEP included: (1) studies in the BALB 3T3 transformation system; (2) transfection of anchorage-independence in NIH-3T3 from transformed cell lines; (3) studies on the role of deoxyglucose uptake in mitogenesis and promotion by phorbol esters in mouse epidermal cell lines; and (4) studies on metabolism, toxicity and transforming activity of arsenic in mouse epidermis.

Several new projects are currently being initiated:

The establishment of an organ culture system from inbred Syrian hamster bronchial

tissue which preserves tissue relationships, together with a replicative epithelial cell culture derived from the same source, will form a hierarchical bridge between the level of the whole animal and the molecular level. Comparative studies utilizing the in vivo and the two in vitro models will be made to investigate the activity of selected carcinogens at the cell, tissue and whole animal levels. Emphasis will be placed on response to carcinogens at the cell level as measured by altered response to serum and growth factors, loss of anchorage-dependence, and morphological and karyotypic changes. Organ and cell cultures will be established from normal and carcinogen-treated hamsters with the aim of identifying early induced changes and in determining their relative importance in multistage carcinogenesis.

Cultured normal human prostatic and bladder epithelial cells will be treated with carcinogens singly and in combination. The normal prostatic epithelial line NP-2s was previously isolated, characterized and transformed by SV40 virus (Kaighn et al. 1980). Several bladder cultures are also available. 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. DNA preparations from available SV40 transformed prostatic epithelial cells will be tested for their ability to induce anchorage-independence and other changes in growth behavior in the normal prostatic epithelial cells; if positive, this model will be used to investigate chemically induced DNA changes.

An important facet in developing replicative epithelial cell cultures involves the use of serum-free, chemically defined media. Defined, serum-free media will be developed for rodent and human epithelial cells. The requirement for serum protein will be reduced or eliminated by substituting known growth factors and by altering the basic nutrients if necessary. Macromolecular factors still required will be isolated and characterized. Other culture parameters such as cell dissociation methods and the culture dish surface will also be optimized. Cultures will be characterized by morphological, ultrastructural, chromosomal and growth criteria. Two human epithelial cells, NP-2s from neonatal prostate and NB128 from adult bladder, as well as epithelial cells from primary Syrian hamster bronchus cultured and mouse keratinocytes will be used. The efficacy of nutrient medium MCDB 402 in the mouse BALB 3T3 transformation assay will also be assessed. The goal is not only to obtain appropriate growth control of target cells for transformation studies, but to provide a baseline for comparison of the growth response to normal and transformed cells.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 04490-06 LEP

PERIOD COVERED

October 1, 1981 through September 30, 1982.

TITLE OF PROJECT (80 characters or less)

Evaluation and Prevention of Carcinogenic Effects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: 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:

0.3

PROFESSIONAL:

0.1

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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, for the evaluation of risk assessment methods, and for the prevention of cancer hazards in the human population. 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 of carcinogenesis and in vitro models for carcinogenesis studies. Quantitative biological variations among individuals, species and experimental systems indicate the need for maximal qualitative comparability of experimental models with their human pathology counterparts. An approach to carcinogenic risk evaluation was proposed on the basis of studies in a series of biological models correlated with each other in a step-by-step sequence, connecting molecular and cellular levels with the corresponding tissues, organs and organisms, both animal and human.

Objectives: To examine current knowledge in the field of chemical carcinogenesis and related fields; to identify 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 evaluation. Organization of and participation in conferences and workshops. Participation in advisory groups for national and international organizations.

Major Findings: A theoretical model was further developed to account for multiple concurrent causative factors in the etiology of cancer in human populations; this model accounts for the following factors: genetic and nutritional factors in all cancers; exposure to carcinogens through diet and through inhalation and exposure to radiations, present in all cancers but effective only in a fraction; exposure to occupational and some other factors present only in a fraction of cases, and effective in a smaller fraction; unknown factors are present in all cases. The composite sum adds up to at least 600-700%, but further fractionation can be considered. Even small increments in any one factor may become determinants of cancer development if the other factors are approaching a permissive set of conditions. Applications of this model to experimental systems are studied.

In order to correlate mechanisms of carcinogenesis, investigated at the cellular and molecular level, with the corresponding events in tissues and organs in animal and human tissues, an approach was outlined that consists of the study of interactions of carcinogens in a series of biological systems of increasing complexity, but related to each other in a step-by-step sequence.

A systematic investigative approach has become possible 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 that has been partly developed in the previous program of LEP and will be further extended in its new 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 counterparts; (c) organ explant culture systems for target epithelia with the possible outgrowth of epithelial cell lines from animal and human sources; (d) mammalian epithelial cell systems for neoplastic transformation in embryo or newborn cells or cell lines; (e) mammalian cell mutagenesis systems; (f) mammalian cell systems for the analysis of DNA damage and repair and other cytotoxic effects; (g) bacterial mutagenesis systems; (h) systems for the analysis of specific molecular targets, such as specific membrane receptors, protein kinases, and their expression in mammalian cell transformation.

Responses to carcinogens in several of these systems, as studied in other projects

of LEP, are evaluated for comparability and for relevance to risk assessment procedures.

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. The experience obtained by analyzing and coordinating different methodological approaches to the study of the carcinogenic process--at the human, animal, cellular and molecular level--provides a strong basis for identifying specific criteria and priorities for the evaluation of carcinogens and their risk assessment and for further research.

Proposed Course: Continuation of these activities, with emphasis on research approaches.

Publications:

Saffiotti, U.: Occupational carcinogens in relation to the multifactorial origin of cancer: Experimental pathology approaches. In Prevention of Occupational Cancer - International Symposium, Occupational Safety and Health Series No.46, I.L.O., Geneva, 1982, p. 17-25.

Saffiotti, U.: The search for criteria to assess the risks resulting from carcinogenic agents. In Castellani, A., and Setlow, R.B. (Eds.): The Use of Human Cells for the Assessment of Risk from Physical and Chemical Agents. Plenum Press. (In press).

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., and Setlow, R.B. (Eds.): The Use of Human Cells for the Assessment of Risk from Physical and Chemical Agents. Plenum Press. (In press).

Saffiotti, U.: Evaluation of mixed exposures to carcinogens and correlations of in vivo and in vitro systems. In Biological Tests in the Evaluation of Mutagenicity and Carcinogenicity of Air Pollutants. Environ. Health Perspect. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 04491-06 LEP

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Quantitative Studies on Concurrent Exposures to Numerous Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Umberto Saffiotti	Chief	LEP	NCI
OTHERS:	M. Edward Kaighn	Expert	LEP	NCI
	Enrico Cortesi	Visiting Fellow	LEP	NCI
	Corrado Ficorella	Visiting Fellow	LEP	NCI
	Margherita Bignami	Guest Researcher	LEP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

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INSTITUTE AND LOCATION

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TOTAL MANYEARS:

1.6

PROFESSIONAL:

1.4

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Experimental studies are conducted on concurrent exposure of appropriate biological targets to numerous carcinogens of different classes, each administered at doses which would be expected to produce low or undetectable effects in the chosen system. Mechanisms of action are studied to determine the most effective conditions of synergism. The biological models selected for these studies include in vitro assays for mutagenesis and neoplastic cell transformation and whole animal models. Analysis of dose-response relationships in carcinogenesis is conducted on data collected in a comprehensive literature survey, and on dose-response results obtained experimentally in the selected biological systems. In mutagenesis tests, mutual inhibition was found for polycyclic aromatic hydrocarbons, additive effects among aromatic amines and mutual synergism for benzo[a]pyrene, benzidine, aflatoxin B-1 and safrole.

Objectives: To determine whether a marked carcinogenic effect can result from concurrent exposure to numerous different carcinogens, each administered at doses which would be expected to produce low or undetectable effects in the chosen target system; and to analyze the mechanisms of such interactive effects and of dose-response relationships in carcinogenesis.

Methods Employed: Biological model systems include the Salmonella mutation assay (Ames) and the BALB/c 3T3 clone A31-1-1 mouse embryo cell line in culture for mutagenesis and neoplastic transformation. Quantitative exposure protocols were used for graded doses of individual carcinogens and their combinations (up to 8 compounds together so far) and for fractionated doses of carcinogen. The results were analyzed quantitatively.

I. Salmonella mutagenesis: New experiments were initiated utilizing the resources of the Microbial Mutagenesis Laboratory at FCRF. Selected resource tests were made to confirm the conditions of synergism previously determined with polycyclic aromatic hydrocarbons, aflatoxin and benzidine. Other carcinogen combinations are under study.

II. Neoplastic transformation: The BALB/c 3T3 clone A31-1-1 embryo cell line system was reestablished under new tissue culture conditions. Culture methods were standardized for reproducibility. The neoplastic transformation system was first set up following the original protocol (Kakunaga, *Intl. J. Cancer* 12:463-473, 1973) with minor modifications; these culture conditions and media (MEM with 10% fetal bovine serum) are being reevaluated in an attempt to identify suitable chemically defined media for transformation assays. Current standards for toxicity determinations (200 cells/dish, 8 days) and neoplastic transformation (1×10^4 cells/dish, 5 weeks) are being reevaluated. Characteristics of type III foci, used for scoring of transformation, and Type II foci are being reexamined. A protocol for concurrent toxicity, mutagenesis (ouabain-resistance) and neoplastic transformation studies on the same treated cell population is being developed. This system is also used for studies of cell density effects in transformation. New culture procedures are investigated for these purposes, particularly the use of chemically defined media and of milder cell passaging techniques.

Major Findings: I. Salmonella mutagenesis. In previous years, mutual inhibition was demonstrated for a mixture of 5 polycyclic aromatic hydrocarbons as well as for each individual combination of components; mutual synergism was demonstrated for a group of compounds belonging to different chemical classes; benzo[a]pyrene (BP), aflatoxin B₁ (AFB), benzidine (BZ) and safrole (SF), and their individual combinations, while ethylenethiourea was inactive. Synergism occurred at low levels of exposure where toxicity is minimal for all compounds. Another class of compounds, the aromatic amines, was studied because of a) data on carcinogenic effects in humans and in animals, b) the synergistic effects of benzidine mentioned above, and c) the recent findings of high carcinogenic activity for benzidine dyes and their metabolic breakdown to several free aromatic amines. Tests in TA 98 and TA 100 for benzidine (BZ), 3,3'-dichlorobenzidine (DCB), 4-amino-biphenyl (4-AB), 2-naphthylamine (2-NA), 1-naphthylamine (1-NA), o-dianisidine (OD), o-tolidine (OT), and aniline (AN), and for their equimolar combinations gave different results, including mixture of the 8 compounds (resulting in inhibition in both strains), mixture of the 3 most active compounds, DCB, 4-AB and 2-NA (inhibitory in TA 100 and additive in TA 98) and permutations of 2 compounds at a time; most of these showed approximate additive effects, a few were

inhibitory (4-AB + OD, in both strains), while the combination BZ + 4-AB resulted in synergism in both strains. Representative points from these studies are being retested under better standardized test conditions at FCRF and the data reevaluated for publication. Additional groups of compounds are under study.

II. Neoplastic transformation in BALB/c 3T3 A31-1-1 cells. These studies have been continued and the culture methods are under reevaluation. Dose-response relationships for cytotoxicity and transformation were determined for the following carcinogens: the direct-acting N-methyl-N'-nitro-N-nitrosoguanidine, (MNNG), as well as BP, 3-methylcholanthrene (MCA), AFB and BZ which require different type of metabolic activation. The relative transforming activity on a molar basis was found to be in the following order: BP>AFB>MCA=MNNG>BZ. Good quantitative reproducibility was obtained in several replicate tests. Optimal duration of exposure was determined. Studies on combined effects in this transformation system have shown synergism of several combinations of carcinogens in cytotoxicity, while preliminary results of transformation assays also show synergism under certain dosage conditions for certain combinations of carcinogens.

III. Studies on cell density in the expression of transformation. A new study was undertaken to determine the dose-response effects of carcinogen exposures in the expression of transformation obtained after replating exposed cells at different cell densities. Kennedy and Little (1980) reported that cultures of the C3H/10T1/2 mouse fibroblast cell line, exposed to a transforming dose of X-radiation, grown to near confluence and then replated at widely different densities, show a nearly constant rate of transformed foci per plate. These studies suggest that nearly all carcinogen-exposed cells behave as potentially transformed cells but that the expression of transformation is inversely proportional to the number of cells replated. A possible explanation is that cells inhibit the expression of transformation in other cells. This behavior is being studied in BALB/c 3T3 clone A31-1-1 cells for chemically induced transformation. An initial study unfortunately showed that trypsinization of the treated cells at 48 hours results in very poor plating efficiency impairing the further conduct of the study. New gentler procedures are being developed to resuspend the cells after the initial plating and treatment; such procedures should make it possible to replat the same treated cell population at different densities and to test concurrently for cytotoxicity, mutagenicity and transformation.

Significance to Biomedical Research and the Program of the Institute: Multiple concurrent exposures to many different carcinogens represent the actual conditions under which the human population is exposed to carcinogens. From prenatal life through childhood and adult life people are exposed to a large number, probably in the hundreds, of environmental carcinogens from different routes. Such "realistic" conditions of exposure have never been reproduced experimentally so far. The definition of the effect of this background low level exposure to individual carcinogens, acting together with specific exposures to many others, is expected to provide information on the mechanisms of carcinogenesis in tissues exposed to multiple hits by different kinds of agents. The role of multiple background exposures will also be studied. Wide-ranging implications can be projected for the understanding of basic chemical biological interactions in carcinogenesis, for mathematical models for dose/response extrapolation and for the evaluation of carcinogenic hazards in public health policies.

Proposed Course: It is proposed to extend these studies in biological systems for mutagenesis, cell transformation and in vivo carcinogenesis, using additional types of carcinogens; mechanism studies will be further developed to elucidate the bases of interactions and their relation to the human response. Current work (Z01 CP 05276-01 LEP), aimed at the establishment and definition of a transformation system based on transfection with DNA from chemically treated cells, is expected to provide a new molecular model for studies on combined effects of carcinogens that may elucidate the basis of the interactions.

Publications

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 B₁, benzidine, benzo[a]pyrene, 3-methylcholanthrene and N-methyl-N'-Nitro-N-nitrosoguanidine. Teratogenesis, Carcinogenesis and Mutagenesis. (In press).

CONTRACT IN SUPPORT OF THIS PROJECT

MICROBIOLOGICAL ASSOCIATES, Contract # N01-CP95637-58

Title: Biochemistry and Cell Culture Resource
Project C: BALB 3T3/c Transformation

Objectives: Propagation, maintenance and storage of BALB 3T3 cells; screening of bovine serum lots; selection of target subclones; cytotoxicity and transformation assays with BALB/c 3T3 cells.

Methods Employed: Procedures as originally described by Kakunaga (1973), modified as needed by agreement with NCI staff.

Major Findings: 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 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 MNN: subclone A31-1-1C was the cell line of choice and was used for all subsequent work. Standard tests were carried out with different compounds at different doses and for different durations of exposure. These findings, together with those obtained in the LEP intramural project, established the baseline for subsequent studies with combined exposures to different carcinogens. These studies are currently underway; preliminary results indicate that synergistic effects occur with certain combinations of exposures.

Proposed Course: Completion of the current studies (contract expires on 2/28/83)

Current Annual Level: \$100,000 from LEP (3/1/82-2/23/83)

Man Years: .44 for LEP (3/1/82-2/23/83)

PERIOD COVERED
 April 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
 Effects of Chemical Carcinogens on Transforming DNA Sequences and Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomas D. Gindhart	Expert	LEP	NCI
OTHERS:	Margherita Bignami	Guest Researcher	LEP	NCI
	Umberto Saffiotti	Chief	LEP	NCI
	M. Edward Kaighn	Expert	LEP	NCI
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INSTITUTE AND LOCATION
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TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0.0
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(a) HUMAN SUBJECTS
 (b) HUMAN TISSUES
 (c) NEITHER

(a1) MINORS
 (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project seeks to determine whether chemical carcinogens interacting differently with DNA induce the same or different agent-specific changes in DNA, necessary for malignant transformation of mammalian cells. DNA's of BALB 3T3 cells, transformed by carcinogens of at least three different classes, are transfected into NIH 3T3 cells and mouse epidermal cells permissive for expression of the transformed phenotype. Transforming DNA's produced by each agent will be compared according to efficiency of expression in permissive indicator cells and patterns of sensitivity to restriction endonucleases. Other studies investigate whether apparently recessive suppressor genes are inactivated early in multistage carcinogenesis in order to permit expression of transforming genes. Cell culture and biochemical facilities have been established. Six MNNG-transformed BALB 3T3 cell lines were isolated. BP and 4NQO-transformed BALB 3T3 cell lines were obtained from T. Kakunaga. Successful transfection of NIH 3T3 cells was confirmed under present laboratory conditions using cloned Moloney sarcoma virus transforming genes. Development of a chemically defined culture medium was initiated to improve reproducibility and efficiency of transfection assays.

Objectives: This project includes two major approaches. Part A: Characterization and comparison of carcinogen-activated transforming genes. 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 tumor lines. Transforming genes of human and rodent origin can now be "fingerprinted" according to their restriction endonucleases pattern (RFP) of sensitivity to inactivation in transfection assays. Little evidence, however, has been obtained as to whether different carcinogenic agents activate identical or different transforming genes in the same cells. The observation that one cell line, NIH 3T3 fibroblasts, has been transformed by at least 14 different transforming DNA's in transfection experiments indicates that a multiplicity of final-step transforming genes could possibly occur and be expressed in a single cell type. Although it was reported that methylcholanthrene activated the same transforming DNA sequence in C3H/10T1/2 cells exposed to the agent in several independent experiments, the DNA's of two out of the five transformed lines could be functionally distinguished by their inability to transform indicator NIH 3T3 cells upon transfection (Shilo and Weinberg, 1981). In a collection of one human and six rodent mammary carcinomas the same transfectable transforming DNA was found in the human tumor induced by unknown agents and in the rodent tumors induced by either DMBA or MMTV, indicating that different agents could all activate the same transforming gene in cells of the same type regardless of mechanism of action (Cooper et al., 1982). 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 carcinogens of different chemical classes in one cell type.

Specific objectives are: 1) to determine whether carcinogens known to damage DNA in very 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 combination; 3) to determine whether transforming genes activated by sequential treatment with carcinogens and tumor promoting agents are the same as those activated by carcinogens alone or in combination; and 4) to identify specific alterations in DNA affecting expression of transforming genes.

Part B: Identification and characterization of onc-suppressor genes. Another important question in chemical carcinogenesis concerns the number and nature of apparently recessive suppressor genes whose inactivation early in multistage carcinogenesis permits expression of the final transforming genes detected in DMGT studies.

The recent DNA transfection experiments have shown that pure DNA fragments can bring about completion of transformation in certain cell lines permissive for expression of transforming DNA sequences isolated from tumor cell lines. Even though transforming DNA can be transfected into many cell lines other than transformation-indicator cell lines, its transforming activity appears suppressed in most transfectable cell lines. Recent cell fusion studies have clearly shown that expression of the transformed phenotype can be suppressed by the genes of normal cells. Only continuous cell lines which show a partially transformed phenotype such as NIH 3T3,

JB6-C130 and CHEF 18 cells have been found to allow expression of "dominantly acting" transforming genes in DNA transfection experiments. Since similar demonstrations have not been made with unmanipulated normal cells, these transforming DNA sequences appear to act at a late stage in multistage carcinogenesis.

Further evidence suggests that certain genes must be inactivated before expression of the transformed phenotype can occur: 1) most carcinogenic agents damage primary DNA and alter secondary DNA structure and 2) the transformed phenotype is characterized by extensive loss of genetic markers and normal regulatory processes. Thus, although carcinogens could inactivate more genes than they activate, only one or a few genes might need to be activated for expression of the transformed phenotype as measured in current transfection assays in selected susceptible cells, such as NIH 3T3 cells. Specific objectives of this project are to identify and characterize DNA sequences which can suppress the transformed phenotype.

Methods Employed: Part A: BALB 3T3 clone A31-1-1 cells have been transformed in this laboratory by several different carcinogenes. Multiple independently derived transformed cell lines are either available or being developed. High molecular weight DNA from each transformed line will be extracted and transfected into NIH 3T3 cells by the calcium phosphate-DNA precipitation technique and will be evaluated for the ability to induce type III foci in NIH 3T3 cells. DNA's which reproducibly transform the indicator cells will then be typed according to their pattern of inactivation by restriction endonucleases. DNA's will also be evaluated for their ability to induce promoter-dependent transformation of the mouse epidermal cell line JB6-C130.

Part B: DNA from normal or known preneoplastic cells will be transfected into an indicator tumor cell line which grows in Spinner suspension culture and is capable of reverting to an anchorage-dependent, nontumorigenic phenotype. This will provide a sensitive, specific system for testing DNA's for the ability to suppress the transformed phenotype. Cotransfection with the Ecogpt dominant selectable marker gene and subsequent coselection for cells which grow in HAT + mycophenolic acid medium with a nontransformed phenotype should increase the efficiency of such a selection procedure by 10^3 - 10^5 fold. This step should also allow distinction of transfection-induced cells which suppress the transformed phenotype from spontaneously arising transformation-defective variants. Procedures exist for selecting more highly transfectable sublines from cell lines which are not initially good recipients in DNA transfection assays (Pearson 1981).

We plan to use indicator cells which have already been shown to revert upon fusion with normal, nontransformed cells such as H. Harris's A9HT subline of L-cells, PG19 melanoma cells, E. Stanbridges' Hela cells, or osteosarcoma cells. Cell lines which grow in Spinner culture and are capable of reversion to anchorage-dependent growth under Spinner conditions, such as Kaighn's DU 145 line, will also be studied as potential indicator cells in this assay.

Major Findings: This is a new project and no findings are yet available for reporting.

Significance to Biomedical Research and the Program of the Institute: If all agents activate the same transforming gene in the same cell, then the preexisting phenotype of the target cell critically determines the transformation response to

carcinogens in general and identity of the agent itself is relatively unimportant; only one transforming gene may exist for BALB 3T3 cells but carcinogens may activate it by different molecular mechanisms; combinations of transforming agents can 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 may occur only at the level of procarcinogen metabolism rather than at the DNA level. If each agent activates different transforming genes in the same cell, then clearly different target transforming DNA sequences probably 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.

Onc-suppressor genes probably need to be inactivated in early stages of multistage carcinogenesis. Detection of this kind of activity should have greater significance in the study of early lesions in cancer in terms of cancer cause and prevention, risk assessment of individuals and early diagnosis of premalignant lesions.

Proposed Course: If DNA's from transformed lines induced by different carcinogens all show the same restriction endonuclease pattern, then the project will seek to determine how and why agents which alter DNA in different fashions can nevertheless activate the same transforming gene in the same cell. On the other hand if the DNA's show different carcinogen-dependent restriction endonuclease patterns, then the project will seek to determine whether DNA's from transformed lines induced by combinations of carcinogens show restriction patterns characteristic of one agent only, a mixture of patterns or a new pattern.

The role of permissive changes in indicator cells will be determined by transfection of DNA's into different recipient cells, particularly the JB6-C130 mouse epidermal cell line which allows conditional induction of the transformed phenotype in the presence of tumor promoters. Isolation of and structural analysis of carcinogen-activated transforming DNA sequences and inactivated onc-suppressor genes by selective gene cloning techniques are long-term goals of this project. The molecular changes induced by carcinogens in these functionally defined DNA sequences should link structural changes in DNA induced by carcinogens to functional changes in cell behavior.

Publications: None.

PERIOD COVERED

October 1, 1981 through September 30, 1982.

TITLE OF PROJECT (80 characters or less)

Molecular Mechanisms in Multistage Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomas D. Gindhart	Expert	LEP	NCI
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	Umberto Saffiotti	Chief	LEP	NCI
	Nancy H. Colburn	Expert	LVC	NCI

COOPERATING UNITS (if any)

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TOTAL MANYEARS:

1.7

PROFESSIONAL:

1.5

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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. a) Receptors for tumor promoting phorbol esters (e.g., TPA) were characterized in normal and malignant human hematopoietic cells. Presently these molecules are being isolated from human platelet membranes. b) 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 postinitiated mouse epidermal cell lines. Clonal variants which undergo a mitogenic response to 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. Mitogenic effects of tumor promoters, which are likely to mediate early stages of preneoplastic progression in vivo, appear to depend on enhancement of hexose uptake and of EGF receptors. c) Alterations in several protein kinase activities associated with malignant transformation will be studied in epithelial cells through different stages of preneoplastic progression.

Project Description

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 models utilized are the mouse epidermal cell system and the hamster respiratory epithelial system; other aspects of both models are studied in other projects of this laboratory. 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 three specific parts: A) Isolation and characterization of cell membrane receptors for tumor promoting phorbol esters from the simplest known human source, platelet membranes; B) determination of the roles of altered hexose transport and EGF receptors in the mitogenic and final transformation responses to tumor promoters and in mouse epidermal cell lines; and c) evaluation of changes in protein kinase (PK) activities as epithelial cells pass through defined stages of preneoplastic progression.

Methods Employed: Part A) The human platelet is the starting material of choice for isolation of phorbol ester receptors because it is the simplest human source known and possesses between 500 and 5,000 receptors for ^3H -phorbol dibutyrate (^3H -PDBU) per platelet. The majority of platelet membrane integral proteins are already known which should facilitate identification of the physiologic system to which the receptor belongs once a protein has been found which specifically binds phorbol esters. Plasma membrane preparations free of nuclear material, mitochondria, microsomes, specific hormone granules and cytosolic proteins have been prepared and their constituents characterized by SDS polyacrylamide gel electrophoresis. Octylglucoside releases nearly 100% of platelet membrane proteins and can be removed by dialysis to allow subsequent binding studies. An assay for specific binding of phorbol esters by solubilized proteins is being developed using dextran coated charcoal to remove unbound ^3H -PDBu.

Part B) To study the roles of hexose transport and EGF receptors in preneoplastic progression clonal derivatives of the post-initiated JB-6 mouse epidermal cell line are used. The effect of TPA on the rate of ^3H -2 deoxy-D-glucose uptake was determined for eight clonal sublines among which the ability to respond to TPA with cell division at plateau density is clearly dissociated from the ability to respond to TPA with promotion of transformation. EGF receptors were measured on intact cells with ^{125}I -EGF.

Part C) Initially the same battery of clonal variants of mouse epidermal cells plus their transformed counterparts are being labeled with ^{32}P -orthophosphate and the phosphoprotein patterns of whole cell lysates analyzed by one and two dimensional gel electrophoresis. Bands are being sought corresponding to EGF receptors, Vinculin, pp60 src, the 50,000mw unit of Na^+/K^+ ATPase, major excreted

protein, pp21 HSV and several known substrates of viral transforming genes and activated growth factor receptors.

Multiple changes in phosphoproteins (pp) are a regular feature of tumors cells, but those changes that are causally important need to be distinguished from those that are secondary.

By analyzing pp changes in just a single step of a multistage sequence, the total number of changes will be minimized and the essential/nonessential ratio maximized. Tumor promoters are known to induce several PK and pp changes in a variety of cell types. By analyzing changes induced by a single, well studied promoting agent in matched pairs of cell lines, which are known to differ in their transformation response to that agent, irrelevant or secondary pp changes can be identified (those which occur in promotion insensitive cell lines) and separated from the relevant pp changes (those which occur only in the promotion sensitive cell lines).

Major Findings: Part A) Specific binding of ^3H -PDBu by membrane proteins separated by polyacrylamide gel electrophoresis has been measured directly in the gels.

Part B) None of eight cell lines showed the decrease in hexose transport at confluency that occurs in mouse 3T3 cells and chick embryonic fibroblasts. Variants with both a mitogenic and promotion response to TPA (M^+P^+) show two-fold enhancement of 2-DG uptake in both log phase growth and at plateau. Variants defective in the promotion response, but still showing the mitogenic response to TPA (M^+P^-), increase 2-DG uptake in response to TPA while in log phase growth but not at plateau. 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 either log or plateau phases of growth. Cell growth is normal in medium containing 10% and 1% of the normal concentrations of glucose, but the mitogenic response to TPA is abolished. EGF receptors are present on 5/5 mitogenically responsive lines showing TPA enhancement of 2-DG uptake and undetectable on 4/4 lines not showing these responses. Growing M^+P^- cells to confluency does not affect EGF receptors even though enhancement of 2-DG uptake by TPA no longer occurs.

Lines resistant to mitogenic stimulation by TPA 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 passage but at a low rate.

Part C) New project: no findings to report to date.

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 indicate possible manipulations of these mechanisms towards cancer prevention and risk assessment as well as possible treatment.

Isolation of the phorbol ester receptor should lead to identification of the other components of the physiologic system which is the primary cellular target

of tumor promoting agents in late multistage carcinogenesis. 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. Isolation and characterization of the phorbol ester receptor should lead to identification of the physiologic system perturbed by phorbol esters and thus provide direct, unambiguous information on the role of this physiologic system in transformation; it should also facilitate identification of the endogenous ligands of this receptor.

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 mitogenic response of mouse epidermal cells to TPA in vitro requires both stimulation of hexose uptake and receptors for EGF. 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.

Protein kinases (PK's) as a class are regulatory enzymes governing the important steps in biochemical pathways wherever they are found. Phosphorylation of tyrosine residues appears to be particularly associated with transformation by the transforming genes of type C viruses and probably their cellular homologues.

Identification of a change in activity of a specific protein kinase associated with either TPA induction or subsequent TPA-independent maintenance of the transformed phenotype in epithelial cells should lead to a far more detailed description of the biochemical reactions underlying these events.

Proposed Course: Solubilized platelet membrane proteins showing specific 3H -PDBu binding in polyacrylamide gels will be eluted and further characterized biochemically. The hypothesis that EGF receptors mediate TPA-stimulated cell division and hexose transport will be tested by reconstituting EGF receptorless M^- cells with EGF receptor containing mouse liver membranes.

Since total cellular pp's may not reflect changes in key regulator subpopulations, several approaches will be applied to study specific PK and pp subpopulations.

Publications:

Colburn, N.H. and Gindhart, T.D.: Specific binding of transforming growth factor correlates with promotion of anchorage independence in EGF receptorless JB6 cells. Biochem. Biophys. Res. Comm. 102: 799-807, 1981.

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 Research 1982. (In press).

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, 1982. (In press).

Seaman, W.E., Gindhart, T.D., Blackman, M.A., Dalal, B.I., Talal, N. and Werb, Z.: Suppression of natural killing by monocytes and leukocytes: requirement for reactive metabolites of oxygen. J. Clin. Invest. 69: 876-888, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05274-01 LEP

PERIOD COVERED

October 1, 1981 through September 30, 1982.

TITLE OF PROJECT (80 characters or less)

Respiratory Carcinogenesis by Chemical and Physical Factors in the Hamster Model

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Umberto Saffiotti Chief LEP NCI

OTHER: Sherman F. Stinson Biologist 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:

1.4

PROFESSIONAL:

1.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on the mechanisms of bronchogenic carcinoma induction by chemical and physical factors, alone or in various combinations of factors, 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 cofactors. Respiratory epithelial tissue responses are characterized by histological, histochemical and biochemical methods, and by their study in organ and cell culture conditions. Long-term studies were completed on the topical effects of N-methyl-N-nitrosourea which resulted in the unusual induction of spindle-cell carcinomas and on 2,6-dimethyl-nitrosomorpholine which induced a variety of tumor types located in all segments of the respiratory tract.

Project Description

Objectives: The main objective of this project is the elucidation of the mechanisms by which bronchogenic 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 particle (Saffiotti, et al., 1964, 1968). In subsequent years the hamster model was extensively studied both in intramural and in collaborative research. Cell kinetics and cellular responses to carcinogens and cofactors were well characterized and shown to be closely similar in the hamster model and in the human counterpart. The cells of origin were identified for bronchogenic carcinomas with various types of differentiation (squamous, mucous, undifferentiated) and differentiation markers were found to shift between basal cells, intermediate cells, mucous granule cells and keratin containing cells. Still lacking is an adequate induction model to elucidate the origin and pathogenesis of small cell undifferentiated carcinomas.

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 different carcinogens and different particulates having different physical characteristics; their pathogenetic mechanisms remain to be further investigated.

Pathogenetic studies of this model received new emphasis with their use in investigating the interaction of carcinogens with target-cells (reviewed by Saffiotti and Harris, 1979). An important development was the establishment of methods for the explant culture of human respiratory epithelia which opened the way to the experimental investigation of the response of human target tissues to carcinogens. This work, which took increasing emphasis in the LEP program in Bethesda in its Human Tissues Studies Section headed by C.C. Harris, has now become the focus of Dr. Harris' recently established Laboratory of Human Carcinogenesis.

With the present new project, LEP resumes work on the experimental animal model *in vivo* and *in vitro*, with emphasis on the study of combined effects of different carcinogens and cofactors. The specific objectives of this project include the following *in vivo* and *in vitro* 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; 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 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; and study of the conditions and mechanisms of synergistic effects of carcinogens in the respiratory tract.

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. Further in vitro studies will be pursued jointly with project No. Z01-CP-05277-01 "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 epithelium by histologic, histochemical, autoradiographic and immunochemical methods and by scanning and transmission electronmicroscopy. Epithelial tissue isolation, fractionation and use for biochemical analysis or for tissue culture. Biochemical analysis of carcinogen localization, metabolism and binding. Carcinogens to be studied include polycyclic aromatic hydrocarbons, N-nitroso compounds, aflatoxin, arsenic (see Project No. Z01-CP-05275-01 "Arsenic metabolism and carcinogenesis"); others will be selected on the basis of preliminary studies. 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. Selection of optimal culture methods for explants from trachea, main bronchi and smaller bronchi and for corresponding cell cultures.

Major findings: The two hamster colonies have been established and animal production has started. Initial groups of experimental animals are under treatment and observation. This new project has been underway for an insufficient period of time for a report of findings.

The literature and personal experience on the role of polycyclic aromatic hydrocarbons in respiratory carcinogenesis, and on various environmental agents in upper respiratory tract carcinogenesis, were reviewed for publication and evaluation as background for new experimental plans.

Long-term in vivo studies were completed to evaluate the respiratory carcinogenic effects of two N-nitroso compounds: a) N-methyl-N-nitrosourea, 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 and adenocarcinomas of bronchiolo-alveolar origin and also adenomas, papillomas and carcinomas of the upper respiratory tract (nasal cavities, larynx and trachea).

Both of these agents showed a sensitivity to factors known to influence differentiation in epithelial carcinogenesis, such as administration of retinoids. Comparative studies on the effects of N-nitroso compounds when given topically or systemically are continuing.

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 and risk to the multiple exposures that concur to lung cancer causation. This project is expected to contribute basic knowledge in the poorly explored field of mechanisms of synergistic effects in epithelial carcinogenesis and to provide an experimental pathology basis to develop optimal culture conditions towards the selection of a cellular model of neoplastic transformation of epithelial cells.

Proposed Course: This new project is designed as a long-term study of several years' duration. It is expected to become 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 G. Reznik and S.F. Stinson (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 G. Reznik and S.F. Stinson (Eds.): Comparative Nasal and Nasopharyngeal Carcinogenesis, Boca Raton, CRC Press. (In press).

Stinson, S.F., Reznik, G., and Donahoe, R.: Effect of three retinoids on tracheal carcinogenesis with N-methyl-N-nitrosourea in hamsters. J. Natl. Cancer Inst. 66: 947-951, 1981.

Stinson, S.F. and Saffiotti, U.: Experimental respiratory carcinogenesis with polycyclic aromatic hydrocarbons. In H.M. Reznik-Schüller (Ed.): Comparative Respiratory Carcinogenesis, Boca Raton, CRC Press. (In press).

PERIOD COVERED

December 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Arsenic Metabolism and Carcinogenesis Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Umberto Saffiotti	Chief	LEP	NCI
OTHER:	Federico Bertolero	Visiting Fellow	LEP	NCI

COOPERATING UNITS (if any)

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Laboratory of Experimental Pathology

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TOTAL MANYEARS:

0.7

PROFESSIONAL:

0.6

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Arsenic (As) carcinogenesis is a unique example of discrepancy between positive human findings and negative animal tests. Two hypotheses are investigated 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 a cocarcinogenic interaction. Preliminary findings show that the hamster is able to methylate inorganic arsenic in vivo. This biotransformation is similar to that in humans and considerably more effective than in the rat, supporting the selection of the hamster as a more appropriate species for metabolic studies and carcinogenesis tests. Carcinogenesis studies are planned on combinations of As with other compounds. Because of the complexity of these studies in vivo, epithelial culture models are selected in which to investigate arsenic metabolism and its interaction with other compounds. Conditions needed for arsenic methylation and the effect of arsenic on epidermal cell differentiation and keratin synthesis in culture are studied. Primary mouse epidermal cell cultures are used and primary hamster epidermal cell and bronchial epithelial cell culture systems are considered for future use. In vivo carcinogenesis protocols will be developed according to the information obtained from in vivo and in vitro metabolic studies.

Project Description

Objectives: Studies are aimed at the development of combined *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. Since 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 nonmelanotic skin cancer following exposure to inorganic arsenic. Animal experiments, however, have so far resulted in negative findings. This discrepancy can be due to several possible reasons. Two main hypotheses will be investigated in the present project: (a) species-specified 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.

The first problem concerns species differences in arsenic metabolism. Recent studies have shown a significantly different metabolism of inorganic arsenic in rats compared with human and other mammals. (Marafante, E., Bertolero, F., Edel, J., Pietra, R. and Sabbioni, E.: Sci. Tot. Environ. 23, 1982, in press). The major species differences are in the different degrees of biotransformation which arsenic undergoes in each species. Once absorbed, inorganic arsenic is methylated *in vivo* through pathways which have not yet been identified. The methylated metabolites were shown to have a very low affinity for macromolecules and to be rapidly excreted. Arsenic methylation is therefore regarded as a detoxification process although its implications for other pathways of cellular metabolism are completely unknown. A further aspect of arsenic metabolism, which may have major implications in carcinogenesis, is the observation that inorganic pentavalent arsenic can be reduced *in vivo* to the more toxic trivalent form. (Ginsburg, J.M.: Am. J. Physiol. 208: 832-840, 1965). This study in dogs was never confirmed, mainly because of analytical difficulties in the separation of the two forms from biological samples. 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; the *in vivo* reduction of pentavalent to trivalent arsenic would significantly limit this assumption. New HPLC methods are now available for the speciation of arsenic compounds and will be used to elucidate this important problem.

The second hypotheses suggested to interpret the epidemiological data is that arsenic might act as a cocarcinogen in combination with other agents present in the work environment. Very limited experimental data, mostly negative, are available so far from animal studies but they were not supported by analysis of metabolic mechanisms.

Carcinogenicity studies are now planned on combinations of arsenic with one or more other compounds selected on the basis of the effects of these compounds on the process of arsenic metabolism or on its biological activity.

Because these *in vivo* processes are complex and difficult to study, the present project is attempting to select defined epithelial culture models in which to undertake metabolic and carcinogenic studies on arsenic compounds and their interaction. Initial studies were conducted using primary cultures of mouse epidermocytes. Other epithelial cell culture models, relevant to *in vivo* studies are considered for further studies; in particular, primary hamster epidermal cell and bronchial epithelial cell culture systems. The mouse model was selected initially for the following reasons: a) primary keratinocytes of different rodents can be grown in culture and can be chemically transformed under controlled conditions, as shown by S.H. Yuspa and coworkers in previous programs of 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 in human studies, occurs in tissues capable of keratinizing, i.e., epidermis and respiratory epithelium.

Methods Employed: I - 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: a) ion-exchange chromatography on AG50 resin that allows the separation of inorganic arsenic (As III and As V) from the monomethylated and the dimethylated metabolites; b) HPLC separation on a C18 reversed-phased column that improves the speciation by permitting the separation of all four arsenic metabolites with a single elution (Brinckman, F.E., et al., *J. Chromat.* 191: 31-46, 1980). C) Detection and quantification of As metabolites by gamma-counting, employing As-74 and As-73 labeled compounds in all metabolic studies.

II - In vivo metabolic studies. Arsenic metabolism is studied in the syrian golden hamster by different routes of administration.

III - Cell culture studies. Primary epidermal basal cell cultures from newborn BALB/c mice are selectively cultivated in low calcium medium and then induced to differentiate by medium containing 1.2 mM ionic Ca. (Hennings, et al. *m Cell* 19: 245-254, 1980). This *in vitro* model is used to investigate both the metabolism and the transforming activity of As compounds in epithelial cells.

Major findings: This new project was started too recently for a report of significant results. Preliminary results indicate that: a) inorganic arsenic is methylated *in vivo* by the hamster, more effectively than by the rat; b) inorganic As, incubated *in vitro* with human keratin, was found to bind to this group of proteins; c) trivalent As was found to be considerably more cytotoxic than pentavalent As for primary mouse epidermal cell cultures; and d) trivalent As was also found more cytotoxic than pentavalent As for BALB 3T3 clone A31-1-1 cells in culture, but these cells were in general much more resistant than the epidermal cells.

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 and provide a basis for risk evaluation and prevention.

Proposed Course: Initial effort will be devoted to developing reliable qualitative and quantitative analytical methods for arsenic metabolites in biological samples. Studies are planned on the pharmacokinetics, the tissue distribution and the biotransformation of arsenic in the Syrian golden hamster. Particular attention will be devoted to the metabolism of arsenic in lung and skin and to the identification of compounds that interfere with arsenic biotransformation in these tissues. In vivo carcinogenesis protocols will be developed accordingly. In vitro studies will investigate the conditions needed for arsenic methylation by primary mouse epidermal cells in culture and the effects of arsenic on epidermal cell differentiation in culture and particularly on keratin synthesis.

Publications:

Bertolero, F. and Litterst, C.L.: Changes in renal handling of platinum in cisplatin-treated rats following induction of metabolic acidosis or aldalosis. Res. Com. Chem. Pathol. Pharmacol. 36(2): 273-285, 1982.

PERIOD COVERED

March 1, 1982 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Growth Control in Epithelial Cells and its Alteration in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Edward Kaighn	Expert	LEP	NCI
OTHERS:	Umberto Saffiotti	Chief	LEP	NCI
	Donald G. Blair	Expert	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Culture Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cultured normal human prostatic and bladder 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 and karyotype. Selected cell lines will be tested for tumorigenicity in athymic nude mice. DNA preparations from SV40 transformed prostatic epithelial cells will be tested for their ability to induce anchorage-independence and other changes in growth behavior in the normal prostatic epithelial cells.

Project Description

Objectives: (1) Development of effective exposure conditions of normal human prostatic and bladder epithelial cultures to chemical carcinogens. (2) Comparison of response of normal and carcinogen-treated cultures to growth factors. (3) Study of the effect of carcinogens on culture lifespan, morphology and karyotype. (4) Investigation of the action of combined sublethal levels of carcinogens on the above cellular properties. (5) Studies to determine whether DNA from a transformed prostate cell line (NP-2s/T2) can induce anchorage-independence and reduced serum response in the parental NP-2s.

Methods Employed: Normal prostate (NP-2s) and bladder (NB128) epithelial cell lines were previously established and are available for this study. The NP-2s has been thoroughly characterized and has been transformed by SV40 virus (Kaignn, et al., 1980). Other, less well-characterized prostate lines are available. Use of additional bladder specimens is planned.

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. The goal of these experiments will be to relate the dose to the number of cells at risk and the eventual tumorigenic response. The prime selection technique will be "escaped from senescence." In addition, media which restrict normal cell growth such as those containing reduced levels of serum, growth factor (EGF) or low molecular weight components (Ca++) will also be investigated as potential selective techniques. Appearance of altered growth properties, topographical and karyological changes will be studied as a function of both time after application and dose level. Growth in soft agar and in nude mice will be used as indicators of tumorigenicity. Transfection experiments utilizing DNA from NP-2s-T2 and the NP-2s normal line will be carried out in collaboration with Dr. Donald G. Blair, LMO.

Major findings: This is a new project and there are no results to report.

Significance to Biomedical Research and the Program of the Institute: Both bladder and prostatic cancer are important forms of human cancer. In addition, very little is known about the etiology of prostatic cancer. The normal bladder and prostatic epithelial cultures systems offer an opportunity to investigate etiology as well as the mechanism of carcinogen action in human epithelial cells.

Proposed Course: Initial work on this new project will be with direct-acting carcinogens in both the prostatic and bladder cell lines. When each system is more firmly established, work will progress to investigate the combined activity of several carcinogens and promoters.

Publications: None.

PERIOD COVERED

March 1, 1982 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Hamster Bronchial Epithelial Culture Carcinogenesis Model

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	M. Edward Kaighn	Expert	LEP	NCI
OTHERS:	Umberto Saffiotti	Chief	LEP	NCI
	Sherman Stinson	Biologist	LEP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

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Tissue Culture Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

0.3

PROFESSIONAL:

0.2

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to develop organ and replicative epithelial cell culture models from fresh bronchial tissue of the inbred Syrian hamster.

Comparative studies utilizing the in vivo and the two in vitro models will be made to investigate the activity of selected carcinogens at the cell, tissue and whole animal levels. Emphasis will be placed on response to carcinogens at the cell level as measured by altered response to serum and growth factors, loss of anchorage-dependence, and morphological and karyotypic changes. Organ and cell cultures will be established from normal and carcinogen-treated hamsters with the aim of identifying early induced changes and in determining their relative importance in multistage carcinogenesis.

Project Description

Objectives: (1) Development of replicative cell cultures from fresh bronchial tissue of inbred Syrian hamsters. (2) Development of organ 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 the cell cultures. (6) Comparison of growth behavior and response to carcinogens in epithelial cells from different segments of the laryngo-tracheo-bronchial tract which show different responses to certain carcinogens.

Methods Employed: Both cell and organ cultures will be initiated by the best available current methodology. Defined media (serum-free) will be developed (Project #Z01-CP-05278-01) for the cell cultures based on successful work with human bronchus (Lechner, et al., 1982). Cultures will be treated with selected carcinogens, utilizing several exposure schedules. Alterations in growth behavior (altered serum and growth factor response), loss of anchorage-dependence, morphological changes and karyotypic changes will be studied. Particular attention will be paid to the order in which phenotypic changes occur as measurable parameters in cultured cells after exposure to carcinogens. Such changes will be compared with preneoplastic changes seen in the hamster in vivo, in order to identify sequential steps in the multistage process leading to neoplasia.

Major Findings: The inbred colony of Syrian golden hamsters 15.16/Bio.EHS has been established at FCRF and control animal tissues have been analyzed morphologically (See Project #Z01-CP-05274-01). Culture facilities have been established. This project is only in its planning stage, and there are no findings to report.

Significance to Biomedical Research and the Program of the Institute: Lung cancer, a leading cause of death, continues to increase in incidence. An animal respiratory carcinogenesis model, which reflects the histopathology of human lung cancer developments, will be used as a source of tissues for the cultures. The establishment of an organ culture system which preserves tissue relationships together with development of replicative epithelial cell cultures completes the organizational hierarchy of biological models. Experimental questions posed at any level can be comparatively investigated at other levels. This system should provide 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: This project is in its formative stage and will be developed as described in "Methods Employed."

Publications: None.

PERIOD COVERED

March 1, 1982 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Serum-free Media and Optimal Conditions for In Vitro Carcinogenesis Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Edward Kaighn	Expert	LEP	NCI
OTHERS:	Umberto Saffiotti	Chief	LEP	NCI
	Corrado Ficorella	Visiting Fellow	LEP	NCI
	Federico Bertolero	Visiting Fellow	LEP	NCI
	Margherita Bignami	Guest Researcher	LEP	NCI

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

0.7

PROFESSIONAL:

0.6

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Defined, serum-free media will be developed for rodent and human epithelial cells. The requirement for serum protein will be reduced or eliminated by substituting known growth factors and by altering the basic nutrients as necessary. Macromolecular factors still required will be isolated and characterized. Other culture parameters such as cell dissociation methods and the culture dish surface will also be optimized. Cultures will be characterized by morphological, ultrastructural chromosomal and growth criteria. Two human epithelial cells, NP-2s from neonatal prostate and NB 128 from adult bladder, as well as epithelial cells from primary syrian hamsters bronchus cultures and mouse keratinocytes will be used. The efficacy of nutrient medium MCDB 402 in the mouse 3T3 transformation assay will also be assessed. The goal is not only to obtain appropriate growth control of target cells for transformation studies but to provide a baseline for comparison of the growth response to normal and transformed cells.

Project Description

Objectives: Development of defined media and culture conditions for rodent and human epithelial cells suitable for carcinogenesis studies. Specific aims include: (1) Development of a serum-free medium for bronchial epithelial cells of the Syrian hamster; (2) development of a serum-free medium for a normal human prostatic epithelial cell line (NP-2s); (3) optimization of a defined medium for human bladder epithelial cells (1527F); and (4) assessment of a defined medium (MDCB 402) for use in the BALB 3T3 transformation assay and for mouse skin keratinocytes.

Methods Employed: Normal human prostatic (NP-2) and bladder epithelial cells (NB T28), previously isolated in Pasadena, are available as frozen stocks in liquid nitrogen. The BALB 3T3 clone A31-1-1 (Kakunaga, et al., 1981) and NIH-3T3 cells are currently in use in this laboratory. Fresh bronchial tissue from Syrian hamsters will be obtained from the inbred colony at FCRF. Fresh human bladder tissue will be obtained from surgical or autopsy specimens from tissue procurement facilities at the University of Birmingham, Alabama or the University of Pittsburgh. Tissue not necessary for pathology and for which informed consent has been obtained will be transported to the laboratory in nutrient medium (PFMR-4) containing 5% fetal bovine serum plus antibiotics. Viable tissue has been successfully cultured four days after dissection when transported under these conditions.

Culture procedures previously shown to be effective with prostatic epithelial cells will be used (Lechner et al., 1980) and modified as required.

Cells will be characterized by histology, electron microscopy, karyology (analyzed by banding methods in critical cases), histochemical and immunohistochemical methods.

Clonal growth assay will be used as a direct and reproducible measure of cell growth. It involves counting the cell number per clone and expressing the resultant growth as the number of population doublings (P.D.s). Selective staining with Weigert's iron haematoxylin, including a mild hydrolysis step (to reduce cytoplasmic RNA), allows automatic counting of cell number per colony using an image analyzer. The influence of mitogen concentration on growth rate, R , is measured in dose-response experiments. The data are analyzed by the Lineweaver-Burk method in which the reciprocal of R is plotted against the reciprocal of concentration. The theoretical maximal growth rate, R_{MAX} is defined as the reciprocal of the Y intercept. The substrate concentration at which half-maximal growth occurred, $K_m^{mitogen}$ is the negative reciprocal of the intercept. Each experiment is analyzed by linear regression according to the formula of Ellem and Gierthy (1977). Student's t -test is used to evaluate the significance of the difference between R_{MAX}^I of $K_m^{mitogen}$ values derived from different experimental groups.

Major Findings: This is a new project and no findings are available to report.

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 with 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 to provide more realistic models than the fibroblastic systems and aneuploid cell lines in current use.

Experience has shown that epithelial cell types capable of sustained multiplication can be isolated from many tissues if serum supplementation is avoided. Thus, development of defined media will make possible isolation of appropriate target cells for carcinogenesis studies as well as eliminating the variability imposed by the use of serum.

This approach will improve extrapolation of comparative risk assessment to carcinogens in animals and humans as well as comparative studies of the mechanism of action of carcinogens.

Proposed Course: Development of the Syrian hamster bronchial epithelial organ and cell culture models is dependent on this project. Organ, primary explant and dispersed cell cultures derived from fresh tissue will be cultured in several test media. Various formulations will be evaluated by histology, ultrastructure and growth properties.

The nutrient medium for prostate epithelium now in use (PFMR-4) will be further modified to eliminate the present serum protein requirement (100 ug/ml). A bladder epithelial line NB 128 can now be passed several times in MCDB 152 with 7 factors. This method will be optimized.

Ham and coworkers have developed a new medium (MCDB 402) from 3T3 cells which requires as little as 125 ug/ml of serum protein for clonal growth. This formulation will be tested for efficacy in the BALB 3T3 transformation assay and for use in a current study of arsenic toxicity and carcinogenicity in mouse keratinocytes.

Publications: None

ANNUAL REPORT OF THE LABORATORY OF HUMAN CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1981, to September 30, 1982

The Laboratory of Human Carcinogenesis conducts investigations to assess (a) mechanisms of carcinogenesis in epithelial cells from humans and experimental animals; (b) experimental approaches in biological systems for the extrapolation of carcinogenesis data and mechanisms from experimental animals to the human situation; and (c) host factors that determine differences in carcinogenic susceptibility among individuals.

The Laboratory was established during this fiscal year. The scientific and managerial strategy of the Laboratory is reflected in its organization into three Sections, i.e., In Vitro Carcinogenesis (IVC), Carcinogen Macromolecular Interaction (CMI), and Biochemical Epidemiology (BE). Scientifically, the emphasis is on the role of genetic and acquired host factors as a determinant 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 (IVC and CMI) devote their major efforts to more fundamental and mechanistic studies. The scientific findings, techniques, and concepts developed by these two Sections and, of course, from the scientific community at large, are utilized by the Biochemical Epidemiology Section in selected and more applied studies of carcinogenesis and cancer prevention. The laboratory-epidemiology studies in this Section require the expertise found in the IVC and CMI Sections 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. Tissues from most of the major sites of human cancers 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 culture medium free of serum. 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 exposure to carcinogens and/or tumor promoters 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.

Metabolism of Chemical Carcinogens. 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 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. Representative chemical procarcinogens of several classes can be activated enzymatically to electrophilic reactants that bind covalently to DNA in cultured human tissues. The studies of activation and deactivation of several chemical classes of carcinogens 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 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. To test the interactive effects of cell types in the metabolic activation of carcinogens and to further assess interindividual differences among people, human tissues 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 V-79 cells, suggests that non-target cells of chemical carcinogens may play an important role in the activation of environmental carcinogens.

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 offers 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 and among various organs within an individual. Whether or not these differences are sufficient to influence an individual's cancer risk and organ site is as yet unknown.

DNA Damage and 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. Human bronchial epithelial cells repair single strand breaks (SSB) in DNA damaged by X-radiation, UV-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines.

SSB in DNA are readily caused by either ionizing radiation or during excision repair of UV-induced DNA damage. The number of SSB and the rate of their removal are nearly equal in both normal epithelial and fibroblastic cells. Asbestos fibers cause neither DNA-protein crosslinks nor a detectible increase in SSB even in the presence of the combination of arabinofuranosyl cytosine and hydroxyurea which inhibit the polymerase step during excision repair and, thus, enhance the sensitivity of the assay by allowing SSB to accumulate.

Both procarcinogens, e.g., 7,12-dimethylbenz(a)anthracene (DMBA), and direct activating carcinogens, i.e., benzo(a)pyrene diol epoxide (BPDE) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), cause DNA damage in bronchial cells. When compared to DMBA, BPDE produced more SSB in DNA. MNNG also caused SSB that were nearly completely repaired by 15 hr.

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-HN-CHOH-R₁). 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 DNA SSB. Human bronchial cells were exposed to X-rays and then incubated with or without the presence of formaldehyde, and the repair of DNA SSB was measured. The presence of formaldehyde significantly inhibited the repair of the X-ray induced SSB correlating with the potentiation of cytotoxicity in human cells and mutation frequency in Chinese hamster V-79 cells by the combinations of the agents. Effects of formaldehyde on other DNA repair pathways is also being investigated. For example, the removal of O⁶-methyl-guanine in human bronchial cells is inhibited by formaldehyde. One can also speculate that during the putative demethylation reaction, O⁶-methyl transferase may be inactivated by the generation of formaldehyde.

Results from studies using experimental animals and our results using cultured human bronchial cells showing multiple effects (DNA-protein crosslinks, DNA SSB, inhibition of DNA, repair and cytotoxicity) of formaldehyde suggest that the major metabolites of N-nitrosamines, i.e., alkyl carbonium ions and aldehydes, may act in concert in producing the toxic, mutagenic, and carcinogenic effect of N-nitrosamines.

The molecular events and their fidelity during DNA repair in human cells is unknown. New techniques in molecular and cellular biology provide new opportunities to investigate the mechanisms of DNA repair. Two approaches have been initiated in the Laboratory. The recent availability of microbial DNA repair genes *uvrA*, *B*, and *C*, isolated by our staff and collaborators at Johns Hopkins University, makes possible the first approach, i.e., to determine if these microbial genes, when transfected into xeroderma pigmentosum cells, will complement the repair deficiency in these human cells. The cloned microbial genes are also being used to probe a human DNA library for similar sequences that may be the equivalent genes in human cells. In the second approach, human DNA fragments are being transfected into xeroderma pigmentosum cells 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) can then be identified and isolated.

Interaction between chemical carcinogens and hepatitis B virus in liver cancer induction

Liver cancer incidence is high in areas with both food contamination by carcinogens, such as aflatoxin B₁ (AFB), a liver carcinogen in experimental animals, and a high incidence of chronic active hepatitis. Due to the insensitivity of epidemiological methods, the role of single agents or combinations of agents is uncertain. In order to get a better understanding of this process, we have initiated a project to develop human model systems in which the interaction can be investigated.

The first step in these investigations is to develop a human cell line that contains a full copy or part of the hepatitis B virus (HBV) genome. The rate of transfection of mammalian cells with the pSV2gpt vector was optimized. Human KD cells and mouse 3T6 cells were used for these experiments. Exposure of the mouse cells to a single 100-200 Rad dose of X-ray 48 hrs after transfection (calcium phosphate precipitation method), caused a 5- to 12-fold increase in transfection frequency. Fluorescence studies with chlorotetracycline-bound, calcium phosphate-precipitated DNA showed that mouse 3T6 cells took the complexes up within 24-48 hrs. However, human KD cells required 3-5 days. An equivalent of 10-30 plasmids/cell were present in 3T6 nuclei within 48 hrs, whereas KD cells required reseeding and 3-5 cell generations before any radioactivity was detected in the nuclei. Our present results indicate that KD cells are capable of at least transient expression of XGPRT activity from pSV2gpt.

Attempts to construct a pSV2gpt vector containing the HBV for transfection have been successful. Hybrid plasmids containing HBV DNA at the Bam HI site have been isolated. Restriction analysis of the plasmid indicates the formation of 4 clones containing the gene for the core antigen, DNA arranged in the antisense direction, and 1 clone containing genes for both the core antigen and surface antigen, DNA inverted in comparison with the original HBV DNA.

The effect of carcinogens on hepatoma cell lines with integrated HBV DNA has been investigated. Human hepatoma cell line PLC/5, established from a primary hepatocellular carcinoma containing at least 4 full copies of HBV, was incubated with different types of chemical carcinogens, and the effect was followed by determination of released surface antigen (HBsAg) and by spot DNA hybridization with [³²P]HBV probe. The amount of HBsAg per 10⁶ cells increased after

treatment with MNNG (2 hrs) in a dose-dependent manner reaching a maximum approximately 7-10 days after exposure and returned to near normal after 17 days. This increase was not accompanied by a significant increase in the number of HBV genomes as measured by spot hybridization. Less effect on the production of HBsAg was observed after treatment of the PLC/5 cells with AFB. This could be due partly to the low level of metabolic activity to convert AFB to its ultimate carcinogenic form. MNNG treatment released a small DNA fragment (30 kb) into the Hirt supernatant. This fragment hybridized to [³²P]HBV DNA. Restriction analysis is currently being performed.

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. The molecular basis for these biological phenomena are essentially unknown. 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, serum, platelet-derived growth factor, or calcium ions (> 1 mM), 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 a 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 pattern of keratins were noted between adenocarcinomas and squamous cell carcinomas.

The cytotoxicity, mitogenicity, and differentiating effects of chemical and physical agents from several classes of carcinogens and cocarcinogens are being tested in human bronchial epithelial cells. Some of these compounds were mitogenic, whereas others induced differentiation. For example, teleocidin induced rapid differentiation with a corresponding decrease in ornithine decarboxylase activity. 2,3,7,8-Tetrachlorodibenzo-p-dioxin, on the other hand, was mitogenic and increased ornithine decarboxylase and aryl hydrocarbon hydroxylase activities. Further, the degree of activity change was specific for each compound. These data are important for designing initiation-promotion studies in long-term in vitro carcinogenesis experiments.

One 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 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. Mesothelial cells from lung pleura were more sensitive than epithelial cells to the cytotoxic effects of asbestos.

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 ($< 12 \mu\text{m}$) were taken up quickly by the cells. Asbestos was present within the cells by 2 hrs after exposure, and by 28 hrs, 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 μml) 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.

Biochemical Epidemiology. Two major projects are now ongoing. 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, we are currently examining specimens from populations known to be exposed to carcinogens, e.g., benzo(a)pyrene, and aflatoxin B₁. 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.

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 (a) culturing human epithelial tissues and cells and (b) enzyme immunoassays to measure carcinogen-DNA adducts and onco-fetal antigens. Members of the staff have also co-organized and/or served on the program committees of both national and international scientific meetings. These meetings include "III World Congress on Lung Cancer," "Use of Human Tissues and Cells in Carcinogenesis Studies," "Control of Growth by Nutrients," "1st International Meeting on Cellular Biochemistry and Function." Members of the staff also serve 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.

PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Metabolism of Chemical Carcinogens by Cultured Human Tissues and Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herman Autrup	Senior Staff Fellow	LHC	NCI
Others:	Roland C. Grafstrom	Visiting Fellow	LHC	NCI
	John F. Lechner	Expert	LHC	NCI
	Susan Schlegel	Expert	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

U. of Md., School of Medicine, Baltimore, MD; Institute for Cancer Research, New York, NY; Cancer Institute, Beijing, PRC; Cancer Research Unit, U. of York, England; Lab. of Environmental Carcinogenesis, Copenhagen, Denmark.

LAB/BRANCH

Laboratory of Human Carcinogenesis (LHC)

SECTION

Carcinogen Macromolecular Interaction Section (CMIS)

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TOTAL MANYEARS:

1.5

PROFESSIONAL:

1

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human bronchus, colon, duodenum, esophagus, and pancreatic duct cultured in a chemically defined medium 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. Epithelial cell cultures initiated from human bronchus, bladder, and esophagus metabolized benzo(a)-pyrene (BP), aflatoxin B1 (AFB), and N-nitrosodimethylamine (DMN) into species that reacted with cellular DNA. The metabolic pathways leading to the formation of DNA adducts in explants and epithelial cell cultures have been defined for BP, 7,12-dimethylbenz(a)anthracene, AFB, and 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.

Project Description

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 culture and epithelial cell cultures of human and animal tissues; quantitative high-resolution light microscopic autoradiography; isolation of cellular macromolecules; high pressure liquid chromatography; enzyme assays.

Major Findings: Cultured human bronchial mucosa can enzymatically activate procarcinogens (polynuclear aromatic hydrocarbons: 7,12-dimethylbenz(a)-anthracene (DMBA), 3-methylcholanthrene, benzo(a)pyrene (BP), 6-nitro-benzof(a)pyrene (6-NO₂ 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 B₁ (AFB₁); 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. The ratio of organic extractable metabolites to water-soluble metabolites was greater than one in hamster, human (patients with lung cancer), and C57B1/6N mouse, but less than one in rat, bovine, and DBA/2N mouse suggesting that determination of both activation and deactivation pathways are important in assessing carcinogenic risk of a chemical. No qualitative difference in the profile of organosoluble metabolites was observed between the different species, tetrols and diols being the major metabolites.

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 (+)-(7 β ,8 α)-dihydroxy-(9 α ,10 α)-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 between 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 metabolism of AFB was studied in rat trachea and human bronchus. The major organosoluble metabolite in both species was aflatoxin M₁, but smaller amounts of aflatoxin P₁ and Q₁ 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 (18/25), and no correlation existed between the binding level to DNA of these two carcinogens (-0.2; 25 cases).

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, 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 of guanine; 2) with DMN, N-7 methylguanine and O-6 methylguanine (0-6 MeG/N-7 MeG = 0.3); and 3) with AFB, 2,3-dihydro-2-(N⁷-guanyl)-3-hydroxy-aflatoxin B₁.

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 carcinogen in rats, a comparative study in human and rat 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-nitrosobenzylmethylamine, an organotrophic carcinogen for the rat esophagus. The binding level was about 100-fold higher than in human esophagus. This compound methylated rat esophageal DNA at positions 7 and O⁶ of guanine. The level of benzylation in rat was one-tenth the level of methylation. Formation of benzaldehyde exceeded that of formaldehyde plus CO₂ by a factor of six indicating that the methylene group was preferentially oxidized. N-Nitrosoethylmethylamine, another unsymmetrical N-nitrosamine, was preferentially oxidized by rat esophagus in the ethyl group, as shown by higher formation of CO₂ 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 was detected to human esophagus. 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. As measured by the formation of both CO₂ and formaldehyde, 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 positions 7 and O⁶ 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 (250 cases). After 24 hrs in culture, explants were exposed to radioactive-labeled carcinogen for another 24 hrs, 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-NO₂BP, DMBA, AFB; DMN, DMH, and 3-amino-1,4-dimethyl-5H-pyrido(4,3-6)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 (21) than in colon (8). A wide interindividual variation was observed. A positive correlation in the binding level of BP between bronchus and colon ($r = 0.95$; $p < 0.001$) and duodenum ($r = 0.83$; $p < 0.001$) from the same individual (15 cases) was seen.

Some quantitative differences in the metabolic profile of BP were observed between the four organs. A significantly higher amount of benzo(a)pyrene 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.

Cultured human pancreatic duct could metabolize BP, DMBA, AFB, and DMN as measured by radioactivity associated with DNA.

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. The development of controlled culture conditions for human tissues furnish 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 carcinogen can be studied at the same level of biological organization in both species, information essential for extrapolation of carcinogenesis data between species. Furthermore, human tissues obtained by immediate autopsy also allow a comparative study in various organs from the same individual hoping that we will be able to identify an easily accessible cell type that can be used for the identification of individuals at high risk of developing chemically induced cancers.

Proposed Course: Identify endogenous and exogenous factors that alter the metabolism of environmental procarcinogens. To continue a combined laboratory-epidemiology study of carcinogen metabolism. To use ultramicrossays

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 interaction will be continued.

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Grafstrom, R.C.: Carcinogen metabolism in intestinal tissues. In Autrup, H. and Williams, G. M. (Eds.): Experimental Colon Carcinogenesis. Baco Raton, CRC Press. (In press)

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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, Vol. 13. Cold Spring Harbor, Cold Spring Harbor Laboratory. (In press)

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. IARC Scientific Publication No. 39, Lyon. (In press)

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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. Proc. VII Internatl. Meetings on N-Nitrosocompounds - Occurrence and Biological Effects. (In press)

PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Carcinogenesis Studies Using Cultures of Human Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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7

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3

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4

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Defined methods to grow replicative cultures of normal human bronchial epithelial cells either without serum or Swiss mouse 3T3 feeder-cells 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. Further, mitotically quiescent cells will differentiate into cells with beating cilia, a characteristic of normal bronchial epithelium. Dissociated single cells form colonies when plated at low density. In vitro carcinogenesis experiments with normal bronchial epithelial tissue and cell cultures have yielded populations of cells which have abnormal characteristics. These phenotypically altered cells (PACs), which have keratin epithelial cell markers, have extended population doubling potentials, normal and abnormal human karyologies and abnormal differentiation control. Amosite asbestos was found to be 100 times more cytotoxic for bronchial epithelial cells compared to bronchial fibroblastic cells. In addition, 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 microanalysis in these lesions.

Project Description

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 progression of carcinogen-induced, phenotypically altered cells (PAC) to malignancy; (6) assess xenotransplantation methodologies in order to attain increased sensitivity for the assay; (7) investigate the mechanism(s) of nickel carcinogenesis at the molecular level; and (8) develop a mesothelial cell culture system to study in vitro carcinogenesis (mesothelioma) of these cells with asbestos.

Methods: 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. Mesothelial cultures are initiated from pleural effusions.

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, then subcultured. These cells are then used in in vitro carcinogenesis, cell nutrition and differentiation studies, carcinogen metabolism and DNA repair experiments, and 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, and 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.

Two types of long-term explant cultures are being used to scrutinize cellular changes induced by exposure to chemical carcinogens. Quasi-quiescent long-term explant cultures are maintained for from 8 weeks to more than 1 year and are exposed to chemicals [7,12-dimethylbenz(a)anthracene (DMBA) or 4-nitroquinoline-1-oxide (4NQO)] or asbestos fibers. Explant cultures are also used to study carcinogenesis in a continually growing epithelium. After epithelial cell migration, the explant is transferred to a new dish. Both the outgrowth and transplanted explant dishes are maintained. After a new outgrowth, the explant is again transferred. Abnormal cells are isolated from the carcinogen-exposed populations by selecting those cells which fail to respond to terminal differentiating signals incorporated into the medium.

Pure populations of bronchial epithelial cells are also exposed for extended periods to chemical carcinogens, and abnormal, mitotic cells are selected using the terminal differentiation inducing protocol.

Growth of carcinogen-induced abnormal cells in athymic nude mice is being tested using several procedures. Cells are being injected both subcutaneously and intracranially into untreated or either estradiol or antilymphocytic serum treated nude mice. Further, the cells are being co-injected with high concentrations of irradiated human fibroblastic and/or tumorigenic cells. In addition, freeze/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 has been developed. Large pieces of bronchus tissue are initially set up as explant cultures and incubated in a rocking chamber for 2 to 3 days to facilitate reversal of ischemia. The explants are then cut into smaller pieces and explanted into Ca^{2+} -reduced M199 medium containing 1.25% fetal bovine serum. This medium with a low serum concentration permits rapid outgrowth of epithelium, but retards growth of the fibroblastic cells. Thus, after 5-10 days incubation, very few fibroblastic cells are present among the epithelial outgrowth. At this time, the tissue explant is transferred to a new dish for reseeding a second wave of epithelial cell outgrowth. Sequential tissue transfer has been 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 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-2) contains transferin, insulin, epidermal growth factor, hydrocortisone, phosphoethanalamine, ethanolamine, and T_3 . In addition, the Ca^{2+} and SO_4 levels were adjusted. Further improvements in in vitro conditions and techniques were obtained by modifying the surface of the petri dish and incorporating urea into the subculturing methodology. 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. In addition, plating efficiency was improved by prewashing explant outgrowth cultures with 0.25M urea prior to trypsin and EGTA dissociation. 3) Supplementation with as little as 0.25% fetal bovine serum results in a decrease in clonal growth rate; 8% supplementation completely inhibits growth by inducing terminal, squamous cell differentiation. Platelet derived growth factor alone, at concentrations which stimulate fibroblastic cell multiplication, also

inhibits DNA synthesis and stimulates cell migration and eventual squamous-like differentiation. On the other hand, plasma-derived serum was significantly less growth inhibitory. 4) Incorporation of 1.25% serum into LHC-2 altered the Ca^{2+} requirement. Whereas no significant change in the Ca^{2+} requirement was noted between 60 and 1000 μM in serum-free medium, 50% inhibition of growth occurred above 700 μM Ca^{2+} with serum supplementation. The calcium concentration of the medium influences colony morphology and cell shape. The cells are small and tightly polygonal when growing in normal (1 mM) levels of calcium. Progressive reduction of the calcium concentration to 0.06 mM causes the cells to become flatter and 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 1mM Ca^{2+} . 6) Mg^{2+} and Ca^{2+} regulation of clonal growth of bronchial epithelial cells was found to be by a mechanism that is significantly different from that which occurs in lung fibroblasts. Whereas for the lung fibroblasts, the role of Mg^{2+} is more proximal to the intracellular events that determine the maximal clonal growth rate, the results with bronchial epithelial cells suggest that the roles of the two ions are equally important and, to a great extent, can be substituted for one another. 7) Serum influenced growth factor requirements. Cholera toxin was growth stimulatory 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) A less defined method to grow human bronchial epithelial cells was developed prior to elucidating serum-free conditions. In this system, Swiss mouse 3T3 feeder-cells are added to the cultures, and the cells are grown in Ca^{2+} reduced M199 medium supplemented with fibronectin improved clonal plating efficiency (from 2 to 15%) and increased the clonal growth rate (2.5-fold). The addition of other factors, e.g., MSA, FGF, and EGGG, were less mitogenic. 9) Cultures of human bronchial epithelium have the normal human karyotype and average 35 population doublings in vitro. 10) 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 have beating cilia. Ultrastructurally, these cells have numerous tonofilaments and desmosomes. 11) The effect of the tumor promoter TPA was found to be dependent upon culture methods. When cocultured with Swiss mouse 3T3 feeder-cells, the response to TPA was biphasic. Maximal stimulation occurred at low TPA concentrations, whereas higher concentrations of TPA were toxic. On the other hand, a first order response was seen when the cells were grown in serum-free medium, without feeder-cells, and the half-maximal stimulation concentration was more than 100-fold greater than was observed with feeder-cells. 12) Normal epithelial explant and cell culture systems for adult bronchial epithelium have now been extensively developed. Thus, in vitro chemical carcinogenesis experiments have become potentially feasible. Large quantities of epithelial cells can be readily obtained permitting experiments using large numbers of cells at risk to be repeated several times. 13) More than 12 replicative cultures of mesothelial cells have been isolated from pleural effusions. These cultures have been identified as mesothelial in origin by transmission electron microscopy.

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. As time progressed, the outgrowth cultures become 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 few successive subcultures. 2) Phenotypically altered cells (PACs) were isolated after repeated treatments of bronchial epithelial cells with Ni^{2+} or DMBA. PACs seemingly arose via two pathways. Ni^{2+} -induced PACs arose as individual clones. These clones appeared 3-7 weeks after the exposed semiconfluent cultures had become mitotically quiescent. Fourteen of the PAC colonies have been isolated from four experiments and expanded. The PAC culture which arose after 6 months of repeated exposure of explants to DMBA apparently developed via a different mechanism. Clonal origin cannot be suggested; escape from senescence developed throughout the outgrowth cell population and a period of mitotic quiescence did not precede their appearance. 3) Five PAC cultures, which arose in different experiments and are autopsy donor unrelated, have been partially characterized. All express the keratin epithelial cell markers and exhibited desmosomes in early passage. Chromosomal studies revealed both normal and aneuploid karyologies. The clonal growth response to ethanolamine and serum and Ca^{2+} differ both among themselves and compared with the normal cells. 4) Differentiation stimulation by serum and 1 mM Ca^{2+} has been found to be useful as a means to select and characterize PACs. Some PACs respond to Ca^{2+} but not serum, and differentiate and *visa versa*, whereas a few cultures fail to respond to either differentiation-promoting signal. 5) Of 5 human lung tumorigenic cell lines tested, 3 were found to grow poorly in serum-free low Ca^{2+} LHC-2 medium which was especially developed for optimal growth of normal human bronchial epithelial cells under defined, clonal conditions. However, whereas supplementation of LHC-2 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; PAC epithelial cells have been detected growing on the lumen after two week's implantation in the nude mouse. 7) The tumorigenicity assay has been made 10 times more sensitive by co-injecting 2 million irradiated human bronchial fibroblastic cells, i.e., 100,000 tumorigenic cells, co-injected with irradiated fibroblasts cause tumors to appear more quickly than 1 million injected tumor cells alone.

Investigations of the mechanism of asbestos carcinogenesis have shown the following. 1) Amosite asbestos (100-1000 $\mu\text{g/ml}$) 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 ($< 12 \mu$) were found in the cytoplasm of the cells within 6 hrs, 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, 0.1-100 $\mu\text{g/ml}$) were added

to human bronchial epithelial cells that had been subcultured 24 hrs 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 using human bronchial fibroblastic cells. 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.

Investigations in the mechanism(s) of nickel carcinogens have just begun. The only observations to date are (1) vanishingly small amounts of nickel adduct with DNA; (2) single strand breaks were not detected after exposure of bronchial epithelial cells with nickel; (3) the accumulation of soluble Ni^{2+} by human cells appears to be passive diffusion and continuous with approximately 25% of the labeled Ni^{2+} being recoverable in the nucleus, 25% in cytoplasmic particulates and 50% in the soluble cytoplasm at any time. Further, nickel passively diffuses out of cells, equally from all compartments with a $t_{1/2}$ of approximately 55 hrs.

Significance to Biomedical Research and the Program of the Institute:

The extrapolation of experimental animal data to man is a major problem in carcinogenesis. One approach to provide a link between these experimental data and human cancer 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 demonstrate the growth characteristics ascribed to transformed cells. Changes in these properties may be indicative of premalignant transformation. Investigations on single and 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:

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. International Agency for Research on Cancer (IARC Scientific Publication No. 39), Lyon. (In press)

Haugen, A. and Harris, C.C.: Asbestos carcinogenesis: Asbestos interactions and epithelial lesions in cultured human tracheobronchial tissues and cells. Recent Advances in Cancer Research. (In press)

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. (In press)

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Lechner, J.F.: Nutrient, hormone, growth factor and substrate interdependent regulation of epithelial cell growth. Fed. Proc. (In press)

Lechner, J.F., Haugen, A., Autrup, H., McClendon, I.A., Pettis, E.W., Trump, B.F., and Harris, C.C.: Clonal growth of epithelial cells from normal adult human bronchus. Cancer Res. 41: 2294-2304, 1981.

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Stoner, G.D., Kato, Y., Foidart, J-M., Trump, B.F., Steinert, P.M., and Harris, C.C.: Cultured human bronchial epithelial cells: Blood group antigens, keratin, collagens and fibronectin. In Vitro 17: 577-587, 1981.

Trump, B.F., Wilson, T., and Harris, C.C.: Recent progress in the pathology of lung neoplasms. In Tine Peereboom (Ed.): Lung Cancer; Excerpta Medica. (In press)

PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Epidemiology Studies Using Monoclonal Antibodies to Aflatoxin B₁-DNA Adducts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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2

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1

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1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 administered dosages 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

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: 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/aminopterin/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 taken 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 recognized only aflatoxin bound to DNA and not free aflatoxin B₁-guanine adducts or six other aflatoxin B₁ metabolites. These assays were performed using both enzyme linked immunosorbent assay (ELISA) and ultrasensitive enzyme radioimmunoassay (USERIA) techniques.

Major Findings: Immune response to aflatoxin B₁-DNA adducts were obtained by injecting methylated bovine serum albumin-AFB₁-DNA conjugate or AFB₁ conjugate emulsified in Freund's complete adjuvant into mice. Hybridoma clones producing monoclonal antibodies against aflatoxin B₁-DNA adducts or AFB₁ have been obtained and characterized. Competitive ELISA using these monoclonal antibodies reliably quantitated aflatoxin B₁ 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 aflatoxin B₁ adducted DNA. Using biotinylated monoclonal antibody and an avidin enzyme conjugate, a competitive enzyme immunoassay has also been developed.

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 which 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 (a) their rate of formation and removal and (b) 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 B₁-modified DNA using monoclonal antibodies.
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C. C.: Monoclonal antibody to aflatoxin B₁ modified DNA detected by enzyme
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PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Repair of Carcinogen Induced Damage in Human Epithelial and Fibroblast Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Roland C. Grafstrom	Visiting Fellow	LHC	NCI
Others:	Herman Autrup	Senior Staff Fellow	LHC	NCI
	John F. Lechner	Expert	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Lab. of Pathology, DCBD, NCI; Dept. of Physiology, Hershey Medical Center, Hershey, PA; Dept. of Pathology, U. of Md. School of Med., Baltimore, MD

LAB/BRANCH

Laboratory of Human Carcinogenesis (LHC)

SECTION

Carcinogen Macromolecular Interaction Section (CMIS)

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 X-ray, 2) rate of excision repair after UV-radiation, 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. DNA-protein crosslinks (DPC) induced by formaldehyde were rapidly removed in all cell types. Low concentrations of formaldehyde inhibited rejoining of X-ray induced SSB and synergistically potentiated X-ray induced toxicity in bronchial cells. Formaldehyde did not have any effect on unscheduled DNA synthesis. Repair replication was more than 2-fold higher in epithelial than in fibroblast cells after BPDE treatment. Asbestos and nickel did not induce any DNA damage and did not affect repair of damage induced by BPDE or UV-radiation.

Project Description

Objectives: To understand the mechanism of repair of DNA damage by environmental agents in human epithelial tissues and cells.

Methods Employed: Culture of human epithelial and fibroblastic cells; alkaline elution technique for detection of DNA single strand breaks and DNA protein crosslinks (DPC); BND cellulose chromatography for measurement of repair replication; ³H-thymidine incorporation in the presence of hydroxyurea for measurement of unscheduled DNA synthesis; isolation of cellular macromolecules; high pressure liquid chromatography.

Major Findings: DNA single strand breaks (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-radiation 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-radiation 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-dimethylbenzo(a)anthrazene, a small number of DNA SSB was seen in both cell types. Also, in the case of anti-benzo(a)pyrene diolepoxide (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 also 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 with either UV-radiation or BPDE. A smaller effect was seen with BPDE in fibroblasts 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-radiation in either cell type. Furthermore, nickel had no effect on the level of excision SSB after UV-radiation. 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. Exposure to formaldehyde resulted in formation of DPC to an equivalent extent in bronchial

cells as well as 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 DNA-protein crosslinks with an approximate half-life of 2 hours independent of dose and cell type. After 8-10 hours incubation, only 5-10% of the DPC remained. Incubation of DNA from the formaldehyde-treated cells in alkaline lysing solution indicated that the DPC were relatively stable ($t_{1/2} \sim 10$ hr). When the crosslinks 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 using the polymerase inhibitor combination (Ara-C/HU), DNA SSB could be accumulated in bronchial cells and skin fibroblast, but were not 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 combined exposure to X-rays and formaldehyde resulted in a synergistic potentiation of the cytotoxicity, human cells, and mutagenicity (Chinese hamster V-79 cells) of these agents. The biological significance of this synergism is being further investigated.

The mechanism of the observed repair inhibition of formaldehyde was further investigated. The "long patch repair" agents, UV-radiation 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-ray. This indicated a higher sensitivity of the ligation step of excision repair to formaldehyde action.

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., Jr., Seres, D.S., Lechner, J.F., and Harris, C.C.: DNA-protein crosslinking by chromium salts. Chem.-Biol. Interact. 36: 345-354, 1981.

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, Vol. 13, 1982. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05193-02 LHC

PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Differentiation of Normal and Transformed Human Epithelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Susan Schlegel	Expert	LHC	NCI
Others:	Curtis C Harris	Chief	LHC	NCI
	John F. Lechner	Expert	LHC	NCI

COOPERATING UNITS (if any)

Laboratory of Pathology, DCBD, NCI; U. of Md. School of Med., Baltimore, MD; VA Hospital, Washington, DC; Litton Bionetics, Rockville, MD; Cancer Institute, Beijing, PRC; U. of Pittsburgh School of Med., Pittsburgh, PA

LAB/BRANCH

Laboratory of Human Carcinogenesis (LHC)

SECTION

In Vitro Carcinogenesis Section (IVCS)

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The program of differentiation in human esophageal epithelium was found to be similar to, yet unique from, that of the human epidermis. With the exception of the absence of high molecular weight keratins, the pattern of keratin proteins in human esophageal cells was found to be qualitatively similar to that of epidermal cells. There were, however, dramatic quantitative differences in the array of keratin species between the two tissues. During in vitro cultivation, epithelial cells from these two tissues were found to maintain a differentiative program remarkably similar to that of the native epithelium. Malignant transformation of esophageal 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 esophageal epithelial cells in culture. Analysis of keratin proteins extracted from a wide variety of tumors indicates that these proteins may prove to be a valuable adjunct in diagnostic pathology.

Project Description

Objectives: The program of growth and differentiation in normal human epithelial cells will be analyzed and compared to that of their neoplastic counterparts. Moreover, we will attempt to establish neoplastic epithelial cells in culture. The growth and differentiated properties of these cells will be compared to their normal counterparts.

Methods Employed: Human tissue is obtained from "immediate" autopsy, autopsy material that is less than 12 hours post-mortem, or from surgical specimens. The epithelium and some adherent connective tissue is 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 cut into explants and used for radiolabeling of proteins. When being used for cell culture, the epithelium 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, and peptide mapping. The terminal differentiation of the cells is triggered by means of ionophore. In the case of human tumor material, the epithelium is not separated from adherent stroma. Otherwise, the tissue is handled identically to that of the normal tissues.

Major Findings: The program of differentiation of human esophageal epithelium is morphologically and biochemically similar to, yet unique from, that of human epidermis. Morphologically, the human esophageal epithelium resembles that of the epidermis, both being stratified squamous epithelia. However, unlike the epidermis, the esophageal epithelium lacks a granular layer and a stratum corneum. Analysis of the keratin protein patterns from these epithelia revealed an absence of the higher molecular weight keratins (63, 65, and 67 kd) from the esophageal epithelium consistent with the morphologic data showing no granular layer or stratum corneum. Although the remainder of the keratins in the molecular weight region from 40-61 kd were qualitatively similar between these two epithelia, there were dramatic quantitative differences in the keratin profiles. In addition, the esophageal cells contained two new proteins (38 and 92 kd) immunoprecipitated with whole keratin antiserum which were not present in the human epidermis. Peptide maps of these proteins are being carried out in order to confirm the identity of these proteins as keratins. When cells from these epithelia are grown in culture, they maintain a pattern of differentiation similar to their in vivo counterparts. The cultured epithelium from the human epidermis is stratified, contains keratohyalin granules in the most superficial layers, and contains a 67 kd keratin by immunoprecipitation with whole keratin antiserum. In contrast, although the cultured esophageal epithelium is stratified, it lacks keratohyalin granules in the outermost cell layers and does not contain a 67 kd keratin.

Analysis of keratin proteins extracted from human esophageal epithelial tumor cells demonstrated dramatic changes in the pattern of keratins. A number of keratin species, especially in the higher molecular weight region, were found to be absent in the tumor cells. Moreover, there was an overall reduction in the amount of keratin present. Injection of these tumor cells into a nude mouse and analysis of that tumor for keratin proteins revealed an even more dramatic shift in patterns of keratins being made.

After repeated attempts at establishing esophageal epithelial tumors in culture, we have succeeded in establishing one tumor cell line. While the cells look typically epithelial in appearance, the colonies which grow out are morphologically very different from those of normal esophageal epithelial cells. The cells tend to pile up on top of one another, and crater-like structures form in the center of the colonies. The cells grow much more slowly than their normal counterparts and more cells can be seen being sloughed into the medium. Although the pattern of keratin filaments within the cells looks very similar to that of normal esophageal epithelial cells, the keratins from these tumor cells are remarkably different from those of the normal cell. First of all, the specific activity of the keratins extracted from the tumor cells is 6 times that of the normal cell. The fact that the amount of keratin protein in both cases was the same suggests that the keratins in the tumor cell must be undergoing a much faster turnover rate. In addition, analysis of the keratins by polyacrylamide gel electrophoresis revealed that a number of keratin species in the tumor cell (especially in the lower molecular weight region) had a slower migration rate than that of keratins extracted from normal cells. The explanation for this finding is unknown at the present time. Surprisingly, immunoprecipitation of the keratins from these tumor cells revealed the presence of a 67 kd keratin typical of human epidermis. These data, however, are consistent with the fact that some well differentiated squamous cell carcinomas of the esophagus show signs of keratinization. Another differentiative function, the ability to make cross-linked envelopes, is also dramatically altered in these tumor cells. When terminal differentiation of these cells is induced by means of a calcium ionophore, only 20% of the cells make envelopes. In comparison, 94% of the normal esophageal epithelial cells make envelopes during ionophore-induced terminal differentiation.

Another area of interest has involved screening various tumors for keratin. Not only have keratins been found to be useful for delineating the epithelial nature of the tumor, but also as a useful 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. Polyacrylamide gel electrophoresis of proteins extracted from various tumors of the human lung indicate that the analysis of keratin proteins may be a useful adjunct in distinguishing squamous cell carcinomas and adenocarcinomas. The pattern of water-insoluble proteins in the molecular weight range of 40 to 60 kd, characteristic of keratins, was remarkably different between the squamous cell carcinomas and adenocarcinomas of the lung. The squamous cell tumors contained a more complex and more abundant array of proteins in the keratin region of the gel. In contrast, proteins extracted from adenocarcinomas of the lung were not found to be concentrated in any particular region of

the gel. At present we are immunoprecipitating the proteins extracted from these various tumors with keratin antiserum in order to identify specifically the keratin species present in these various tumor types.

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: Continuation of studies aimed at understanding the control of differentiation and the sequence of events involved in malignant transformation of epithelial cells.

Publications:

Banks-Schlegel, S.P.: Keratin alterations during embryonic epidermal differentiation: A presage of adult epidermal maturation. J. Cell Biol. 93: 551-559, 1982.

Banks-Schlegel, S.P., Schlegel, R., and Pinkus, G.S.: Keratin protein domains within the human epidermis. Exp. Cell Res. 136: 465-469, 1981.

Stoner, G.D., Pettis, E.W., Haugen, A., Jackson, F., and Harris, C.C.: Explant culture of rat esophagus in a chemically defined medium. In Vitro 17: 681-688, 1981.

PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cell Surface Antigens Associated with Neoplastic Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Curtis C. Harris Chief LHC NCI

Others: Dean L. Mann Senior Investigator I NCI
John F. Lechner Expert LHC NCI

COOPERATING UNITS (if any)

NCI-Naval Medical Oncology Branch, DCT

LAB/BRANCH

Laboratory of Human Carcinogenesis (LHC)

SECTION

Biochemical Epidemiology Section (BES)

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:

0.5

PROFESSIONAL:

0.25

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human cells which have undergone neoplastic transformation may express an array of cell surface structures not found on their normal counterparts. Over the past several years, a number of monoclonal antibodies to human cell surface antigens have been produced detecting antigens controlled by the major histocompatibility complex as well as other cell surface structures. These sera were reacted with human tumor cells growing in tissue culture. The enzyme linked immunosorbent assay (ELISA) technique was modified in order to detect antigen-antibody reactions on surface adherent tumor cells. The studies thus far indicate that the assay system is applicable to the examination of cell surface determinants detected by these monoclonal antibodies. This technique will be applied to the assessment of antigens on normal epithelial cells and cells treated with chemical carcinogens.

Project Description

Objectives: Monoclonal antibodies detecting a variety of cell surface structures have been developed in various laboratories. These monoclonal antibodies have been procured by this laboratory and will be used to study the cell surface changes that occur with neoplastic transformation. It is the goal of these studies to examine qualitatively and quantitatively the cell surface changes that occur with neoplastic transformation comparing the neoplastic and preneoplastic cells with normal counterparts.

Methods: Monoclonal antibodies have been obtained from a number of different investigators. These monoclonal antibodies by and large were raised by specific immunization of mice with normal or neoplastic cells, subsequent screening against the target cells, and in some instances characterized by isolation of the cell surface determinant with which this antibody reacts. The initial studies will concentrate on human lung cancers. Normal and neoplastic tissues are maintained and grown in culture. The cells are tested with the monoclonal antibodies by enzyme linked immunosorbent assay (ELISA). The cells are placed in 96-well plates and grown to confluency, and monoclonal antibodies are added. After incubation for a period of 1 hour, each well is thoroughly washed, and a solution and media containing 0.5% gluteraldehyde is added. After 5 minutes of incubation, this solution is removed, the cells are washed, and an anti-immunoglobulin conjugated with alkaline phosphatase is added. After an additional hour of incubation, the anti-immunoglobulin conjugate is removed by washing and enzyme substrate is added. This combination is then incubated for 4 hours, and the development of colometric change is determined.

Major Findings: Studies thus far have indicated that human tumor cells can be grown in 96-well test plates to confluency. The combination of plates and cells effect no increase in background on the ELISA reader. Various methods of addition of antibodies as well as incubation times have been explored. It has been determined that gluteraldehyde fixation of the cells after attachment of the antibody is an effective method to maintain the cells in condition so that the ELISA test is applicable.

Significance to Biomedical Research and the Program of the Institute: Identification of cell surface changes that occur with neoplastic transformation is fundamental to our understanding of the neoplastic process. The use of reagents that characterize cell surface determinants, such as monoclonal antibodies, provide the means for examination of these cell surface changes. Identification of new markers represented as cell surface antigens may add to our basic understanding of this differentiation that occurs with neoplastic transformation.

Proposed Course: Studies are underway to examine a number of well characterized monoclonal antibodies detecting cell surface determinants. Normal bronchial epithelium will be explanted to tissue culture and treated with chemical compounds known to be associated with carcinogenesis. Having established a "phenotype" of the neoplastic lung tumor, the phenotypically altered cell (those cells treated with chemical carcinogens) will be examined to determine if a similar phenotype is induced by the chemical carcinogen.

Publications: None.

Project Description

Objectives: To develop a human model system to study the possible interaction between chemical carcinogens and hepatitis B virus in the induction of liver cancer.

Methods employed: Cell culture; DNA transfection; recombinant DNA techniques; Southern blot and spot hybridization; radioimmunoassays.

Major findings: Liver cancer incidence is high in areas with both high level of food contamination of aflatoxin B₁ (AFB), a liver carcinogen in experimental animals, and a high incidence of chronic, active hepatitis. Due to the insensitivity of epidemiological methods, the role of single agents or combinations of agents is unknown. In order to better understand this process, we have initiated a project in human-model systems in which these interactions can be investigated. The first component of this model is the creation of a human cell line with an integrated partial or full copy of hepatitis B virus (HBV). As a vector for DNA transfection, we have been using the pSV2gpt vector. HBV genome was obtained from pAM6 and was restricted with Bam HI to separate the pBR322 moiety from HBV. Hybrid plasmids containing HBV DNA have been isolated and are currently being used to transfect normal human fibroblasts (KD). Alternative integration sites in the pSV2gpt are currently being investigated. The rate of transfection of mammalian cells with the pSV2gpt vector has been optimized. Mouse 3T6 cells were used for these experiments. Exposure of the cells to a single 100-200 Rad dose of X-ray 48 hrs after transfection (calcium phosphate precipitation method) caused a 5- to 12-fold increase in transfection frequency. Fluorescence studies with chlorotetracycline-bound, calcium phosphate-precipitated DNA showed that mouse 3T6 cells took up the complexes within 24-48 hrs. However, 3-5 days was required for human KD cells. Using [³H]-TdR labeled E. coli DNA, we have shown that an equivalent of 10-30 plasmids per cell were present in 3T6 nuclei within 48 hrs, whereas KD cells required reseeded and 3-5 cell generations before any radioactivity was detected in the nuclei. Our present results indicate that KD cells are capable of at least transient expression of XGPRT activity from pSV2gpt. Small colonies were observed 3-4 weeks after selection was applied.

Another approach has been to investigate the effect of carcinogens on hepatoma cell lines with integrated HBV DNA assuming that the effect of the carcinogen would be a gene amplification phenomenon. Human hepatoma cell line PLC/5 established from a primary hepatocellular carcinoma containing at least 4 full copies of HBV was treated with different types of chemical carcinogens. The cells were examined for levels of released HBV surface antigen (HBsAg) and copy number of HBV by spot hybridization with [³²P]HBV probe. The amount of HBsAg per 10⁶ cells increased after treatment with MNNG (2 hrs) in a dose-dependent manner, reaching a maximum approximately 7-10 days after exposure, returning to near normal levels after 17 days. This increase was not accompanied by any significant increase in the number of HBV genomes as measured by spot hybridization. Less effect on the production of HBsAg was observed after treatment of the PLC/5 cells with AFB. This could partly be due to the low level of metabolic activity to convert AFB to its ultimate carcinogenic form. However, the AFB-DNA adducts formed were similar to the ones formed in rat liver DNA in vivo.

Repeated treatment of PLC/5 with MNNG produced cells displaying altered morphology which produced a several-fold higher level of HBsAg than the control cells. These cells appear to have a finite lifespan. Treatment of the PLC/5 cells with MNNG released a small DNA fragment (30 Kb) into the Hirt supernatant. This fragment hybridized to [³²P]HBV DNA. Restriction analysis is currently being performed.

Significance to Biomedical Research and the Program of the Institute: The role of hepatitis B virus and aflatoxin B₁ in the induction of liver cancer has been established from epidemiological investigations. As these two factors both prevail in the same area of the world, it is difficult to identify the "initiating" agent of hepatocellular carcinoma. Furthermore, the other agent may act as a cocarcinogen or as a tumor promoter. The development of a human model system will eventually allow us to study the role of hepatitis B and aflatoxin B₁ in human hepatocellular carcinoma. A better understanding of this disease would help the measures to prevent it.

Proposed Course: To attempt to introduce portions of HBV genome into normal human cells and to study morphological and phenotypical changes; to study the effect of carcinogens on cells containing HBV; to elucidate the role of hepatitis B virus and AFB in the induction of liver cancer in man.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05290-01 LHC

PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Enzyme Immunoassays to Measure DNA Adducts Caused by Nitrosourea Anticancer Drugs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Glennwood Trivers	Research Scientist	LHC	NCI
Others:	Dean L. Mann	Senior Investigator	I	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

NCI, Immunology Branch; School of Public Health, Boston University, Boston, MA;
Laboratory of Molecular Pharmacology, DCT, NCI

LAB/BRANCH

Laboratory of Human Carcinogenesis (LHC)

SECTION

Biochemical Epidemiology Section (BES)

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The principle nitrosourea anticancer agents (1,3-bis (2-chlorethyl)-1-nitroso-urea, BCNU; 1-(2-chlorethyl)-3-cyclohexyl-1-nitrosourea, CCNU; methyl-CCNU), used with some success in treating humans with cancer, cause delayed and cumulative bone marrow toxicity and secondary tumors in some patients. A property of these compounds which has been correlated with toxicity, antitumor activity, and carcinogenesis is their ability to alkylate cellular DNA. In physiological conditions, nitrosoureas decompose to alkyl diazohydroxides and chlorethyl carbonium ions that alkylate nucleic acids, and cause breaks in DNA. There are currently no simple methods available to detect and monitor damage induced by such compounds. One newer agent, chlorozoticin (CLZ) (chlorethyl nitrosourea attached to glucose), is more therapeutic, less myelosuppressive and produces 2-fold greater alkylation of RNA and DNA than BCNU or CCNU and no carbamoylation. Therefore, CLZ-modified calf thymus DNA has been injected intramuscularly and intradermally into rabbits to obtain antibodies for an ultrasensitive enzymatic radioimmunoassay (USERIA) to study macromolecular alterations in patients clinically exposed to these compounds and to experimentally study the mechanisms of their cellular effects.

Project Description

Objectives: Develop enzyme radioimmunoassays for the study of DNA adducts formed by chlorozoticin and similar compounds used to treat cancer patients; to use anti-chlorozoticin (CLZ) immunoreagents to experimentally study macromolecular interactions induced by CLZ in vitro in cultured human tissues and cells.

Methods Employed: Identification of adducts in CLZ-modified calf thymus DNA using high pressure liquid chromatography (HPLC), rabbit antisera to CLZ-DNA (immunization by the Vaitukaitis intradermal procedure for small amounts of antigen), preparation of monoclonal antibodies by hybridoma technology, modified enzyme-linked immunosorbent assay (ELISA) to establish assay conditions, comparative ELISA and USERIA to optimize conditions for competitive USERIA; antiserum characterization for specificity; cultured human cells for metabolism and studies; intact rodents (mice and rats) and athymic, immune-deficient mice for in vivo studies.

Major Findings: This is a new project. HPLC analysis of mouse L1210 cells exposed by CLZ revealed 3 major chloroethyl and hydroxyethyl guanine adducts, whereas analysis of DNA modified by CLZ in a cell-free system produced a greater number of detectable adducts. Mice and rabbits are currently being immunized, and antisera with specificity for CLZ-modified DNA have been demonstrated.

Significance to Biomedical Research and the Program of the Institute: The results of successful chemotherapy with nitrosoureas and other carcinogenic anticancer compounds that are relevant to this project are mainly (1) an increasing number of humans are being and will continue for some time to be necessarily subjected to substances with long-term side effects including secondary neoplasms and (2) a percentage of the long-term survivors of this type of therapy, including, in particular, young adults and children, will quite likely suffer therapy-induced tumors. This project has the potential to establish a simplified test for detection and quantitation of the accumulative, carcinogen-related toxicity of these compounds via short- and long-term studies of the specific decomposition metabolites associated with macromolecules especially DNA. Once available, a USERIA for specific CLZ-induced DNA alterations could both benefit cancer patients and facilitate our knowledge of the carcinogenic process in humans. With the ability to quantitate cellular interactions during therapy, cellular damage could be routinely monitored to capitalize on this unique opportunity to document carcinogen-macromolecular interactions in humans given quantitated exposure to known and suspected carcinogens. Potentially, there is the opportunity to study low and high responders at the molecular level with relatively simple methods through the full course of therapy, periods of remission, and the development or failure to develop secondary tumors. Specifically, this study could provide correlations between host metabolic response, therapeutic efficacy, dosage, DNA alterations, and development of a second tumor in long-term survivors.

Proposed Course: Continue preparing antisera to CLZ-modified DNA using rabbits for polyclonal antibodies and mice for the subsequent production of monoclonal antibodies. We will proceed with characterization and preliminary tests of its usefulness.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05291-01 LHC
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PERIOD COVERED
October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)
DNA Adducts in People Exposed to Benzo(a)pyrene

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	A.K.M. Shamsuddin	IPA	LHC	NCI
	Nuntia Sinopoli	Visiting Fellow	LHC	NCI
	Dean L. Mann	Senior Investigator	I	NCI

COOPERATING UNITS (if any)
Mt. Sinai School of Medicine, New York, NY; NCI-Naval Medical Oncology, DCT, NCI; LIVCTP, DCCP; University of Maryland School of Medicine, Baltimore, MD

LAB/BRANCH
Laboratory of Human Carcinogenesis (LHC)

SECTION
Biochemical Epidemiology Section (BES)

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER: 0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Exposure to benzo(a)pyrene (BP), an 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), foundry workers, and DNA from lung tissue, bronchial washing and alveolar macrophages of lung cancer patients, smokers, etc., are being investigated. BP-DNA antigenicity has been detected in several of the high risk individuals. Preliminary data on sera of some of these individuals also suggest presence of anti BP-DNA antibodies. These data suggest the activation of BP to its ultimate carcinogen as well as formation of adducts with DNA occurs in humans.

Project Description

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 of activation and mechanism of carcinogenesis in humans.

Methods Employed: Twenty-five to 40 ml of peripheral blood were 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 μ Cl₂; 0.05 m MG Cl₂) using glass homogenizer. The homogenate was centrifuged for 10 minutes at 300 x g at 4°C. The pellet was suspended in HKM-sucrose buffer containing 0.5% Triton 100 and centrifuged (Triton X-100) for 10 minutes at 4°C. The pellet was suspended in HKM-sucrose buffer and recentrifuged. The final pellet was resuspended in 5 ml HKM-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 minutes followed by centrifugation at 10,000 x g for 10 minutes. The aqueous epiphase was removed and treated with RNase (100 μ g/ml) at 37°C for 20 minutes. 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 was determined by absorbance at 260 nm and 280 nm using a Beckman DU8 spectrophotometer. Final volume of DNA solution was adjusted to 1 mg DNA/ml water 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 were received in purified form, ready to be tested.

Competitive enzyme immunoassays, 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 were determined from the standard curves. All tests and assays were done in duplicate, and the standard deviation was less than 10%.

Major Findings: Seven of 28 tested DNA samples from asphalt workers, several Lung cancer patients, and foundry workers were positive for BP-DNA antigenicity. The weakest antigenicity detected is 2 fm/50 μ g which is equivalent to 1 BP-DNA adduct in 75 x 10⁶ bases. Assays for anti-BP-DNA antibodies in human demonstrated a competitive inhibition of rabbit anti-BP-DNA antibody in some humans.

Significance to Biomedical Research and the Program of the Institute: Demonstration of carcinogen-DNA interaction in the 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 us with an opportunity to screen high risk individuals using 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. We are also further characterizing the anti-BP-DNA antibodies using various other antigens. Once consolidated, these results would form the basis for conducting biochemical epidemiology studies of high risk individuals.

Publications:

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. (In press)

Hsu, I-C., Poirier, M.C., Yuspa, S.H., Grunberger, D., Weinstein, I.B., Yolken, R.H., and Harris, C.C.: Measurement of benzo(a)pyrene-DNA adducts by enzyme immunoassays and radioimmunoassay. Cancer Res. 41: 1091-1096, 1981.

Hsu, I-C., Yolken, R., and Harris, C.C.: Ultrasensitive enzymatic radioimmunoassay. In Langone, J. J. and Van Vunakis, H. (Eds.): Methods in Enzymology, Vol. 73B. New York, Academic Press, 1981, pp 383-394.

Yuspa, S. 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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05292-01 LHC
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PERIOD COVERED
October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Effects of Tumor Promoters and Cocarcinogens on Growth and Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: James C. Willey Medical Staff Fellow LHC NCI

Others: John F. Lechner Expert LHC NCI
Curtis C. Harris Chief LHC NCI

COOPERATING UNITS (if any)
University of Maryland School of Medicine, Baltimore, MD; Litton Bionetics, Kensington, MD; Georgetown University School of Medicine, Washington, DC

LAB/BRANCH
Laboratory of Human Carcinogenesis (LHC)

SECTION
In Vitro Carcinogenesis Section (IVCS)

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER: 0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Models were developed using human bronchial epithelial (HBE) 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 using concentrations that were maximally mitogenic or that gave no more than 50% cell death by observing effects on cells exposed for 6 hrs. They were observed for morphologic changes under light microscopy and assayed for change in ornithine decarboxylase (ODC), plasminogen activator (PA), aryl hydrocarbon hydroxylase (AHH) activity, and cross-linked envelope (CLE) formation. Initial findings indicate that 12-O-tetradecanoylphorbol-13-acetate (TPA) or teleocidin (1-100 nM) induce differentiation which is accompanied by an increase in PA and CLE formation while other compounds tested thus far cause some mitogenicity with increased AHH formation or no observable effect. When these initial studies are completed, selected compounds will be used for in vitro carcinogenesis experiments and to investigate their mechanisms of action.

Project Description

Objectives: To evaluate biochemical, morphological, growth, and differentiation effects of cocarcinogens/promoters on HBE.

Methods Employed: Outgrowths of human bronchial epithelial cells were subcultured and plated at 100,000 cells/well into 24 well plates in MCDB-151 media minus epidermal growth factor (LHC-0), or MCDB-151 media plus thymoxine (T³), pituitary extract (PEX) (LHC-4). Twenty-four hours later, media was replaced with fresh media containing cocarcinogen, and the cells were exposed for 6 hours. At the end of 5 hours, highly purified human plasminogen was added to each well in which plasminogen activator (PA) was to be tested. After an additional hour of incubation, the following were done (1) photographs were taken of all cells representing cells under each condition; (2) aryl hydrocarbon hydroxylase (AHH) media was replaced with 1 ml of media containing ³H-benzo[a]pyrene (B[a]P) at 32 μ Ci/ml. After a 6 hour exposure to B[a]P, media was removed along with 1 ml of a PBS wash. A 200 μ l aliquot was then placed on a charcoal-alumina column, eluted twice with 1 ml of distilled H₂O (dH₂O), and the eluent containing ³H-H₂O was counted; (3) ornithine decarboxylase media was removed, and the cells were frozen at -70°C later to be assayed with ¹⁴C-ornithine according to the method of Lichti; (4) plasminogen activator media was removed to microfuge tubes on ice and centrifuged for 1 min to remove any loose cells; 90 μ l were added to 12 x 100 glass tubes and incubated with 10 μ l benzyloxy carbonyl-glycyl-L-prolyl-L-arginyl-anilide [aniline-¹⁴C]. Glycine-L-proline-L-arginine [¹⁴C]anilide. During this incubation, any plasmin formed would cleave ¹⁴C-aniline from the original substrate. The ¹⁴C-anilide partitions into lipophilic phase which was counted; (5) cross-linked envelopes. The cells were incubated with 20% urea for 3 minutes, then exposed to 1% trypsin for 5-10 min. at room temperature. An aliquot of cells were counted on the hemocytometer, and the rest were exposed to 2% sodium dodecyl sulfate (SDS) and 10 mm dithiothreitol (DTT) for 10 min. An aliquot was then counted on the hemocytometer for cross-linked envelopes.

Major Findings: This is a new project. Cytotoxicity-mitogenicity data have been obtained for phorbol, 12-O-tetradecanoylphorbol-13-acetate (TPA), teleocidin, 2,7-dichlorodibenzo-p-dioxin (DCDD), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (10⁻⁷ M, 10⁻⁸ M, and 10⁻⁹ M) and for CSC (0.1, 1, 10 μ g/ml) and catachol, B[a]P, B[e]P, and pyrene (1.5 x 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. Phorbol had no apparent effect on colony forming efficiency or population doubling time relative to DMSO control. TPA and teleocidin induced rapid differentiation at all concentrations tested. DCDD and TCDD induced an increase in colony forming efficiency and colony size in the LHC-4 media. At 1.5 x 10⁻⁶ M, B[a]P, B[e]P, and pyrene induced differentiation and cessation of division as did cigarette smoke condensate at 10 μ g/ml. At 1.5 x 10⁻⁸ M catechol and 0.1 μ g/ml CSC had significant mitogenic effects, while the other compounds had little effect compared to the DMSO control. ODC, aryl hydrocarbon hydroxylase, plasminogen activator (PA), and CLE information have been obtained for phorbol, TPA, teleocidin, DCDD, and TCDD at 10⁻⁷ M and for cigarette smoke condensate at 10 μ g/ml and B[a]P, B[e]P, catechol, and pyrene at 1.5 x 10⁻⁶ M. The values within the parentheses will indicate percent relative to DMSO control. TPA or teleocidin cause a decrease in

ornithine decarboxylase (60% and 80%, respectively), and TPA, a decrease in aryl hydrocarbon hydroxylase (60%), and they both effected an increase in PA had little effect. 2,7-Dichlorodibenzy-p-dioxin and TCDD effected an increase in ornithine decarboxylase (180% and 190%, respectively), an increase in AHH (150% and 300%, respectively), an increase in PA (210% and 240%, respectively), and an increase in CLE (21% and 240%, respectively). 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.

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 media 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 HBE and select compounds which demonstrate effects potentially relevant to human carcinogenesis for further investigation.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05293-01 LHC
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PERIOD COVERED
October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)
DNA Repair and Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George Yoakum	Senior Staff Fellow	LHC	NCI
Others:	Brent Korba	Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Herman Autrup	Senior Staff Fellow	LHC	NCI
	John F. Lechner	Expert	LHC	NCI

COOPERATING UNITS (if any)
Department of Biochemistry, Johns Hopkins University School of Public Health, Baltimore, MD

LAB/BRANCH
Laboratory of Human Carcinogenesis (LHC)

SECTION
Carcinogen Macromolecular Interaction Section (CMIS)

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.33	PROFESSIONAL: 1	OTHER: .33 (SIS)
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have initiated a study of the role of the initial step of DNA repair in human carcinogenesis. This research program utilizes recombinant DNA technology and DNA repair genes from *E. coli*. Having successfully amplified *E. coli* uvr-genes to high levels, we are producing antibodies to uvr-proteins for detection of cross-reacting antigens in human cells. In addition, we have developed a method to genetically identify DNA repair gene products by inactivation mapping and specific labeling of plasmid encoded gene products.

Project Description

Objectives: The primary goal of this research project is to understand the genetic and enzymatic mechanism(s) of human DNA repair processes, their relationship to carcinogenesis, and potential for cancer prevention.

Methods Employed: 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 LHC. Selection schemes to isolate revertants of UV-sensitive mutant human cells in culture and selection of gpt^+ transfectants after $CaPO_4$ -DNA treatment of human cell cultures will utilize adaptations of those methods previously reported for selection of transfectants in mouse cell cultures.

Major Findings: We have developed a method during the preceding year to construct plasmids which amplify DNA-repair gene products to several percent of total cellular protein by weight. In addition, we have identified *uvr*-gene products by combining gene inactivation by insertion mapping with specific labeling of plasmid-encoded polypeptides. This provides the means to obtain large amounts of highly purified *uvr*-gene products for 1) enzymatic characterization, 2) physical studies; and 3) production of antibodies for detection of cross-reacting repair antigens in heterologous systems (i.e., human cells).

In addition, the development of plasmids carrying well characterized segments of *E. coli* DNA with *uvr*-genes which are expressed from a novel promoter, i.e., pGHY5003, provides the starting plasmid for development of a unique vector capable of expressing heterologous genes for complementation of *E. coli* mutants lacking a similar function. A segment containing a portion of the *amp* gene and a poly-G 3'-end will serve as a selectable adaptor, and an oligonucleotide insert into a 95% deletion of the *uvr*-gene with a restriction site which leaves a 3' end (SstI) will provide a site of hybridization for polyadenylated message for DNA polymerization. This plasmid should be capable of expressing and amplifying heterologous genes inserted at the SstI site in a fashion similar to that observed for *uvr*-gene inserts in pGHY5003.

Significance to Biomedical Research and the Program of the Institute:

Isolation and study of the genes and gene products which respond to UV-damage after UV-irradiation of human cells will permit us to determine the role that DNA repair plays in human carcinogenesis after treatment with a variety of UV-mimetic agents. An understanding of the role which DNA repair plays in carcinogenesis will provide the means to alter the repair capacity of cells to appropriately respond to DNA insults which may cause permanent genetic changes and ultimately lead to carcinogenesis. In addition, development of heterologous vectors capable of use for genetic and physiologic studies of human genes in *E. coli* will provide a tool for broad application to the study of human genetics. Similarly, the improvements in gene transfer technology required to express transfected genes at a good frequency in human cells in culture will prove generally useful to the study of human genetics.

Proposed Course: 1) Isolation of human DNA repair gene(s). 2) Construction of an heterologous vector capable of regulated expression of human genes in *E. coli*. 3) Genetic and physical characterization of human DNA repair genes.

4) Enzymatic characterization of human DNA repair gene products. 5) Production of antibodies against DNA repair gene products for epidemiologic studies to determine the relationship of population variations of DNA repair enzyme levels and predicted risk for eventual carcinogenesis among low-level variants in human populations.

Isolation of human DNA repair genes will begin with construction of a vector capable of expressing human genes for selection in *E. coli*, and *uvr*-gene hybridization to human genomic libraries to detect homologous human genes. Initial characterization of the heterologous vector described above will include insertion of the HBV surface antigen gene as a test signal to determine the extent of P1-control over expression and potential for amplification of heterologous inserts for polypeptide production. After isolation of human DNA repair genes, insertion inactivation will be used to identify gene products of interest and protein purification schemes worked out to isolate large quantities of repair proteins for enzymatic and physical study. In addition, antibodies against purified human repair proteins will be produced for epidemiologic studies.

Publications:

Yoakum, G.H. and Grossman, L.: Identification of *E. coli* *uvrC* protein. Nature 292: 171-173, 1981.

Yoakum, G.H., Yeung, A.T., Mattes, W.B., and Grossman, L.: Amplification of the *uvrA* gene product of *E. coli* to 7% of cellular protein by linkage to the P₁ promoter of pKC30. Proc. Natl. Acad. Sci. USA 79: 1766-1770, 1982.

CONTRACT INDEX

LABORATORY OF HUMAN CARCINOGENESIS
DIVISION OF CANCER CAUSE AND PREVENTION
NATIONAL CANCER INSTITUTE

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CONTRACT NARRATIVES
LABORATORY OF HUMAN CARCINOGENESIS
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1982

EXPERIMENTAL PATHOLOGY LABORATORIES, INC. (N01-CP-95645/3 and 4)

Title: Resource for Microscopic and Autoradiographic Technology

Contractor's Project Director: Dr. Beverly Y. Cockrell

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: Routine preparation of biological material for light and electron microscopy.

Methods Employed: Standard electron microscopic techniques have been employed. Various embedding media have been utilized in the processing of many different sample types including tissue culture monolayers in plastic dishes, cell pellets, large tissue pieces, whole fetal kidneys, and tumors.

Major Findings: All data from this contract are in the form of either one micron section on glass slides or electron micrographs which are returned to NCI originators for interpretation. In those cases where a short report of electron microscopic findings is returned to NCI, that report is a microscopist's impression of each sample, and any project findings are made at NCI.

Significance to Biomedical Research and the Program of the Institute: Electron microscopy and related techniques are valuable tools in the study of carcinogenesis and in the field of experimental pathology. Service efforts in these areas continue to hasten the advancement and enhance studies within the Institute.

Proposed Course: This basic ordering agreement was terminated as of December 13, 1981.

Date Contract Initiated: January 1, 1977.

Current Annual Level: Minimum amount - \$25,589; Maximum amount - \$50,645.

Man Years: N/A

GEORGETOWN UNIVERSITY (N01-CP-05707)

Title: Human Tissue Collection Resource

Contractor's Project Director: Dr. Henry Yeager

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: To provide NCI with a source of human lung and bronchial tissue

taken at surgery, human alveolar macrophages from fiberoptic bronchoscopy, and bronchial lavage of normal subjects, both smokers and nonsmokers.

Methods Employed: Tissue portions of human bronchus and/or peripheral lung are taken in sterile fashion from specimens available in the Surgical Pathology Division, Georgetown Hospital. Tissues are placed in sterile Lebowitz medium and transported on ice to NIH. Primary pleural tumors and benign pleural growths are sent when available. Pleural fluids are transported in sterile plastic containers or plastic screw-top jars on ice to NCI.

Lavages. Volunteers are screened for normal lung function using standard pulmonary function tests and lung X-rays (PA and lateral). Bronchoscopy and saline bronchial lavage are performed; peripheral blood specimens are obtained for harvesting of autologous mononuclear cells. The centrifuged and washed bronchoalveolar cells are assayed for viability.

Lung macrophages are harvested from normal volunteers by fiberoptic bronchoscopy and bronchial lavage. Volunteers are periodically screened for normal lung function using standard pulmonary function tests and lung X-ray (PA and lateral). Macrophage viability is assessed using trypan blue exclusion. Cells are suspended in RPMI-1640 supplemented with fetal calf serum, penicillin, and streptomycin, placed on ice, and sent directly to NIH.

Major Findings: In this period, the contractor provided a total of 20 bronchial and peripheral lung samples, 17 pleural fluids (both malignant and nonmalignant), and pleural plaques taken at surgery from a patient with asbestosis to NCI for ongoing experiments here.

Significance to Biomedical Research and the Program of the Institute: A supply of human bronchial tissues and pleural fluid cells on an ongoing basis is vitally required for continued experiments in carcinogenesis. The establishment of cultures of human mesothelial cells is poorly understood and is one of the recognized complications of asbestos exposure in human beings.

Proposed Course: 1) to continue to provide human lung tissue and alveolar macrophages to NCI for the remainder of the contract period; 2) to recompute the contract (terminates February 1983) for future acquisition of these resources.

Date Contract Initiated: May 1980.

Current Annual Level: \$52,000.

Man Years: 1.25

LITTON BIONETICS, INC. (N01-CP-43274)

Title: Resource for Xenotransplantation and Evaluation of Human Tissues in Athymic Nude Mice

Contractor's Project Director: Dr. Marion G. Valerio

Project Officer (NCI): Dr. Glennwood Trivers

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.

Methods Employed: Human epithelial tissues or cells are exposed to carcinogens either prior to or after transplantation and maintained in vivo in the athymic mouse as xenografts. Human cells are injected in various sites. A self-sustained breeding colony of athymic nude mice is maintained in a modified barrier facility.

Major Findings: Human bronchus, pancreatic duct, colon, breast, prostate, and esophagus can be maintained for long periods of time as xenografts in the nude mouse as evidenced by a viable-appearing epithelium with normal histology and the incorporation of tritiated thymidine into epithelial cells of the grafts.

Techniques for the use of epithelium-denuded rat tracheas as a ground substance for human bronchus cells have been developed. The bronchus cells have attached to and layered the luminal surface of the tracheas. Human esophageal xenografts have shown good epithelial growth and cyst formation.

Malignant transformation has not been observed in the xenografts. Explants treated in vitro with carcinogens have shown epithelial abnormalities that, when xenografted, have frequently reverted to normal in the nude mouse. Squamous metaplasia has occurred in grafts treated in vivo to several carcinogens, but these lesions have not as yet progressed to malignancy under current treatment regimens.

Significance to Biomedical Research and the Program of the Institute: The establishment of an animal host for human tissue xenografts which have been treated with carcinogens in vitro and/or in vivo will provide a model more predictive of the effects of carcinogens on human tissues than extrapolation from an animal tumor or tissue culture model system. This system provides an opportunity to study chemical carcinogenesis in target tissues for human cancer.

Proposed Course: 1) Continue long-term testing of the effects of various carcinogens and treatment regimens upon the human tissue xenografts in the athymic nude mouse. 2) Determine tumorigenic potential of human cells exposed to chemical and physical carcinogens in vitro. 3) Studies of other investigators suggest high natural killer (NK) cell activity in the nude mouse. Since NK represent a potentially inhibitory effect on the formation of tumors in xenografts, considerable emphasis is being placed on further immunosuppression of the nude mouse to enhance its xenotransplantation capabilities. X-irradiation, anti-mouse interferon antibodies, coinjection of human fibroblasts, antilymphocyte serum, and various monoclonal antibodies are being explored for this purpose.

Date Contract Initiated: June 30, 1981.

Current Annual Level: \$240,000

Man Years: 2.43

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Contractor's Project Director: Dr. Benjamin F. Trump

Project Officer (NCI): Dr. Glennwood Trivers

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.

Methods Employed: Neoplastic and non-neoplastic tissues from human bronchial epithelium, pancreas, colon, and liver are collected at time of surgery and autopsy including immediate autopsy. Samples are processed for morphological study including light and electron microscopic study, histochemistry, and immunocytochemistry. Samples are also collected for organ (explant) and cell culture, metabolic studies, and measurement of ion ratios.

Major Findings:

Bronchus:

1) Morphological and histochemical characterization of human primary lung carcinomas are continuing. Epidemiologic data are being analyzed to study 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) Immunohistochemical characterization of the human tissues was continued using the peroxidase-antiperoxidase method to demonstrate the presence or absence of various antigens. Lung tumors, normal fetal lung and normal, abnormal and preneoplastic adult bronchus were examined for beta human chorionic gonadotropin (HCG), calcitonin, adrenocorticotrophic hormone (ACTH), serotonin, alpha-fetoprotein (AFP), keratin, somatostatin, neuron specific enolase (NSE), calmodulin, and tubulin. Normal and abnormal (but non-neoplastic) adult bronchus contained only mucosubstances, keratin, calmodulin, and tubulin. Keratin was shown only in aldehyde-fixed bronchus, if the epithelium was metaplastic. However, keratin was found in the basal layer of ethanol-fixed normal bronchus, epithelium (including bronchial glands), and occasionally in columnar cells that reached the lumen. Calmodulin was apparently at increased levels at the cell borders. Characterization of lung tumors by these methods revealed greater heterogeneity than observed in the bronchial epithelium. Each marker was found in at least an occasional lung tumor. HCG (80%) and keratin (75%) were demonstrated in virtually all nonsmall cell tumors. Somatostatin was commonly seen in the centers of areas undergoing keratinization and more diffusely distributed in a smaller proportion of adenocarcinomas. Somatostatin immunoreactivity was granular in endocrine tumors. Keratin immunoreactivity in tumors with a well-differentiated epidermoid pattern was present in nearly all cells with variable intensity and a tendency toward perinuclear rings. In other tumors, it was less variable cell to cell, diffuse cytoplasmically, and generally less intense. NSE and serotonin were observed in endocrine tumors only. Appearance of other markers showed less predilection for particular types of lung tumors, although HCG seemed to follow glycogen distribution.

3) Indirect immunofluorescent detection of tubulins was performed on cellular outgrowths of bronchial explants involved and noninvolved 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.

Pancreas:

1) Pancreatic tissues from immediate autopsy are being examined by morphological techniques, histochemistry, immunohistochemistry, and freeze fracture. Pancreatic ducts are maintained using the contractor's organ explant and cell culture techniques. Routine autopsy and surgically derived tissues are examined to elucidate cellular alterations in pancreatic cancer. Presently 40 cases (18 autopsy cases and 22 surgical cases) are under study including both primary and metastatic tumors. Over 90% of these nonendocrine tumors appear as duct cell adenocarcinomas in routine light microscopy; however, selected cases are being studied by electron microscopic mucosubstances, keratin, CEA, alpha-fetoprotein, HCG, and calcitonin.

2) Eighteen pancreatic ducts (routine medical examiner's autopsies) have been cultured in a feasibility study of their usefulness. Viable tissues were present in only 2 of these cases. The time interval following death to culture ranged from 3 to 14 hours. Ducts successfully cultured for 2 weeks came from earlier periods.

Colon:

1) Morphological (LM, TEM, SEM) and histochemical examinations of normal, premalignant and malignant human epithelium were continued. The much needed comprehensive description of the morphology of normal human colon is not yet complete. Indeed, that some of the previously reported morphological markers of premalignant changes may actually represent normal features of the different segments is indicated in these data, i.e., by electron microscopy there are 3 cell types (undifferentiated, endocrine, and mucous), and in ascending segments, apical vesicles are electron microscopy dense, but in the rectum they are electron microscopy lucent. By light microscopy, using histochemical stains, there are differences seen in ascending, transverse, 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 stain 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.

Liver: 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 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 according to

organ:

	<u>Medical Record Abstracted</u>	<u>Patient Interviewed</u>	<u>Data Coded</u>
Bronchus	344	255	305
Colon	235	190	206
Total	579	445	511

Significance to Biomedical Research and the Program of the Institute: Of significance to NCI's program and biomedical research is the pressing need to develop systems in which data can be obtained on the response of important epithelial tissues to toxic and carcinogenic stimuli. This is essential in order to provide methods of interspecies comparisons and extrapolation of animal data to man. It is essential to continue the development and characterization of systems for long-term maintenance and study of interactions of various environmental stimuli with normal human tissues obtained from individuals with non-neoplastic disease. Characterization of such tissues from a variety of standpoints is necessary to assess viability, determine reversibility, assess development of markers for normal, preneoplastic, and neoplastic tissues and to examine a variety of influences on carcinogen metabolism. Such developments will not only improve the study of human tissues but will assist in future development of improved animal models.

Proposed Course: During the coming year, emphasis will continue to be placed on providing this unique resource to NCI.

Date Contract Initiated: February 1, 1981.

Current Annual Level: \$408,001.

Man Years: 9.17

MARYLAND, University of (N01-CP-75909)

Title: Resource for Human Esophageal Tissues and Cells from Donors with Epidemiological Profiles

Contractor's Project Director: Dr. Benjamin F. Trump

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: To provide specimens of human esophagus to the Laboratory of Human Carcinogenesis for carcinogenesis studies.

Methods Employed: Normal human esophagus from immediate autopsies at the University of Maryland, from autopsies by Baltimore City Medical Examiner, and surgically resected esophagi that include carcinoma are collected and distributed to NCI. Morphologic, histomorphometric, kinetic, and immunohistochemical characterizations are performed on normal esophagus from surgical specimens at the time of collection and at subsequent time points in organ culture. Similar techniques are used to investigate one- and two-stage carcinogenesis in the organ culture system.

Major Findings: A total of 56 specimens have been collected and characterized

by the contractor: 39 from the Baltimore County Medical Examiner's autopsy facility, 10 from immediate autopsies, and 7 from surgical resections of esophageal cancer at the University of Maryland. An epidemiological profile of the donors has been provided whenever possible. This arrangement with the Medical Examiner has provided the experience that tissues from this source possess chance viability in organ cultures which decreases rapidly with time after death. Esophagi collected more than 18 hours postmortem are unlikely to be sufficiently viable to survive in vitro, and survival of explants from a particular donor esophagus is either all or none. The contractor examines the baseline (zero-time) morphological characteristics of normal, premalignant, and malignant esophageal epithelium. By light microscopy, the tumors were diagnosed as squamous cell carcinomas with varying degrees of differentiation.

The contractor has previously established the morphological characteristics of normal human esophageal epithelium prior to and for extended periods of time in organ culture (7-181 days). In this culture system, the squamous epithelial characteristics were retained as demonstrated by light and electron microscopy. Efforts to optimize conditions show that CMRL is a more suitable medium than MEM, that rocker cultures and CO₂ incubator cultures are equivalent, and that no additives used increased the prickle cell layer.

In addition, in this period the contractor has contributed the following:

1) Baseline cell kinetic analysis. Experiments indicate that about 4 days, after most of the differentiated portion of the epithelium has sloughed, a vigorous wave of cell replication through day 9 is followed by establishment of kinetically active esophageal epithelium.

2) Organ explant outgrowth analysis. Approximately 70% of outgrowth epithelium labels with tritiated thymidine in 24 hours and appears to maintain epitheloid differentiation.

3) Tumor markers. Normal and malignant human esophagi have been examined for a variety of tumor markers using mainly the immunoperoxidase technique. Normal adult esophagus is uniformly negative for HCG, HPL, CEA, and AFP, while majority of tumors, boundary zones between tumor and normal epithelium are the positive. Approximately 75% of specimens were positive for HCG and HPL in tumors and junctions, with CEA, positive tumors were higher than positive junctions.

Significance to Biomedical Research and the Program of the Institute: The major task of this contract is to provide well characterized tissues from donors with an epidemiological profile to NCI for study of carcinogenesis in human esophageal epithelium. Such studies are important not only for understanding the detailed pathogenesis of the human lesion and for development of methods for the testing of carcinogens in vivo and in vitro, but will also serve as models for the development of similar methodologies in other tissues. The methods and concepts developed in this program can then be used by other groups to foster the further development of carcinogenesis research in human tissues.

Proposed Course: Continuation of current tasks and recompetition of the contract (terminates September 30, 1982) for continuation of these resources.

Date Contract Initiated: September 30, 1977.

Current Annual Level: \$54,000.

Man Years: 1.0

Title: Biochemistry and Cell Culture Resource

Contractor's Project Director: Dr. Rodger D. Curren

Project Officer (NCI): Dr. Glennwood Trivers

Objectives: The purpose of this contract is to provide the Laboratories of Human Carcinogenesis, Cellular Carcinogenesis and Tumor Promotion, and Experimental Pathology with resources for investigations of chemical carcinogenesis in general, and the mechanisms of human carcinogenesis in particular. Tasks supporting the Laboratory of Human Carcinogenesis are listed here.

Methods Employed: This is a multifaceted contract serving three different Laboratories in the Carcinogenesis Intramural Program. The methods used are provided by the individual program personnel and involve synthesizing biologically active molecules, culturing cell lines from rodent and human sources, analyzing the in vitro effects of known and suspected carcinogens, and determining the biochemical nature of carcinogen metabolites.

Major Findings:

Human Bronchus Culture

1) The contractor grows and cryopreserves human bronchial epithelial cells and human bronchial fibroblasts. In order to expand the epithelial population to a size at which it can be frozen, large amounts of Swiss mouse 3T3 cells are needed for feeder layers. To date the contractor has produced, irradiated, and delivered 7.4×10^9 cells (50 biweekly deliveries) for use at MCI, plus approximately 10-20% of that amount for use in the contractor's facility.

2) The contractor cryopreserved 198×10^6 bronchial epithelial cells from 10 different sources and used bronchial explants from 4 of these sources to generate fibroblasts (388×10^6) which are now frozen at various passage levels. Large pieces of intact explant have been cryopreserved and used to initiate new epithelial cultures following in storage up to 9 months.

3) Two tasks initiated this year were 1) continuous passage of a carcinogen altered bronchial epithelial cell line and 2) culturing of human mesothelial cells for asbestos studies. The carcinogen-altered cells were passaged nine times and samples were cryopreserved. The mesothelial cells were difficult to grow and to clone. Recently, however, cloning efficiencies of up to 20% were obtained which allowed the initiation of cytotoxicity assays with various types of asbestos.

Cell-mediated Mutagenesis

Experiments to establish a lymphoblastoid cell line as target cells in a cell-mediated mutagenesis assay were attempted. 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) analysis of BP metabolites, separation of BP conjugates on alumina columns, and extracting DNA from tissue

samples to measure the H³-BP binding levels were continued. The contractor has analyzed 180 media samples by HPLC, processed 90 media samples on alumina columns, and extracted DNA from 127 tissue samples to measure the bound BP. Methodologies were studied for separating various organic extractable aflatoxin B₁ metabolites using both thin layer chromatography and high pressure liquid chromatography. The result was successful separations of the major metabolites, including AFQ₁, AFM₁, and AFP₁. Other experiments employed alternative methodologies for separating aqueous soluble metabolites of AFB₁ metabolism, and are still in progress.

Significance to Biomedical Research and the Program of the Institute: These support services represent a significant contribution to the ability of these laboratories to achieve more extensive programs and investigations that deal primarily with mechanisms of in vitro chemical carcinogenesis in animal and human tissue. An understanding of the in vitro relationship between these species may lead to answers to the more important questions concerning their in vivo responses to chemical carcinogens.

Proposed Course: Contract will terminate in February 1983.

Date Contract Initiated: July 1, 1980.

Current Annual Level: \$100,000.

Man Years: 2.64

NATIONAL NAVAL MEDICAL CENTER (Y01-CP-80204)

Title: Procurement of Human Tissues

Contractor's Project Director: Dr. R. Zajtchuk

Project Officer (NCI): Dr. Glennwood Trivers

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, and the capability to metabolize carcinogens to mutagens.

Methods Employed: Using the operating room schedules for the following day, a medical biology technician contacts the attending physician(s) for permission to seek informed consent and medical history information from subject patients. On the day of surgery, the technician notifies NCI of a pending specimen, arranges with the pathologist for assistance in collecting the tissue and provides a sterile container and cold L-15 medium. With the surgeon's permission and assistance, the technician obtains human tissue, transfers the specimens to the pathologist for sterile dissection of the nontumorous tissue, then immediately transfers the material to NCI researchers for final dissection and subsequent culturing.

Major Findings: Since the initiation of this agreement, the National Naval Medical Center (NNMC) has delivered a total of 157 surgical specimens of human lung (34) and colon (123) to NCI. After an hiatus in availability of a

technician due to the hiring freeze, 6 lung and 21 colon specimens were collected. Appropriate clinical history has been obtained on all patients, and the specimens have been properly processed through the Pathology Department of the NNMC. Another 23 candidates presented at surgery, but did not have cancer or were not resectable, and, therefore, no specimens were available.

Significance to Biomedical Research and the Program of the Institute: Lung and colon cancer are among the most frequent types affecting the human population. They are also among the forms of cancer most likely to be influenced by environmental factors such as chemicals; therefore, it is important to determine whether or not persons with diagnosed disease or who are suspected of preneoplastic changes suffer a predisposition or increased susceptibility to the transformational effects of chemical carcinogens. Research of this nature has potential for both cancer detection and cancer therapy.

Proposed Course: Additional specimens will be obtained at Walter Reed Army Hospital and, thus, increase the volume.

Date Interagency Agreement Initiated: March 31, 1978.

Current Annual Level: \$15,150.

Man Years: 1.0

UNIFORMED SERVICES UNIVERSITY OF HEALTH SCIENCES (Y01-CP-00502)

Title: Hybridoma Resource

Contractor's Project Director: Dr. John Martin

Project Officer (NCI): Dr. Dean Mann

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 bronchial 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.

Methods Employed: Immunization of experimental animals, preparation of mouse and human hybridomas, production of monoclonal antibodies, and enzyme immunoassays of antigens and antibodies.

Major Findings: This is a new contract. Preparation of monoclonal antibodies to chemical-DNA adducts are in progress.

Significance to Biomedical Research and the Program of the Institute: Cancer of the lung and related tissue is one of the most frequent types in humans. It is also the one most suspected to be chemically induced. Successful development of these reagents could contribute to early detection of persons at risk for tumor development and will provide invaluable data on the relationship of cellular and macromolecular damage and the development of neoplasia

of the lung.

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.

Date Contract Initiated:

Current Annual Level: \$87,000

Man Years: .75

VETERANS ADMINISTRATION HOSPITAL (Y01-CP-60204)

Title: Resource for Procurement of Human Tissues from Donor with an Epidemiological Profile

Contractor's Project Director: Dr. Paul W. Schafer

Project Officer: Dr. Glennwood Trivers

Objectives: The general objectives of this interagency agreement are 1) to effect a better morphologic and biochemical characterization of normal, pre-malignant, 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.

Methods Employed: An abstract of the clinical record has been prepared for each patient whose tissues were entered into the collaboration. The patient's specific problem requiring operative intervention is therein identified. Results of specific preoperative diagnostic procedures are included together with such assessments of pulmonary functions as were undertaken to evaluate the patient's ability to withstand surgery. Pulmonary disease other than that for which the surgery was performed has also been identified. Sputum, brush, and lavage specimens and biopsies have been characterized morphologically.

Gross pathology encountered at surgery together with pertinent aspects of the operative procedures are entered in the input protocol. The operative procedures employed range from local excisions, through segmental or complete lobectomy and bi-lobectomy, to total pneumonectomy. Only those tissues are resected that are required for diagnostic and therapeutic purposes. With a tissue proven preoperative diagnosis, a definitive resection without further biopsy is performed when technically possible. In the absence of a positive preoperative diagnosis, the diagnostic procedure of choice is a total local excision; when this is not technically impossible, incisional biopsy is used. No tissues are obtained solely for the purposes of investigation.

All resected specimens are aseptically dissected promptly upon removal from

the patient. Parts of the specimen critical to the requirement of the Veterans Administration Hospital laboratory service, particularly those tissues at the line of resection, are left untampered. As much of the bronchial tree as otherwise could be obtained is dissected free. Adequate samples are taken of all disease processes encountered. Representative samples of the foregoing are variously prepared for light and electron microscopy. The remainder are placed in L-15 medium and stored at 4°C and transported to NCI for culturing and subsequent xenotransplantation studies.

Major Findings: The contractor obtains tissue samples of lobar, segmental and sub-segmental bronchi from 108 patients who underwent the following operations:

Right pneumonectomy	11
Left pneumonectomy	10
Right upper lobectomy	34
Right middle lobectomy	4
Right lower lobectomy	13
Left upper lobectomy	12
Lingulectomy	2
Left lower lobectomy	10
Bilobectomy	3
Local excision	9
Total	<u>108</u>

By light microscopy, it has been ascertained that 10 of the 88 patients did not harbor a malignancy, but rather had aspergillosis, cryptococcosis, sequestration, bronchiectasis, and granuloma. The pulmonary malignancies were identified as follows:

Squamous cell carcinoma	61
Adenocarcinoma	17
Undifferentiated carcinoma	11
Broncho-alveolar carcinoma	3
Carcinoid	3
Carcinoma <u>in situ</u>	1
Metastatic	<u>2</u>
Total	<u>98</u>

Tissues provided also included 11 colon and 8 esophageal specimens.

Significance to Biomedical Research and the Program of the Institute: Model systems incorporating human tissues in culture or in experimental hosts are promising targets for experimental carcinogenesis by suspect classes of environmental agents. Ultimately, they may constitute the basis for the development of new prophylactic and definitive therapies.

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.

Date Contract Initiated: October 1, 1982

Current Annual Level: \$51,136.

Man Years: 1.6

ANNUAL REPORT OF THE LABORATORY OF MOLECULAR CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1981 to September 30, 1982

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 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. These processes are studied in biological preparations and cells from experimental animals and humans.

Although the objectives of the Laboratory have been unchanged during recent years, the projects and the technology must and do change as new concepts and methodologies emerge. From transfection experiments that have been reported from other laboratories, it appears that many oncogenes are normal genes that have gone astray--genes whose expression has been either misplaced in time or been augmented inappropriately. It is a central hypothesis of the research of this Laboratory that such changes are frequently produced by chemical carcinogens acting on genomic DNA. The transfection experiments do not rule out the possibility that cancer may arise by modification of elements other than genomic DNA, but in our judgement such considerations are less likely to be fruitful at present.

We consider it important, therefore, to continue efforts to characterize the damage that is produced in the DNA genome by such carcinogens and the mechanisms by which such damage may be repaired. We also consider it important to study, generally, the factors which control gene expression in order to identify the means by which oncogenes (or their logical equivalents) ultimately produce malignant disease.

In the Section summaries which follow, progress in these areas is outlined. These reports discuss several successes in identifying, with considerable precision, the nature of mutations resulting from the action of chemical carcinogens. In these and other reports, increasing use of the new DNA technology has been the major factor in finding new and specific information about the action of carcinogens. Our recent emphasis on recruitment of younger investigators skilled in the new arts of recombinant DNA has led to an infusion of these methods throughout the Laboratory.

The experience gained with the use of monoclonal antibodies in projects to characterize enzymes involved in polycyclic hydrocarbon metabolism has led to very major advances in the knowledge of these enzymes and in our ability to study the human genetics of carcinogen and drug metabolism. In addition, the introduction of the hybridoma technology has and will have further impact on many other areas of our laboratory research program.

Cell Genetics Section - Studies (1) the molecular mechanism of initiation of malignant transformation of cells by chemical carcinogens and irradiations, (2) the cancer prone genetic diseases, (3) the structure and function of cellular genes and their products involved in the expression of the transformed state. 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 molecules or metabolism directly involved in these crucial processes of cell transformation.

Current primary areas of research in this Section are 1) development of an efficient system for characterizing the malignant transformation of cultured rodent and human cells by chemical carcinogens and irradiation; 2) investigation of the genetic and physiological factors affecting cell transformation with special attention to DNA repair function and promoter function; 3) mechanism of cellular hypersensitivity of cancer prone genetic disease patients and its relation to clinical abnormalities; 4) cellular and molecular effects of psoralen plus long wavelength ultraviolet radiation (UV-A); 5) mechanism of the alteration of gene expression associated with the malignant transformation and identification of macromolecules involved in the expression of the transformed state and 6) mechanism of enhancement by promoting agents of carcinogen-initiated cell transformation.

Attempts are being made to develop a rapid, reliable, convenient, and quantitative system for assay of malignant transformation of cells. Success in this project will lead to discovery of the crucial factors involved in cell transformation as well as an improved assay system for assessment of human risk from environmental carcinogens. Improvements in the methods for DNA transfection and in the human diploid cell transformation assay system have been made. The conditions required for maximum enhancement of carcinogen-initiated transformation by tumor promoters have been established using the Balb/3T3-A31-1-1 line. The optimum conditions were very similar to those required for the promotion of skin carcinogenesis. The potencies of various promoters in enhancing transformation in vitro generally paralleled their potential to promote skin carcinogenesis. These observations, combined with other data on the properties of promoters in A31-1-1 cells, indicate that the enhancement by promoters of transformation of A31-1-1 cells represents at least some part of the in vivo tumor promotion process and that the in vitro system may serve as a model system to study the action of promoters. Systems to detect cocarcinogens, "weak carcinogens" and presumptive "pure initiators" are being developed using A31-1-1 cells. The mechanism of cell transformation was investigated by examining the genetic and physiological factors which affect cell transformation induced by chemicals and irradiation. With nine phorbol ester derivatives and two teleocidin derivatives, close correlation was observed between the enhancement of the carcinogen-initiated transformation, the induction of early biochemical changes (such as a

stimulation of DNA synthesis, glucose uptake in the G0-arrested cells, stimulation of arachidonic acid release inhibition of binding of epidermal growth factor to membrane receptor), and the promotion of carcinogen-initiated skin carcinogenesis in mice. The phorbol ester derivatives showed strong activity in inducing early biochemical effects but weak activity in enhancing transformation. The weak transformation-enhancing potentials of these two derivatives have been ascribed to the short life of their biochemical effects. The accumulated biochemical effects induced by the promoter derivatives were closely correlated with their transformation-enhancing potencies. These results indicate that continuity of the biochemical effects of promoters is essential for the fixation of enhancement of transformation. In the cell mutant which is highly sensitive to chemically induced transformation, the biochemical effects of promoters last longer compared to those in the resistant cells. Further molecular studies on the differences in continuity of biochemical effects between promoters and between cell mutants are underway.

Studies have shown that alternating purine-pyrimidine sequences in DNA can form a left-handed helix called Z-DNA. The presence of such sequences in natural genomes would have great implications in the regulation of gene expression and in the processes of cell differentiation and carcinogenesis. Various eukaryotic genomes have been surveyed for potential Z-DNA-forming sequences. A high copy number of a dT-dG alternating sequence was found in several eukaryotic genomes, including yeast and human. Two different cloned human actin genes contained the sequences (dT-dG)₂₅ and (dT-dG)₁₅. A comparison of the nucleotide sequence of the dT-dG alternating region and its flanking region in various genes indicated that the repeated element consists only of (dT-dG)_n (designed the Z1-element). Another purine-pyrimidine alternating sequence, (dT-dC), was found to be moderately repeated in some eukaryotic genomes, but not detected in others (the repeated element with dG-dC alternating sequence is designated the Z2-element). These results provide direct evidence for the general and abundant occurrence of potential Z-DNA-forming sequences in eukaryotic genomes. Experiments are now underway to test the hypothesis that Z-DNA sequences may be regulatory elements for gene expression and even a key element in carcinogenesis.

Because it is unstable in aqueous solution, it is not clear how the active metabolite of benzo(a)pyrene (BP), benzo(a)pyrene diol epoxide (BPDE I) survives transport from the cytoplasm where it is formed, to the nucleus, where it reacts with DNA. By using an exogenous plasmid DNA method to assay the binding activity of BPDE I to DNA it was found that the microsomal fraction of the cell homogenate has the ability to stabilize BPDE I in aqueous solution. However, the inability of partially purified P-450 to stabilize BPDE I indicates the importance of a lipid moiety in protecting BPDE I from hydrolysis. Translocation of BPDE I from microsomes to nuclei was demonstrated in human cell culture (KD) and in rat hepatocytes, indicating that an active carcinogen metabolite can be transferred from its activation site in the cytoplasm to the target molecule in the nucleus. A portion of BPDE-I remained active in cells by a cellular stabilizing mechanism. Thus, secondary BPDE-I DNA adducts were formed upon cell lysis during the isolation of DNA. This property introduced errors in the estimation of the amount of carcinogen actually bound to DNA, which in turn led to errors in the estimation of the repair rate in the cells.

As another approach to identify the genetic factors involved in cell transformation, human cancer-prone genetic diseases are being studied with special attention to 1) identifying groups of people with an increased susceptibility to environmental carcinogenesis, 2) correlation of cellular hypersensitivity with clinical abnormalities, and 3) the molecular basis for the cellular hypersensitivity. Patients with xeroderma pigmentosum (XP), ataxia telangiectasia (AT), and other diseases caused by ultraviolet (dysplastic nevus syndrome) were studied. Detailed examinations of the clinical features of affected individuals were made. A registry of XP patients is being established. Cultures of skin and blood are being established and the effects of cell survival, mutagenesis, and DNA damaging agents are being examined. An attempt to clone the genes responsible for UV sensitivity in XP cells is in progress. These studies may give insights into mechanisms of cancer induction and suggest modes of cancer prophylaxis. In addition, these diseases serve as models for studies of human environmental carcinogenesis.

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. An in vitro assay has been developed to measure the effects of UV-A mediated psoralen-DNA binding in human lymphoid cells. Parameters monitored included 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 changes in immune reactivity. These studies indicated 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. Studies are in progress to evaluate whether lymphoid cells from patients with cancer-prone genetic diseases have increased sensitivity to psoralen plus UV-A induced cell killing and mutagenesis. These studies are aimed at understanding the mechanism of cell damage induced by psoralen plus UV-A so that toxicity to human cells during therapy may be minimized and to identify individuals with abnormal sensitivity.

Concentrated attempts are being made to clarify the nature of the cellular molecules responsible for the malignant phenotype of chemically transformed human cells. The mutated beta-actin gene, which we had found previously in chemically transformed human fibroblasts, was found to be further mutated in subclones of the transformed cells. Variations in expression of the mutated beta-actin gene were accompanied by corresponding changes in the malignant potential of the subclones. The expression of this mutated beta-actin was also associated with the expression of the in vitro transformed phenotypes in hybrid cells formed between the transformed and normal parent human fibroblasts. These observations suggest that the mutated beta-actin has an important role in the expression of the transformed phenotypes. A human cardiac muscle actin gene was isolated from a human DNA library and its molecular structure and evolutionary origin was determined.

Computerized microdensitometry of autoradiographs of two-dimensional gel electrophoresis of the polypeptides from transformed and normal cells revealed that, as a consequence of the neoplastic transformation of human fibroblasts by a chemical carcinogen, less than 2% of the genes were activated or shut off, but at least 32% were modulated quantitatively. Classes of "highly variable" and "marginally variable" polypeptides were established.

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 susceptibility and sensitivity, (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 detoxification forms or to active carcinogenic forms. During evolution, humans have been exposed to foreign chemical compounds (including carcinogens) and have developed systems for their detoxification and elimination. 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 known sequence of PCH metabolism is as follows: The first step is oxygenation by the microsomal mixed-function oxygenases. Aryl hydrocarbon hydroxylase (AHH) activity is one indicator of this step. Very sensitive methods have been developed for AHH activity which are applicable to human tissues. Secondly, epoxides are hydrated to dihydrodiols by epoxide hydratase. The epoxides are also metabolized by glutathione S-transferases. The phenols and dihydrodiols are conjugated to UDP glucuronic acid or to sulfates. Sensitive assays for each of these systems, as well as a method for total metabolite analyses by high pressure liquid chromatography (HPLC), have been developed.

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 multileveled 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.

Earlier, we introduced the use of HPLC for the analysis of polycyclic aromatic hydrocarbon metabolism. We are continuing this development in an attempt to improve the separation efficiency and reproducibility of analysis of polycyclic aromatic hydrocarbon metabolism. We have found that the addition of anti-oxidants prevents oxidation of low levels of metabolites and we have used this improvement to analyze low levels of metabolism in human cells.

We have successfully made one of the major advances of the last decade in the study of the genetic basis of human variability in carcinogen and drug metabolism. We have successfully prepared monoclonal antibodies to four different forms of cytochrome P-450, the major receptor enzyme for drugs, carcinogens, and environmental chemicals. We have used one of these MABs to prepare an atlas of cytochrome P-450 in human tissues sensitive to the MAB. Cytochrome P-450 (P-450) dependent AHH and 7-ethoxycoumarin deethylase (ECD) in human tissues were differentially inhibited by MAB's that were prepared to, and completely inhibited the activity of MC-induced rat liver cytochrome P-450. The AHH and ECD of placentas from individual women who smoked was inhibited by the MAB's by 83-90% and by 34-74% respectively. Benzo(a)anthracene (BzA)-induced AHH and ECD in lymphocytes were inhibited 18-65% and 30-78% respectively. These enzyme activities in both control and BzA-induced human cells in culture were inhibited to different extents. Both the AHH and ECD in control and BzA-induced monocytes and in normal liver were largely unaffected by the MAB. Thus, we have with the MAB's (1) identified P-450's with a common antigenic site in placenta, lymphocytes and human cells in culture; (2) identified two forms of hydrocarbon-induced P-450s in lymphocytes, at least one of which is common with the induced P-450 of placenta and with a P-450 form present in uninduced lymphocytes; (3) identified two forms of P-450 responsible for ECD activity in smoking-induced placenta, one of which is also responsible for AHH activity; (4) shown that the P-450s of liver and basal and BzA-induced monocytes are different than the MAB-sensitive P-450s of placenta and lymphocytes; (5) quantitated in several human tissues the percent of control and inducible AHH and ECD dependent on the MAB sensitive P-450; and (6) defined by HPLC the contribution of the MAB-sensitive P-450 to the formation of specific benzo(a)pyrene metabolites.

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

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 successfully constructed recombinant bacterial plasmids carrying cDNA complementary to the mRNA for one MC-type cytochrome P-450 from rat. We are now isolating recombinant plasmids carrying phenobarbital-type cytochrome P-450 mRNA sequences as well. We have already used the MC-P-450 mRNA clone to demonstrate that MC-P-450 mRNA is four- to six-fold elevated in liver from MC-treated rats and that there are two size classes of mRNA which code for MC-induced cytochrome P-450. One class of mRNA consists of RNA chains approximately 2000 nucleotides long; the other is 2700 nucleotides long.

We have recently used this cDNA clone to isolate the complete native gene for a MC-P-450. We have carried out restriction analysis and hetero-duplex mapping, showed that this gene is about 5600 nucleotides long, has several intervening sequences, and has structural sequences totalling about 1900 nucleotides. The cDNA clones for other cytochromes P-450 will be used in an analogous fashion to isolate and characterize the native genes for those proteins.

We have analyzed a series of flavone compounds for their specificity toward different forms of cytochrome P-450. Many of these compounds have extraordinary inhibitors or activating activity towards different forms of cytochrome P-450. One of these, L-Maackianin, we found to have activity the reverse of 7,8 benzoflavone, an inhibitor we discovered several years ago. Thus we have two inhibitors with unique specificity to different P-450s. 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.

Nucleic Acids Section - Studies (1) interaction of chemical carcinogens with nucleic acids and their actions on the genetic functions of DNA, (2) establishment of highly sensitive and accurate methods for mutation assay which are applied to the study of molecular mechanisms of mutation, (3) the relationship between genetic defects in DNA repair and human cancer, (4) effect of DNA methylation on gene expression, and (5) detection of oncogenes in chemically and virally transformed rodent cells.

Highly sensitive and accurate methods for the detection of mutations caused by chemical carcinogens have been developed. A plasmid was constructed using recombinant DNA technology, which consisted of two well-defined genetic functions. The plasmid was then modified by reaction with the diol epoxide of benzo[a]pyrene. By exposure of the plasmid to the carcinogen, before the plasmid was introduced into the bacterial cell, problems of toxicity to the cell were minimized. A mutation frequency of two percent (exceptionally high) was observed. The mutated plasmid DNA was recovered from the cells, permitting a determination of changes in the DNA sequence. It was found that the mutations caused by BP diol epoxide were not located at random but tended to cluster in regions of identical nucleotide sequence. The selection of the "hot spot" may depend on specific DNA conformations of an unknown nature.

A new method has been developed to investigate DNA conformational changes arising from drug interaction and various other physico chemical effects in solution. Primary changes introduced by such agents generate higher order changes in the DNA helix. The target sites for covalent binding of chemical carcinogens are determined in part by the structure of the DNA helix and thus subject to dynamic change under various local conditions.

Human tumor cell strains and SV40-transformed human fibroblast strains identified in this laboratory as unable to repair DNA damaged by certain alkylating agents were studied further. Such repair-deficient strains (Mer⁻) are more sensitive than Mer⁺ strains to being killed by certain carcinogens and chemotherapeutic agents and may provide information relating the role of DNA repair both to cancer induction and to cancer therapy. The earlier finding that the Mer⁽⁻⁾ phenotype is often produced during transformation was strengthened by the finding

that Rous sarcoma virus produced Mer⁽⁻⁾ transformed human strains. Mer⁽⁻⁾ strains are deficient in repairing a damaged base, O-6-methylguanine. While able to relax their DNA in preparation to repair DNA damage as well as Mer⁺ strains, Mer⁻ were found unable to introduce supercoiling into their DNA. One exceptional Mer⁻ strain was unable to relax its DNA in which O-6-methyl-guanine was produced, showing that the relaxation step is cellularly controlled and, therefore, likely to be enzymatic. Ethidium bromide titration analysis of nucleoids, prepared from several cell strains during attempted repair, supported this notion. DNA repair in cells damaged by UV or certain alkylating agents was markedly blocked by inhibitors of either DNA synthesis or topoisomerases implicating the involvement of these DNA metabolic processes in DNA repair.

An attempt is being made to define the relationship between methylation of DNA and two important genetic functions, transcription and gene rearrangement and/or amplification. DNA containing 5-methyl cytosine can be identified using restriction endonucleases that are sensitive to methylated DNA sequences. A large number of repeated sequences which showed a concerted methylation were found in chicken DNA. Within long methylated regions, there were specific under-methylated areas which appeared to be an insertion. The insertion is composed of a tandem repeat sequence flanked by unrelated DNA sequence, thus suggesting that this region arose by transposition.

In a project recently started, it is hoped to identify oncogenes generated as a consequence of treating cells with chemical carcinogens. Normal mouse cells will be transfected with DNA from chemically induced rat tumor cells. The DNA from cells in positive foci will then be used as a donor in a second round of gene transfer. DNA isolated from cells of positive foci will be analyzed for the presence of rat DNA sequences using the probe DNA described below. As a first requirement, different rat-repeated sequences have been cloned and labeled ³²P by nick translation. This probe was used to screen recombinant DNA libraries of rat and mouse cells. For detection of chemical oncogenes the donor DNA will be prepared from chemically transformed rat cells. DNA transformation will be performed by CaPO₄ precipitation method.

Protein Section - Studies (1) protein-nucleic acid interactions which may regulate or coordi the production of cellular proteins, (2) specific alterations in DNA sequences which may modify control functions or lead to altered levels of regulatory proteins, (3) mechanisms which modify genetic information in DNA by post-transcriptional processing of RNA.

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 errorfree 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 in 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 is being sequenced.

A system for studying gene stability and rearrangement in normal and tumorigenic cells has been devised. A plasmid shuttle vector has been constructed. 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 will replicate 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 is introduced into a bacterial host which permits the detection of the presence or absence of a functional galactokinase gene. With this assay it is possible to quantitatively assess the stability of the plasmid in the mammalian cells. In our first experiments we have used a permanent cell line of African green monkey kidney cells as the mammalian host and find that about 1% of the replicated plasmids have lost a functional galactokinase gene. Analysis of the defective plasmids indicate that molecules both larger and smaller than the starting material appear. Experiments designed to assay the plasmid stability in tumorigenic cells are in progress.

The influence of the structure of the chromatin fiber on the binding of the chemical carcinogen, benzo(a)pyrene diol epoxide-1 (BPDE-1), to the genome was studied. Antibodies specific to DNA modified by BPDE-1 were elicited and used to visualize the binding sites of the carcinogen to DNA in intact cells and in polytene chromosomes by immunofluorescence and in SV40 minichromosomes by immunoreplication techniques. The results indicate that in polytene chromosomes an apparent "hot spot" for antibody binding corresponds with certain transcriptionally active loci and in the SV40 minichromosomes the non-nucleosomal origin of replication shows a 1.4 increase in BPDE-1 binding as compared to the rest of the chromatin. It is concluded that the packing of the cellular DNA into nucleosomal conformation does not significantly affect the binding of BPDE-1 to DNA.

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin is studied. Antibodies against histones and nonhistone chromosomal proteins have been elicited. Part of the antibodies to protein HMG-1 are directed against sequential determinants. Antibodies to the globular region of the differentiation-related protein H1^o have been elicited and characterized. Functional antibody and antibody fragments have been microinjected into the nuclei of oocytes from *Pleurodeles* and into the cytoplasm and nuclei of human KD fibroblasts. The intact IgG cannot transverse the nuclear membrane, but the F(ab)₂ fragment can. Microinjection of control IgG molecules did not inhibit transcription. In contrast, microinjection of affinity-purified antibodies to HMG-17, histone H2A and H3 did inhibit transcription. The monovalent Fab fragment also inhibited transcription, suggesting that these proteins are present along the transcribable DNA.

A new method for two-dimensional analysis of DNA is under development. The DNA under study is first cleaved with a restriction enzyme; the cleavage products are separated by electrophoresis on specially treated agarose under conditions which permit a second restriction enzyme easy access to the separated fragments.

After a suitable incubation, the fragments cleaved by the first enzyme are cleaved again, this time at new sites specified by the second restriction enzyme. The electrophoresis is repeated, but in a direction perpendicular to the first run. The result is that the number of DNA fragments that can be resolved is approximately the square of the number that can be resolved in a one-dimension analysis. The method is expected to be of great utility in locating polymorphic genes and in identifying segments of the gene that may be rearranged during carcinogenesis. The method will also facilitate analysis of heterogeneous nuclear RNA.

Two minor seryl-tRNAs from mammalian cells suppress the nonsense codon UGA in protein synthesis. They are the first authentic nonsense suppressor tRNAs found in mammals. Furthermore, they form phosphoseryl-tRNA in the presence of an extract from lactating mammary tissue. The phosphoseryl-tRNAs recognize UGA in a ribosome binding assay which suggest that these isoacceptors donate phosphoserine directly to protein.

Transfer RNAs from mammary glands of mice were isolated and separated by two-dimensional electrophoresis to determine changes that may occur in the developing tissue *in vivo* and *in vitro*. Quantitative and qualitative differences in isoacceptor patterns of transfer RNAs obtained from mammary tissues of mice in early and late pregnancy were observed. In contrast, patterns from late pregnant and lactating tissue were very similar. To determine whether patterns of tRNAs might be altered in concert with synthesis of milk protein mRNAs, explants from mammary glands of mice in mid-pregnancy were incubated in synthetic medium with insulin and hydrocortisone in the presence or absence of prolactin. Patterns of tRNAs from prolactin-stimulated and -unstimulated explants labeled with ^{32}P -Pi were similar to tRNAs from late pregnant tissue and to each other.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04496-05 LMC
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Structure and Function of Chromosomal Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION
Protein Section

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TOTAL MANYEARS: 4.8	PROFESSIONAL: 4.0	OTHER: 0.8
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin is studied. Antibodies against histones and non-histone chromosomal proteins have been elicited. Part of the antibodies to protein HMG-1 are directed against sequential determinants. Antibodies to the globular region of the differentiation-related protein H1^o have been elicited and characterized. Functional antibody and antibody fragments have been micro-injected into the nuclei of oocytes from Pleurodeles and into the cytoplasm and nuclei of human KD fibroblasts. The intact IgG cannot transverse the nuclear membrane, but the F(ab)2 fragment can. Microinjection of control IgG molecules did not inhibit transcription. In contrast, microinjection of affinity-purified antibodies to HMG-17, histone H2A and H3 did inhibit transcription. The mono-valent Fab fragment also inhibited transcription, suggesting that these proteins are present along the transcribable DNA.

Project Description

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.

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 in HMG proteins, 3) studying the tissue distribution of H₁^o, and 4) initiating production of monoclonal antibodies against various chromosomal proteins.

Methods Employed: Microinjection, enzyme linked immunoassays, immunofluorescence, immunoreplica, enzyme digestions, electrophoresis, centrifugation, chromatin preparation, monoclonal antibodies, and autoradiography.

Major Findings: 1) Involvement of chromosomal proteins in transcription studied by microinjection of functional antibody fragments into living cells. The major goal of this study is to understand the in vivo function of certain chromosomal proteins. We have found that microinjected proteins do not always migrate to their true cellular location. Cytochrome C, a distinct cytoplasmic protein, when injected into the cytoplasm migrates to the nucleus. Therefore, we explored the possibility that antibodies can be introduced into living cells and neutralize the biological function of the protein. Purified IgG and affinity-purified antibodies to histone H₃ and H₂A and to nonhistone proteins HMG-1, 2 and 17 were fluoresceinated and injected into either the nucleus or cytoplasm of human KD fibroblasts. The location of the injected molecules was followed under fluorescence optics. The IgG molecule did not transverse the nuclear membrane but the F(ab)₂ fragment derived from the molecules did. The F(ab)₂ fragment derived from anti-H₂A antibodies, when injected into the cytoplasm, rapidly migrates into the nucleus. It is concluded that the antibody can bind to the appropriate antigen under in vivo conditions. The F(ab)₂ fragment

derived from anti-HMG-1 stayed at the site of injection regardless of whether it was injected into the nucleus or the cytoplasm of the cell. It is concluded that HMG protein is found both in the nucleus and cytoplasm of cells. To study the influence of antibodies on transcription, the cells were incubated with ^3H uridine after microinjection. One hour after the addition of ^3H uridine the cells were fixed and processed for autoradiography. Examination of the cells reveal that microinjection of control IgG, F(ab) $_2$ or Fab did not affect uridine incorporation into RNA in the nuclei of the injected cells. Antibodies to H $_2$ A, H $_3$ and HMG-17, however, inhibited transcription. Antibodies to HMG-1 as well as monoclonal antibodies against unidentified nuclear components did not inhibit transcription. The F(ab) $_2$ fragment against histone H $_2$ A and H $_3$ also inhibited transcription. Furthermore, the monovalent Fab prepared from anti-H $_2$ A IgG also inhibited transcription, suggesting that the inhibition is not due to cross linking of the chromatin strands by the antibody but rather to physical blocking of the passage of the RNA polymerase by the Fab attached to a histone present along the transcribable DNA. These findings, together with previous results, indicate that histones are associated with the transcribed chromatin fiber.

2) Definition of antigen 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 $_1^\circ$ is induced. We have elicited antibodies to peptide GH $_5$ and demonstrated that it cross-reacts immunologically with protein H $_1^\circ$. By trypsin digestion of H $_1^\circ$, an analogue of peptide GH $_5$ was isolated from H $_1^\circ$. 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 to cell differentiation. Chromosomal proteins HMG-1 and HMG-2 have been digested with pepsin and trypsin. The peptides generated were separated by gel electrophoresis and then transferred to DBM paper. Immunoreplica blots with anti-HMG-1 and anti-HMG-2 followed by ^{125}I Protein A and autoradiography indicated that a large portion of the antibodies present in the anti-HMG sera are directed against sequential antigenic determinants. This situation maximizes the chance that the antibodies will recognize the protein regardless of its exact organization in the cells.

3) The tissue specificity of histone H $_1^\circ$. In certain cells committed to differentiation or in which replication has stopped, a new histone called H $_1^\circ$ is induced. In an effort to see whether the appearance of H $_1^\circ$ is a cause for, or consequence of, cessation of DNA replication and differentiation, the occurrence of this protein in several human cell lines was examined. The protein is not present in lymphoblast cells or in A549 cells (lung carcinoma). It is present, however, in HeLa, A 427 (lung carcinoma) and HT-29 (colon carcinoma) cells. Quantitative and qualitative variations in other chromosomal proteins were also observed. The results suggest that the occurrence of H $_1^\circ$ in a cell does not correlate in any obvious way with tissue specificity, cell differentiation or the rate of cell division.

4) Monoclonal Antibodies Against Various Chromosomal Proteins. In the chromatin fiber the DNA is complexed with several types of groups of proteins. One of these groups, the so-called tight binding proteins, remains associated with the DNA even after extraction with 2.5M NaCl and 5M urea. Studies using polyclonal antibodies reveal that the complex of DNA and tight binding proteins elicit antibodies which display tissue, species and developmental stage specificity. Since presently the tight binding proteins cannot be purified and isolated, a monoclonal antibody approach to study their structure-function relation seems justified. So far we have set up the preparation of the DNA tight binding protein complex, immunized mice and obtained several positive hybrids.

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 carcinogen binding 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) elucidating the in vivo role of defined chromosomal proteins by microinjecting functional antibody fragments into living cells, 2) producing monoclonal proteins against certain chromosomal proteins, and 3) understanding the function of H₁^o and HMG proteins.

Publications:

Bustin, M., Neihart, N.K. and Fagan, J.B.: m-RNA of chromosomal proteins HMG-1 and HMG-2 are polyadenylated. Biochem. Biophys. Res. Commun. 101: 833-897, 1981.

Bustin, M., Reisch, J., Einck, L. and Klippel, J.H.: Autoantibodies to nucleosomal proteins: Antibodies to HMG-17 in autoimmune diseases. Science 215: 1245-1247, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04516-06 LMC						
PERIOD COVERED October 1, 1981 - September 30, 1982								
TITLE OF PROJECT (80 characters or less) Cellular and Molecular Effects of Psoralen Plus Ultraviolet Light								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table border="0" style="width: 100%;"> <tr> <td style="width: 33%;">PI: K.H. Kraemer</td> <td style="width: 33%;">Research Scientist</td> <td style="width: 33%;">LMC NCI</td> </tr> <tr> <td>OTHER: M. Perera</td> <td>Visiting Fellow</td> <td>LMC NCI</td> </tr> </table>			PI: K.H. Kraemer	Research Scientist	LMC NCI	OTHER: M. Perera	Visiting Fellow	LMC NCI
PI: K.H. Kraemer	Research Scientist	LMC NCI						
OTHER: M. Perera	Visiting Fellow	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: 0.25	PROFESSIONAL: 0.25	OTHER: 0						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords) <u>Psoralen plus long wavelength ultraviolet radiation (UV-A)</u> is being investigated as a model system for clinically relevant <u>photochemical carcinogenesis</u> . 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 <u>psoralen-DNA binding</u> on human lymphoid cells. Parameters monitored include the rate of <u>DNA synthesis</u> , induction of <u>DNA-psoralen cross-links</u> , induction of <u>sister chromatid exchanges</u> , alterations in the rate of cell proliferation and survival and in <u>immune reactivity</u> . 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. Presently, we are investigating whether lymphoid cells from patients with <u>cancer-prone genetic diseases</u> have increased sensitivity to cell killing and <u>mutagenesis induced</u> by psoralen plus UV-A. These studies are aimed at understanding the mechanism of cell damage induced by psoralen plus UV-A so as to minimize the toxicity to human cells during therapy and to identify individuals with abnormal sensitivity.								

Project Description

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.

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 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 related to in vivo carcinogenesis.

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., Levis, W.R., Cason, J.C. and Tarone, R.C.: Inhibition of mixed leukocyte culture reaction by 8-methoxypsoralen and long wavelength ultraviolet radiation. J. Invest. Dermatol. 77: 235-239, 1981.

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Kraemer, K.H., Waters, H.L.: Effects of psoralens plus ultraviolet radiation on human lymphoid cells in vitro. J. Natl. Cancer Inst. (In press).

Z01 CP 04517-06 LMC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

DNA Repair In Human Cancer-Prone Genetic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	R. Tarone	Mathematical Statistician	BB	NCI
	M. Bustin	Visiting Scientist	LMC	NCI
	H.V. Gelboin	Chief	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; P. Kohn, Div. of Genetics, U. of FL, Gainesville, FL; W.H. Clark, Dept. Derm., Hosp. U. of Penn., Phila., PA

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

4.9

PROFESSIONAL:

2.9

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 determine 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 and X-ray sensitivity, respectively, and patients with familial malignant melanoma (dysplastic nevus syndrome) are being studied. Detailed examinations of the clinical features of affected individuals are being made. A registry of XP patients is being established. Cultures of skin and blood are being established and the effects on cell survival, mutagenesis, DNA synthesis and repair, histone synthesis and chromosome integrity after treatment with DNA damaging agents are being examined. We are using recombinant DNA technology to attempt to clone the genes responsible for UV sensitivity in XP cells. These studies may give insights into mechanisms of cancer induction and suggest modes of cancer prophylaxis. In addition, these diseases serve as models for studies of human environmental carcinogenesis.

Project Description

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, and (3) to understand the molecular basis of their cellular hypersensitivity.

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 familial malignant melanoma (dysplastic nevus syndrome, DNS) have been studied clinically. The English language medical literature on XP is being reviewed comprehensively, and information on individual patients is being abstracted and entered into a computer for analysis. Physicians treating patients with XP are invited to fill out a registry questionnaire about their patients. Histological sections of pigmented lesions in XP are being examined for microscopic abnormalities. Cultured 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 transfection experiments and recombinant DNA technology are being utilized in an 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 almost 500 XP patients described in the literature have been entered into the computer. In a 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 an 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 dietary carcinogens. We are presently assessing the sensitivity of XP cells to dietary carcinogens *in vitro*. A registry of XP patients is being established in collaboration with W.C. Lambert, A.D. Andrews, and J.L. German. The question of the possible role of environmental mutagens in internal neoplasia is being approached by attempting to determine if XP patients have increased frequency of internal as well as cutaneous neoplasms.

AT, an autosomal recessive cancer-prone disease with cutaneous, neurological, and immunological abnormalities has X-ray sensitivity. We are studying the ability of cultured cells from AT patients and their parents to survive DNA damage induced by the chemotherapeutic agent, bleomycin. We have found that AT homozygous and heterozygous lymphoblastoid cell lines have a range of sensitivity to

killing by bleomycin. Further, this agent induced an abnormally large increase in chromosome breakage (but not in sister chromatid exchanges) in AT homozygous lymphoblastoid cells but not in AT 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 lesion, the dysplastic nevus, is being examined in 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. Lymphoblastoid cell lines from selected patients are being examined for evidence of sensitivity to DNA damaging agents as measured by cell survival and mutagenesis and examined for possible DNA repair defects.

Patients with psoriasis, a benign disease with a strong genetic component, have rapid, but non-neoplastic, proliferation of lesional epidermal cells. We are studying the level of aryl hydrocarbon hydroxylase (AHH) activity, an important enzyme in carcinogen metabolism, in lymphocytes and monocytes from psoriasis patients in collaboration with the Metabolic Control Section, LMC. This Laboratory is examining the patients clinically and obtaining blood for analysis. Age-related changes in AHH activity were found in monocytes and lymphocytes. In addition, topical therapy with steroids and/or tar appeared to induce AHH activity in circulating leukocytes.

Significance to Biomedical Research and the Program of the Institute: These studies may identify persons with increased risk of cancer and by revealing the mechanism of cancer induction, 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:

Kraemer, K.H.: Cancer-prone genodermatoses and DNA repair. Progress In Dermatology 15: 1-6, 1981.

Kraemer, K.H., Lee, M.M., and Scotto, J.: Diseases of environmental-genetic interaction: Preliminary report of a retrospective survey of neoplasia in 268 xeroderma pigmentosum patients. In Sugimura, T., Kondo, S. and Takebe, H. (Eds.): Environmental Mutagens and Carcinogens (Proceedings of the Third International Conference on Environmental Mutagens). Tokyo, University of Tokyo Press, 1982, pp. 603-612.

Kohn, P.H., Kraemer, K.H. and Buchanan, J.K.: Influence of ataxia telangiectasia gene dosage on bleomycin-induced chromosome breakage and inhibition of replication in human lymphoblastoid cell lines. Exp. Cell Res. 137: 387-395, 1982.

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.: 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 (In press).

Tucker, M.A., Green, 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).

Kraemer, K.H.: Cellular hypersensitivity to physical and chemical agents in patients with ataxia telangiectasia: Disorders in cell growth and chromosomal integrity. In Waldmann, T.A., Nelson, D. and Kraemer, K.H. (Eds.): Ataxia Telangiectasia: A Hereditary Disease With Immunodeficiency, Impaired Organ Maturation, DNA Repair Defects and a High Incidence of Neoplasia. Ann. Intern. Med. (In press).

Elder, D.E., Greene, M.H., Guerry, D., Kraemer, K.H. and Clark, W.H.: The dysplastic nevus syndrome. Am. J. Dermatopathology (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04518-05 LMC
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
AA-tRNAs in Mouse Mammary Tissue and Phosphoseryl-tRNAs in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT
PI: Dolph Hatfield Research Biologist 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: 1.0	PROFESSIONAL: 1.0	OTHER: 0
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CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
 An analysis of the tRNA population of mouse mammary tissue showed very similar patterns of fractionation of isoacceptors during development and in response to prolactin in mouse mammary explants. Two minor seryl-tRNAs from mammalian cells suppress the nonsense codon UGA in protein synthesis. They are the first authentic nonsense suppressor tRNAs found in mammals. Furthermore, they form phosphoseryl-tRNA in the presence of an extract from lactating mammary tissue. The phosphoseryl-tRNAs recognize UGA in a ribosome-binding assay which suggest that these isoacceptors donate phosphoserine directly to protein.

Project Description

Objectives: The objectives are 1) to examine the aa-tRNA population of mouse mammary tissue in order to determine if specific isoacceptors may be enriched during lactation; 2) to determine the cellular function of three minor Ser-tRNAs, two of which read the nonsense codon UGA in protein synthesis and form phospho-seryl-tRNA; and 3) to determine the effect of specific base modifications in isoacceptor aa-tRNAs on protein synthesis and whether misreading genetic code words by isoacceptor aa-tRNAs during protein synthesis may be involved in the expression of cancer.

Methods Employed: Transfer RNA was prepared from mouse mammary tissue at different stages of development, from mouse mammary explants treated and untreated with prolactin, and from mouse liver by standard techniques. tRNA was labeled with ^{32}P in mouse mammary explants grown in the presence and absence of prolactin. tRNA was extracted from each tissue by standard techniques and the tRNA populations compared by two-dimensional polyacrylamide gel electrophoresis. This project is a collaboration with Drs. M. Green and A. Peacock, Protein Section, LMC, NCI (Project No. Z01 CP 04782-12 LMC).

Three minor seryl-tRNAs, two of which specifically recognize the nonsense codon UGA, were isolated from bovine liver by previously described procedures. Extracts from mouse liver, rooster liver, and bovine mammary tissue were prepared by procedures similar to those of other investigators to examine the phosphorylation of the minor seryl-tRNAs. ^{32}P -phospho- ^3H -seryl-tRNAs were prepared with labeled ATP (gamma labeled ^{32}P) and ^3H -serine and their binding to ribosomes in response to UGA determined. This project is a collaboration with Drs. A. Diamond and B. Dudock, Department of Biochemistry, Stony Brook University in New York.

Large quantities of normal tRNA^{Phe} from mouse liver and large quantities of tRNA^{Phe} lacking the Wye base from neuroblastoma tissue were isolated and purified by Sepharose 4-B and reverse phase chromatography. The ability of each isoacceptor to donate phenylalanine to protein at specific sites will be determined. This project is a collaboration with Dr. J.F. Mushinski, LCBGY, NCI (Project No. Z01 CB 08727-05 LCBGY) and Dr. D.W.E. Smith, Department of Pathology, Northwestern University.

Major Findings: Comparison of tRNA populations from mouse mammary tissue seven days into pregnancy, eighteen days into pregnancy, ten days into lactation, from mouse mammary explants treated with and without prolactin and from a control tissue, mouse liver, by two-dimensional gel electrophoresis revealed 1) several differences in the pattern of fractionation of tRNA from early and late pregnant mice; 2) several differences in the pattern of fractionation of tRNA from late pregnant mice and mouse liver; and 3) very similar patterns of fractionation of tRNA from each of the other stages of development. Fractionation of ^{32}P -labeled tRNA from mouse mammary explants confirmed these observations. These studies show that the tRNA population of the mouse mammary gland is established by late pregnancy and remains stable throughout the remaining stages of development.

Sequences of the minor seryl-tRNAs from bovine liver which recognize UGA were determined previously by Drs. A. Diamond and B. Dudock of Stony Brook University in New York. Both tRNAs suppress the nonsense codon UGA in protein synthesis and, therefore, are the first authentic suppressors found in mammalian cells. Incubation of total tRNA with an extract of rooster liver, of bovine mammary tissue, and of mouse liver, subsequent isolation of each serine isoacceptor, and determination of phosphoserine formation demonstrated that the minor seryl-tRNAs which recognize UGA were phosphorylated most extensively. The level of phosphorylation ranged from about 65% with mammary extract to about 4% with mouse liver extract. ^{32}P -phospho- ^3H -seryl-tRNA responded to the nonsense codon UGA in a ribosome-binding assay.

Significance to Biomedical Research and the Program of the Institute: Major unresolved questions in biology are whether tRNA may play a role in cellular regulation and in carcinogenesis. Approaches to understanding these problems are 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 minor seryl-tRNAs which suppress UGA and to compare the ability of normal phenylalanyl-tRNA and phenylalanyl-tRNA lacking the Wye base to read their cognate codons and to misread other codons in protein synthesis.

Publications:

Diamond, A., Dudock, B. and Hatfield, D.: Structure and properties of a bovine liver UGA suppressor serine tRNA with a tryptophan anticodon. Cell 25: 497-506, 1981.

Hatfield, D., Rice, M., Hession, C. and Melera, P.: Aminoacyl-tRNAs from Physarum polycephalum: Patterns of codon recognition. J. Bacteriol., 1982 (In press).

Hatfield, D., Varricchio, F., Rice, M. and Forget, B.: The aminoacyl-tRNA population of human reticulocytes. J. Biol. Chem. 257: 3183-3188, 1982.

Smith, D.W.E., McNamara, A., Rice, M. and Hatfield, D.: The effects of a post-transcriptional modification on the function of tRNA^{LYS} isoaccepting species in translation. J. Biol. Chem. 256: 10033-10036, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on Electrophoretic Techniques for Protein, RNA, and DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.C. Peacock	Chief, Protein Section	LMC	NCI
OTHERS:	M. Seidman	Senior Staff Fellow	LMC	NCI
	S.L. Bunting	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:

2.2

PROFESSIONAL:

1.2

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new method for two-dimensional analysis of DNA is under development. The DNA under study is first cleaved with a restriction enzyme; the cleavage products are separated by electrophoresis on specially treated agarose under conditions which permit a second restriction enzyme easy access to the separated fragments. After a suitable incubation, the fragments cleaved by the first enzyme are cleaved again, this time at new sites specified by the second restriction enzyme. The electrophoresis is repeated, but in a direction perpendicular to the first run. The result is that the number of DNA fragments that can be resolved is approximately the square of the number that can be resolved in a one-dimension analysis. The method is expected to be of great utility in locating polymorphic genes and in identifying segments of the gene that may be rearranged during carcinogenesis. The method will also facilitate analysis of heterogeneous nuclear RNA.

Project Description

Objectives: To investigate the influence of primary structure and conformation on the electrophoretic and biological properties of proteins and nucleic acids and to use this information in devising improved methodology for analysis and purification. To study the role of hnRNA in cellular control in normal and malignant cells by these techniques. To develop suitable methodology for the preparation of hnRNA in undegraded and native form.

Methods Employed: Cultures of HeLa cells, assay of radioisotopes, isolation and electrophoresis of RNA and DNA, optical scanning of stained and unstained gels, preparative electrophoresis, optical measurement of melting of nucleic acids, and ultracentrifugal analysis of RNA and DNA.

Major Findings: Resolution of DNA fragments generated by restriction enzymes is greatly improved by electrophoretic separation prior to the use of a second restriction enzyme. Although this idea has been appreciated for some time, it has been technically very difficult to achieve. Some success has been forthcoming as the combined result of removing inhibitors from the agarose matrix in which the second digestion is performed and providing the second restriction enzyme easy access to the separated DNA. For removal of inhibitors from agarose, chemical purification by selective preparation, electrophoretic separation, and chromatographic methods have all been of some value; chromatographic techniques removed almost all of the inhibitory material. DNA from lambda phage has been used in the experiments performed to date. Complete cleavage, first by Hind III and second by Eco RI has been achieved, with an electrophoretic display of all the expected fragments clearly resolved, even though some differ by less than 3% in nucleotide length.

Efforts to improve the characterization of heterogeneous nuclear RNA (hnRNA) have continued in two directions: a) In an effort to minimize the effect of conformation on the migration of hnRNA, glyoxal treatment of the sample was attempted, but degradation of large hnRNA was a serious consequence of this approach. There were reasons for supposing that the use of gradient gel might minimize the effect of conformation, but this was found to be incorrect. b) Studies are in progress to simplify the calculations used to estimate chain-lengths of hnRNA. The computer facilities required by the present approach makes the method difficult to apply in many circumstances. The simplification consists in generating suitable subfractions of the original population, such that the range of sizes is much reduced.

Significance to Biomedical Research and the Program of the Institute: The ability to estimate chain lengths of polydisperse ribonucleic acids by methods dependent on molecular properties very different from those previously available (centrifuge, electron microscope, etc.) provides a check on these methods. The improved electrophoretic separation of these large molecules presents them in a form suitable for further characterization by defined probes. Such methods should lead to improvements in understanding the control of gene expression in normal and malignant cells.

The heterogeneous RNA is the primary transcript of the gene and, in addition to specific messengers for specific proteins, presumably contains all RNA that might be involved in the control of genetic expression. Analytical methods for studying this RNA and assessing its role may be fundamental to understanding the alterations which occur in malignancy.

The ability to sharpen the characterization of genomic DNA by two-dimensional electrophoresis permits direct evaluation of the extent to which rearrangements in the genome may play a role in malignancy. In addition, such methods will improve the identification of genes whose transcription may vary with circumstances, such as in the progression of tumors and differentiation.

Proposed Course: 1) Efforts to develop a conformation-insensitive method for the electrophoretic analysis will continue. Efforts will be directed towards methods which do not accelerate degradation. 2) Properties other than size which may characterize the RNA population will be studied. These include self-association and ability to form duplexes with other molecules. 3) Work on the two-dimensional DNA analysis will be continued. The technique will be applied to the study of rearrangements in the genome of chemically transformed cells and other cell types. The application to the detection of changes in the mRNA population will be investigated.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04554-08-LMC
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development of an In Vitro System for Studying Chemical Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: T. Kakunaga	Chief, Cell Genetics Section	LMC NCI
OTHER: T. Hirakawa	Visiting Fellow	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:

0.9

PROFESSIONAL:

0.4

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop 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. This project may also lead to finding crucial factors involved in cell transformation. The conditions required for maximum enhancement of carcinogen-initiated transformation by tumor promoters have been established using Balb/3T3-A31-1-1 line. The optimum conditions were very similar to those required for the promotion of skin carcinogens. The potency of various promoters in enhancing transformation generally paralleled their potential to promote skin carcinogenesis. These observations, combined with data on the numbers and properties of the receptor for promoters and the early biochemical response to promoters in A31-1-1 cells, indicate that enhancement by promoters of transformation observed with A31-1-1 cells serves as a model system to study the action of promoters. The systems to detect cocarcinogens, weak carcinogens, and presumptive "pure initiators" are being developed using A31-1-1 cells. Improvements of the system for DNA transfection transformation assay system and in human diploid cells have been made.

Project Description

Objectives: To establish an optimum system of assay for transformation of human and mouse cells for studying the mechanisms of chemical carcinogenesis. To obtain an assay system and information on the assessment of human risk from environmental carcinogens, with special attention to the separation of each step of the multistep processes of malignant transformation. Current interests are: 1) to develop a culture system for studying the mechanisms of tumor promotion; 2) to develop a sensitive system to detect extremely weak carcinogens and/or presumptive pure initiators, compounds which have initiating potential but require subsequent promoter treatment for the induction of transformation/tumor; 3) to establish a culture system for studying the action of "cocarcinogens;" and 4) to develop a method to transfect human cells efficiently with mammalian cell DNA.

Major Findings: 1) Development of a system to study the mechanisms of enhancement of transformation by tumor-promoters. One of the subclones (A31-1-1) derived from Balb/3T3-A31 line has been shown to produce transformed foci at a high frequency when the cells are initially exposed to a nontransforming dose of carcinogen and then repeatedly exposed to tumor-promoting agents. Transformation-enhancing effects of tumor-promoting agents are reversible to some point, dose-dependent, and require post-carcinogen treatment. Pretreatment or simultaneous treatment with promoters did not enhance the carcinogen-induced transformation. Long intervals between the repeated treatment with promoters resulted in reduced enhancement of transformation. Conditions required for the maximum enhancement of transformation by promoters were very similar to those required for the promotion of skin carcinogenesis. Furthermore, the ability of 11 derivatives of phorbol esters to enhance the carcinogen-induced cell transformation was found to correlate with their potential to promote skin carcinogenesis, with minor exceptions. A31-1-1 cells have a large number of presumptive receptors for phorbol ester. In comparison to other cells which have been used for the study of the action of promoters, A31-1-1 cells showed a relatively higher sensitivity to the transformation-enhancing effects of promoters and to the induction of various early biochemical changes by promoters, such as stimulation of DNA synthesis and glucose uptake under a G₀-arrested condition, stimulation of arachidonic acid release, and down-modulation of membrane receptors for epidermal growth factor.

2) Development of a sensitive system for detecting weak carcinogens and initiators. A marked enhancement of carcinogen-induced transformation of A31-1-1 cells by promoters, as described above, has been applied to develop an assay system for transforming the potential of weak carcinogens or initiating potential of chemicals. Teleocidin, a naturally occurring promoter isolated from mycelia of Streptomyces, was strongest in enhancing carcinogen-induced transformation among many chemicals examined and, therefore, used as a promoter. The effective dose of carcinogens required for transformation was reduced by a factor of 100 by subsequent treatment with teleocidin for two weeks compared to those without teleocidin treatment. Treatment with 3-methylcholanthrene and benzo(a)pyrene at a concentration of as low as 0.01 ug/ml was sufficient to induce transformation under the conditions described above. Treatment

with a very weak carcinogen, 6-carboxy-4-nitroquinoline-1-oxide, followed by teleocidin treatment induced transformation, whereas no transformation was observed in the culture exposed to 6-carboxy-4-nitroquinoline-1-oxide alone. Other weak carcinogens and noncarcinogens are being tested for their initiating or transforming activities in this system.

3) Development of a system for detecting cocarcinogenic activity of chemicals using a Balb/3T3 cell line. The culture system for the assay of co-carcinogenic activity of chemicals has been explored by using a subclone of Balb/3T3 cell line as target cells, an indirect-acting carcinogen, benzo(a)pyrene, and a direct-acting alkylating carcinogen, beta-propiolactone as carcinogens, and catechol as a cocarcinogen. The rate of transformation was notably higher in groups treated with benzo(a)pyrene and catechol or beta-propiolactone and catechol than in groups treated with the carcinogen alone. The increase in the transformation frequency by catechol treatment was dose-dependent. Catechol alone did not induce any transformation. All the cells isolated from the transformed loci showed characteristics of malignantly transformed cells. Thus, chemical cocarcinogenesis in vitro was similar to the in vivo studies reported earlier on mouse skin.

4) Rapid and quantitative transformation of human diploid fibroblasts by chemical carcinogens. Eagle's minimum essential medium (EMEM), supplemented with serum supported well the clonal growth of normal human fibroblasts and approximately half of a group of human transformed or tumor cell lines. However, the other half of the human transformed or tumor cell lines showed very low efficiencies of clonal growth in EMEM. Extensive nutritional examination revealed that addition of pyruvic acid, aspartic acid, and serine to EMEM greatly enhanced the clonal growth of the transformed cells and shortened the doubling time of both normal and transformed cells. These nutritional supplements did not affect the growth of normal or transformed mouse cells, suggesting the possibility that the ease of transforming mouse cells and the difficulty of transforming human cells may be due to their different nutritional requirements. The attempt to develop a rapid and quantitative system for transformation of human diploid cells is in progress.

5) DNA transfection in human diploid fibroblasts. Biochemical transformation of human diploid fibroblasts was too inefficient to obtain quantitative data. Further attempts to increase the efficiency of transfection are in progress, using a plasmid DNA containing the SV40 early transcription promoter and the bacterial xanthine-guanine phosphoribosyltransferase gene as a transfecting DNA and a modified calcium-phosphate method.

Significance to Biomedical Research and the Program of the Institute: This project will provide a new or improved system for studying the mechanisms of cell transformation. Particularly, it will enable us to investigate each process isolated from the multistep processes of neoplastic transformation and in the long run provide us with a clearer picture of the whole transformation process. In addition, it may make it possible to detect "incomplete" carcinogens which have not been detected or have not been precisely assessed for risk by conventional assay systems such as animal carcinogenesis and short-term

assay systems. This project will also provide information on differences in the response to the carcinogens between species, organs, tissues, and cell systems. The information thus obtained would be helpful for extrapolating the results of animal experiments or in vitro systems into an assessment of human risk from environmental carcinogens.

Proposed Course: 1) To continue the attempt to increase the efficiency of DNA transfection in human cells. To detect and isolate cellular transforming or normalizing genes and their products which control or induce the expression of transformed or normal phenotype of human cells. 2) To continue to examine whether many "weak carcinogens" or presumptive "pure initiators" are detected by the transformation system which has integrated the treatment with an extremely potent promoter, teleocidin, as a promoter. The "weak carcinogens" will be classified into several categories according to their spectrum of biological potential in inducing each separate conversion of the multistep process of malignant transformation. 3) To continue to develop a quantitative and well-characterized assay system for transformation of human diploid cells by chemical carcinogens. The cells derived from the patients who are genetically predisposed to a higher incidence of cancer will be examined whether or not they are highly sensitive to the transformation in culture. If they are more sensitive than normal cells, they will be used as a target cell in the quantitative system for transformation assay by chemical carcinogens. 4) To pursue the goals outlined in Objectives and to publish results obtained.

Publications:

Kakunaga, T.: Assay of chemically-induced transformation of human cells. In Stich, H.F. and San, R.H.C. (Eds.): Short Term Tests for Chemical Carcinogens. New York, Springer-Verlag, 1981, pp. 338-349.

Kakunaga, T.: Cell transformation as a system for studying mechanisms of carcinogenesis. In Heidelberger, C., Inui, N. and Kuroki, T. (Eds.): Mutation, Promotion and Transformation In Vitro: Tokyo, Japan Scientific Societies Press, 1981, pp. 231-242.

Atchison, M., Chu, C.S., Kakunaga, T. and Van Duuren, B.L.: Chemical cocarcinogenesis using a subclone derived from Balb/3T3 cells with catechol as a cocarcinogen. JNCI (In press).

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. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04555-08 LMC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Factors Affecting Malignant Transformation and Mutation by Chemical Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakunaga	Chief, Cell Genetics	LMC	NCI
OTHER:	T. Hirakawa	Visiting Fellow	LMC	NCI
	K. Shimomura	Guest Researcher	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:

2.3

PROFESSIONAL:

1.7

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to clarify the mechanism of cell transformation by examining the genetic and physiological factors which affect cell transformation induced by chemicals and irradiation. With nine phorbol ester derivatives and two teleocidin derivatives, close correlation was observed between their potential to enhance carcinogen-initiated transformation, to induce early biochemical changes, and to promote carcinogen-initiated skin carcinogenesis in mice. The two phorbol ester derivatives showed weak activity in enhancing transformation in spite of their strong biochemical effects. The weak transformation-enhancing potential of these two derivatives was ascribed to the short life of their biochemical effects. The accumulated biochemical effects induced by all the promoter derivatives were closely correlated with their transformation-enhancing potencies, indicating that the continuity of the promoters' effects is essential for the fixation of enhancement of transformation. In the cell mutant which is highly sensitive to chemically induced transformation, the biochemical effects of promoters lasted longer than those in the resistant cells. Molecular studies on the differences between cell mutants are in progress.

Project Description

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.

Current interests are 1) to determine the molecular mechanisms of tumor promotion by promoting agents using cell culture model systems and (2) to clarify the cause of different susceptibilities to transformation induced by chemicals and irradiation in the cell variants which we isolated. Special attention is paid to the process of expression of the transformed state, which is 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 (see Project No. Z01 CP 04554) using 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 [³H]-thymidine or [¹⁴C]-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 [³H]-phorbol dibutyrate and [¹²⁵I]-epidermal growth factor, respectively. Finally, we used separation on a thin layer chromatograph and measurement of [³H]-arachidonic acid released into culture medium from the cells which were prelabeled with [³H]-arachidonic acid.

Major Findings: Relationship between the induction of early biochemical changes and the enhancement of cell transformation by various promoter derivatives. Eleven derivatives of phorbol ester and two teleocidin derivatives were compared with respect to their early biochemical effects and transformation-enhancing effects in Balb/3T3-A31-1-1 cells. These cells have been shown to serve as a model cell system for tumor promotion. In general, close correlation was observed between the potencies of the chemicals in enhancing carcinogen-initiated transformation and inducing early biochemical changes; i.e., stimulation of DNA synthesis and glucose uptake in cultures arrested at G₀-phase, stimulation of arachidonic acid release, and inhibition of epidermal growth factor binding to its membrane receptor. The potencies in inducing these biological and biochemical effects of chemicals in A31-1-1 cells were also correlated with their potencies in promoting carcinogen-initiated skin carcinogenesis. Two derivatives of phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate (TPA) and phorbol-13-decanoate (P13D) did not fit this general rule. Both compounds showed high activity in inducing early biochemical changes but weak in enhancing transformation.

2. Recruitment of continuous effects of promoters for enhancing transformation. TPA and P13D, which have low activity in enhancing transformation and high activity in inducing early biochemical change, showed a tendency for their biochemical effects to diminish earlier than those of other promoters. The remaining

ability to induce early biochemical effects during exposure of the cultures was rapidly lost in these two compounds compared to other promoters. The accumulated biochemical effects, i.e., stimulation of glucose uptake and down modulation of the receptors for epidermal growth factor induced by 11 derivatives of phorbol ester and teleocidin, were closely correlated with their potentials in enhancing transformation without exception. These results indicate that the early biochemical changes induced by promoters themselves are not sufficient to enhance transformation and are not the optimum assay criteria for screening the environmental promoters.

3. Analysis of differential susceptibilities of cell mutants to transformation. Cell mutants showing different susceptibility to transformation induced by chemical carcinogens, which we had previously isolated from Balb/3T3-A31-1 subclone, had a similar number of membrane receptors for phorbol ester responses to promoter-exposure such as increased glucose uptake across the cell membrane and down modulation of the receptor for epidermal growth factor. However, these biochemical responses lasted longer in the sensitive mutant than in the resistant mutant. Detailed studies are underway.

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, cocarcinogens 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 made on the clarification of the mechanisms of the requirement of continuous effects of active promoters to the cells for fixation of enhancement of transformation for which data is unavailable at present. 2) To continue to investigate the molecular mechanisms of 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:

Kakunaga, T.: Cell transformation by chemical agents. In Yamane, I., Okada, Y., Horikawa, M. and Kuroki, T. (Eds.): Somatic Cell Genetics. Tokyo, Rikoqakusha Publishing, 1981, pp. 305-327.

Kakunaga, T.: Cell transformation as a system for studying mechanisms of Carcinogenesis. In Heidelberger, C., Inui, N. and Kuroki, T. (Eds.): Mutation, Promotion and Transformation in Vitro, Japan Scientific Societies Press, Tokyo 1981, pp. 231-242.

Kakunaga, T.: Mechanisms of cell transformation by chemical carcinogens - Relation to the mutation. Tissue Culture 7: 147-154, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Genes and Proteins Responsible for the Transformed Phenotype

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kakunaga	Chief, Cell Genetics Section	LMC NCI
OTHER:	H. Hamada	Visiting Associate	LMC NCI
	T. Hirakawa	Visiting Fellow	LMC NCI

COOPERATING UNITS (if any)

J. Leavitt, Linus Pauling Institute of Science and Medicine, Palo Alto, CA.

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.7

PROFESSIONAL:

1.0

OTHER:

0.7

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is our long-range goal to clarify the nature of the cellular molecules responsible for the malignant phenotype of chemically transformed human cells by using new techniques in molecular biology. The mutated beta-actin, which we had found previously in a chemically transformed human fibroblast cell line, was further altered in subclones of the transformed cells. Variations in the mutated beta-actin expression were accompanied by incremental increases of the malignant potential in the subclones. The expression of this mutated beta-actin was also associated with the expression of in vitro transformed phenotypes in hybrid cells between transformed and normal parent human fibroblasts, suggesting an important role for the mutated beta-actin in the expression of transformed phenotypes. Computerized microdensitometry of autoradiographs of two-dimensional gel electrophoresis of the polypeptide revealed that less than 2% of the genes were activated or shut off, but at least 32% were modulated quantitatively as a consequence of neoplastic transformation of human fibroblasts by a chemical carcinogen. A human cardiac muscle actin gene was isolated from a human DNA library and its molecular structure and evolutionary origin was determined.

Project Description

Objectives: To learn about the genetic regulation of the expression of the transformed phenotype and to determine the biochemical nature of the macromolecules controlling the expression of the transformed phenotype. Current interests are 1) to understand the molecular mechanism of the polypeptide changes associated with the transformation of human cells, 2) to determine the relationship between the particular polypeptide changes and the expression of the transformed phenotype in transformed cells, 3) to determine whether the transformed phenotype is expressed in a complete hybrid cell between a normal human diploid fibroblast cell and its chemical transformant, and 4) to know the molecular structure and regulation of the expression of human actin genes.

Methods Employed: We 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, and DNA transfection. Computerized microdensitometry is performed by scanning autoradiographs of two-dimensional gel electrophoresis with an Optronics 1000-HS microdensitometer and measuring and image-analyzing is done with a PDP 11/60 computer equipped with a DeAnza IP5000 array processor.

Major Findings: 1. Quantitative assessment of changes in gene expression in the chemically transformed cells using a computerized image analyzer. The modulation of gene expression accompanying neoplastic transformation was assessed by computerized microdensitometry of autoradiographic patterns of [³⁵S]-methionine-labeled polypeptides separated by two-dimensional polyacrylamide gel electrophoresis. Nearly 1000 polypeptide species of parent diploid human fibroblasts and clonally derived neoplastic fibroblasts (Hut-14) were compared. Of the 700 more abundant polypeptides measured, 13 were lost and 14 were gained following this neoplastic transformation. It is estimated that less than 2% of the genes expressing abundant polypeptides were either activated or shut off, but at least 32% were modulated quantitatively as a consequence of this neoplastic transformation. Classes of "highly variable" and marginally variable polypeptides were assigned. Among the "highly variable polypeptides" two related species (barely detectable in normal human fibroblasts) were synthesized at a 25-31 fold higher rate in the transformed cells.

2. Changes in mutant beta-actin associated with incremental increases in tumorigenesis. Previously, we discovered a point mutated beta-actin in one of two functional beta-actin genes in human fibroblasts (Hut-14). This mutation occurred during in vitro induction of neoplastic transformation with a chemical carcinogen. Many subclones were isolated from Hut-14 and their transformed phenotypes and expression of beta-actin were examined. Among the subclones examined, only three subclones showed increased tumorigenicity in nude mice and increased ability to grow in soft agar, while other subclones did not show any changes in these properties. The phenotypic cellular changes

in these three subclones were accompanied by a biochemical and functional change in the mutant beta-actin polypeptides. These more variant mutant actins differed from the original mutant actin by one additional negative net charge, a short life in the cell, a greatly diminished ability to incorporate into the cytoskeleton, a decrease in affinity for deoxyribonuclease I and a faster rate of synthesis. The new beta-actin species were translated in vitro correctly from mRNA of the highly malignant subclones, suggesting a second-site mutation in the beta-actin. The increased rate of synthesis of mutant beta-actin was accompanied by an approximate doubling in the relative amount of translatable mutant beta-actin mRNA. These results indicate that the variations in beta-actin expression are accompanied by incremental increases of malignant potential in these subclones.

3. Somatic cell genetics analysis on the expression of the mutated beta-actin gene and the transformed phenotypes. The 1:1 hybrid cells were obtained by fusing the transformed human fibroblast, which expresses the mutated beta-actin and is resistant to both β -thioguanin and ouabain with the normal parent cells, selecting the hybrids by growing the fused cells in the presence of ouabain, hypoxanthin, aminopterin, and thymidine and analyzing chromosome banding patterns of all the presumptive hybrids isolated. Most of the isolated hybrid cells expressed the mutated beta-actin and in vitro characters of the morphologically transformed cells, such as the ability to grow in soft agar and neoplastic alteration. However, one hybrid cell strain showed a very low level of expression of the mutated beta-actin and a normal-like phenotype. All the hybrid cells failed to produce tumors when injected into nude mice, whereas the transformed parent cells produced tumors at high frequencies. These results indicate the close correlation between the expression of the mutated beta-actin and the expression of the in vitro transformed phenotypes and the recessiveness of the expression of tumorigenic properties.

4. Isolation, characterization, and evolutionary origin of human cardiac muscle actin gene. During the course of the experiment designed for the isolation of a human beta-actin gene, two recombinant phages which contain cardiac muscle actin genes were isolated from a human DNA library. DNA sequencing of cloned DNA showed that the entire coding sequence perfectly matched the amino acid sequence of cardiac muscle actin. The initiation codon is followed by a cysteine codon that is not found at the amino-terminal site of any actin isoform. There are 5 introns, interrupting exons at codons 41/42, 150, 204, 267, and 327/328. Surprisingly, these intron locations are exactly the same as those of rat skeletal muscle actin gene but different from those of non-muscle beta-actin gene. Nucleotide sequences of all exon-intron boundaries agree with the GT-AG rule. The 3' untranslated sequence has no homology to that of non-muscle beta- or gamma-actin gene but has considerable homology to that of one of the other actin genes. These results indicate that the recombinant phages which we have isolated contain cardiac muscle actin genes and that cardiac muscle actin genes and skeletal muscle actin genes were derived from their ancestral gene at a relatively recent time in evolutionary development.

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 the expression of the transformed state and will also contribute to the understanding of the mechanism of carcinogenesis. Thus, this project is directly aimed at that aspect of the program of the Institute that 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 transfection and gene cloning techniques; 2) to determine the structure and function of human actin genes, 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., Leavitt, J. and Kakunaga, T.: Mutated β -actin gene: Co-expression with an unmutated allele in the chemically transformed human fibroblast cell line. Proc. Natl. Acad. Sci. USA 78: 3634-3638, 1981.

Leavitt, J., Bushar, G., Mohanty, R., Kakunaga, T. and Ennis, F.A.: Interaction of the structural polypeptides of influenza virus with the cellular cytoskeleton during productive infection of human fibroblasts. In Bishop, D. and Compans, R. (Eds): Negative Strand Viruses. North Holland/New York, Elsevier, 1981, pp. 233-244.

Kakunaga, T.: Protein phenotypes characteristic of the transformed cells. Metabolism 18: 910-911, 1981.

Leavitt, J., Goldman, D., Merrill, C. and Kakunaga, T.: Differential control of gene expression in normal and neoplastic human fibroblasts: Analysis of the quantitative and qualitative polypeptide differences in two-dimensional gels by computerized microdensitometry. Carcinogenesis 3: 61-70, 1982.

Leavitt, J., Bushar, G., Kakunaga, T., Hamada, H., Hirakawa, T., Goldman, D., Merrill, C.: Variations in mutant β -actin expression accompanying increased human fibroblast tumorigenicity. Cell 28: 259-268, 1982.

Kakunaga, T., Hamada, H., Leavitt, J., Lo, K.-Y., and Hirakawa, T.: Evidence both for mutational and non-mutational processes in chemically induced cell transformation. In Cerruti, P. and Harris, C. (Eds): Molecular Mechanisms of Chemical Carcinogenesis. New York, Alan R. Liss. (In press).

Hamada, H., Petrino, M.G. and Kakunaga, T.: Molecular structure and evolutionary origin of human cardiac muscle actin gene. Proc. Natl. Acad. Sci. USA. (In press).

Kakunaga, T.: A point mutation in actin gene and neoplastic transformation of human fibroblasts. Fed. Proc. (In press).

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Hormones and Breast Tissue Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.R. Green	Research Chemist	LMC NCI
OTHERS:	D. Hatfield	Research Chemist	LMC NCI
	A.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:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 failure of or aberrant response to hormones by hyperplastic or neoplastic breast tissue. Among the topics studied are the response of mammary tissue in culture to hormones in terms of nucleic acid and protein synthesis. Transfer RNAs from mammary glands of mice were isolated and separated by two-dimensional electrophoresis to determine changes that may occur in the developing tissue in vivo and in vitro. Quantitative and qualitative differences in isoacceptor patterns of transfer RNAs obtained from mammary tissues of mice in early and late pregnancy were observed. In contrast, patterns from late pregnant and lactating tissue were very similar. To determine whether patterns of tRNAs might be altered in concert with synthesis of milk protein mRNAs, explants from mammary glands of mice in mid-pregnancy were incubated in synthetic medium with insulin and hydrocortisone in the presence or absence of prolactin. Patterns of tRNAs from prolactin stimulated and unstimulated explants labeled with ³²-Pi were similar to tRNAs from late pregnant tissue and to each other.

Project Description

Objectives: 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.

Methods Employed: 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.

Major Findings: Several methods for extraction of total RNAs and transfer RNAs from mammary tissue were compared. These were evaluated by electrophoretic separation of RNAs on composite agarose, polyacrylamide gels. A micro modification of the method described by Roe was found to be suitable for the subsequent separation of transfer RNAs by two-dimensional electrophoresis. ³²P-labeled transfer RNAs did not require prior separation by column chromatography because DNA and higher molecular weight RNAs were well separated from transfer RNAs. Two-dimensional gel electrophoresis separated transfer RNAs into approximately 50 distinct zones. Quantitative and qualitative differences among tRNAs from liver and mammary gland in early and late pregnancy were observed. tRNAs from explants of mice in mid-pregnancy, stimulated to synthesize milk proteins and milk protein mRNAs by the addition of prolactin to a synthetic medium, were compared to tRNAs isolated from explants not synthesizing milk proteins. Except for minor variations, the patterns were similar.

Significance to Biomedical Research and the Program of the Institute: A correlation between levels of isoacceptor amino acyl tRNAs in cells and their cognate codons in corresponding mRNAs has been observed in bacteria and eukaryotic cells. These studies suggest that tRNA levels in cells are adapted to the requirements of protein synthesis. Differences in a limited number of tRNAs have been demonstrated previously in lactating bovine mammary tRNA preparations compared to virgin mammary tRNA. Alterations in tRNA patterns observed in those tissues may reflect changes in ratios of epithelial cells to adipose and connective tissue cells. We also observed quantitative and qualitative changes in the patterns of tRNAs from early versus late pregnant and lactating tissue. When identical cell populations were induced in vitro to synthesize milk proteins, little change in tRNA patterns was observed. At present it is not known whether regulation at the transcriptional or translational level controls the adaptation of isoacceptors required for protein synthesis. A culture system capable of being induced to produce a significant amount of protein without changes in cell population should be useful in obtaining answers to these questions. Minor but consistent variations between stimulated and unstimulated glands were observed.

Proposed Course: Stimulation of mammary glands by appropriate hormones results in the synthesis of a large number of proteins that are modified by post-translational modification. Protein kinases phosphorylate many of these proteins. However, it has been difficult to phosphorylate caseins completely in vitro using protein kinase. This may be because the appropriate kinases have not been isolated or because a special primer must be present for further phosphorylation to occur. Phosphoseryl transfer RNA recently studied by Dr. Hatfield and coworkers might have a function in protein phosphorylation. When this becomes available it will be of interest to determine whether prolactin stimulates its synthesis and whether it has a role in protein phosphorylation.

Modification of proteins plays an important role in cellular interactions, hormone responsiveness, viral transformation, and enzyme function. A histochemical method developed in this laboratory recognizes phosphorus and sialic acid groups on proteins in gels and on tissue sections without the use of a radioactive label. This is particularly useful in the analysis of fluids and tissues from humans when isotopic labeling may not be feasible. In conjunction with in vitro studies of rodent tissue, studies aimed at the elucidation of post translational modification of proteins during carcinogenesis in rodents and humans will continue. Both hydrocortisone and prolactin are required for the full expression of milk protein production in mammary explants. Glucocorticoids interact with a receptor in the cytoplasm that is activated and translocated to the nucleus and binds to DNA. Using the mammary explant system it may be possible to determine whether one of the responses to the addition of hydrocortisone is a translocation of a phosphorylated receptor from the cytoplasm to the nucleus. It is known that MMTV replication is stimulated by glucocorticoids; however, the manner in which the virus interferes with or potentiates receptor functions in the mammary cell is unknown. This may play a role in the carcinogenic process. The manner in which prolactin regulates cellular responses following interaction with its receptor is also not known. Levels of control at the transcriptional and translational level will be examined. Protein synthesis and modification in response to hydrocortisone and/or prolactin in nuclear and cytoplasmic fractions will be assessed to determine which proteins, in addition to the major milk proteins, are modified.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04785-12 LMC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

DNA Repair Studies on Normal Human, Human Tumor, and Transformed Human Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.S. Day, III	Research Physical Scientist	LMC	NCI
OTHER:	M.R. Mattern	Senior Staff Fellow	LMC	NCI
	D.B. Yarosh	Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI - NIH - Bethesda, Maryland 20205

TOTAL MAN-YEARS:	5.0	PROFESSIONAL:	3.0	OTHER:	2.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
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 (17) and SV40-transformed (7) strains deficient in the repair of 0-six-methylguanine (0-6-MeG, a modified DNA base made by certain methylating agents) was identified earlier in this project. Such strains were called MER (-). Like SV40, Rous sarcoma virus was found to produce MER (-) strains. After treatment to produce 0-6-MeG, MER (-) strains, while able to relax the supercoiling of their DNA as well as MER (+) strains, were unable to restore supercoiling to their DNA, whereas MER (+) strains could. One MER (-) strain was found to be unable to relax DNA supercoiling after such treatment, leading to the idea that the relaxation process is enzymatic. Ethidium bromide titration techniques were employed as a further probe into the nature of the initial relaxation step characteristic of cells proficient and deficient in the repair of both ultraviolet- or methyl-damaged DNA. An activity that removes the methyl group from 0-six-methylguanine contained in DNA was detected in MER (+) but not MER (-) lines.

Project Description

Objectives: To learn more about DNA repair mechanisms in human 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 and chemotherapeutic agents in altering DNA.

Methods Employed: 1. Nucleoid sedimentation assay: Following the procedure of Cook and Brazell (Nature 263: 679, 1976), cells were lysed on neutral sucrose gradients containing 2M NaCl and sedimented at about 15,000 rpm for a few hours. The rapidly sedimenting structures that contain DNA (nucleoids) lack most chromosomal proteins (including histones) and behave as if their DNA is supercoiled in that increasing concentrations of ethidium bromide in the gradients causes changes in nucleoid sedimentation (Mattern and Painter BBA 563: 293, 1979) reminiscent of those caused in the small covalently closed supercoiled circular DNA. Changes in the rate of nucleoid sedimentation velocity are interpreted due to alterations in DNA supercoiling which ultimately arise from either the production of strand breaks or to enzymatic coiling or uncoiling. Thus, cellular response to DNA damaging agents can be studied by following the time course of changes in sedimentation rate of nucleoids prepared from cells as a function of time after the damage has been introduced.

2. Ethidium bromide titration curves: Using the nucleoid sedimentation assay it is possible to estimate the amount of nucleoid supercoiling by employing ethidium bromide to cause quantifiable changes in sedimentation rate. By stacking between the base pairs (intercalation) of small covalently closed supercoiled circular DNA molecules, ethidium bromide at low concentrations decreases the DNA sedimentation rate, in part due to concomitant unwinding and opening of the twisted covalently closed DNA structure. Increasing the ethidium bromide concentration further decreases the rate of sedimentation until a minimum rate (correlating with the formation of untwisted open circular DNA structures) is observed. Further increases in ethidium bromide concentration force the molecules into a twisted structure again, but the twists are now in the direction opposite from the original. It is not known whether mammalian cell nucleoids behave as do the small supercoiled DNAs, but upon treatment with increasing ethidium bromide concentrations, the nucleoid sedimentation rate decreases, goes through a minimum, and then increases in a way suggesting that nucleoid supercoiling is very similar to that of twisted supercoiled DNA. Using this method to analyze nucleoids whose sedimentation rate has been altered by DNA damaging agents, it is possible to differentiate between DNA strand breaks and DNA unwinding as the cause for the alteration. The sedimentation rate of noncovalently closed DNAs (containing a strand break) is altered only, very slowly, by ethidium bromide (by extending the molecule, not unwinding) and does not go through a minimum. DNA which is partially unwound requires less ethidium bromide to produce the minimum sedimentation rate.

3. DNA synthesis: DNA synthesis was assayed in intact cells by measuring the incorporation of radioactively labeled thymidine (TdR) into TCA-insoluble material. Repair DNA synthesis was measured according to the BND-cellulose method of Scudiero et al.

4. The survival of cells treated with ultraviolet light or chemicals was assayed by growth of the cells into colonies of at least 50 cells.

5. Plasmid production and purification, together with genomic transfer to *E. coli* and human cells and assays for their presence, followed published protocols.

6. Cellular extracts and partially purified fractions were assayed for O^6 -methylguanine repair activity using as substrate either DNA methylated by [3H -methyl]-methylnitrosourea or a synthetic double-stranded polymer (kindly supplied by Dr. S. Mitra, Oak Ridge National Laboratory) containing [3H -guanine]- O^6 -methylguanine. Reaction mixtures were incubated at $37^\circ C$, 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.

7. 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 nontreated adenovirus. The infected cells are then incubated 12-14 days with feeding by means of periodic overlaying with a nutrient agar. Nontreated virions or those treated ones which have been "reactivated" by cellular repair mechanisms form countable plaques of dead, lysed cells.

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 17 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.

1. Identification of the Rem^- phenotype. In collaboration with Dr. D. Scudiero (FCRF), a group of four Mer^+ human tumor cell strains having a sensitivity to MNNG-produced reduction in colony forming ability between that of Mer^- and Mer^+ strains was identified. The phenotype of this group, high sensitivity of MNNG produced killing and normal ability to repair MNNG-treated adenoviruses, was provisionally been termed " Rem^- ." Rem^- strains have been studied and found sensitive compared to other Mer^+ strains in other assays. With respect to MNNG-treated Rem^+ Mer^+ strains, MNNG-treated Rem^- Mer^+ 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 [3H -methyl]-MNNG "tester dose." On the other hand, compared to $Rem^+ Mer^+$ strains, $Rem^- Mer^+$ strains tend to be more resistant to the killing action of methyl methanesulfonate, a methylating agent that produces much fewer oxygen methylations than does MNNG.

2. Mer^- human tumor cell strains. Studies with Mer^- cell strains have been continued this year in order to define more precisely the nature of the defect that results in their inability to repair O^6 -methylguanine. Both MNNG-treated Mer^+ and Mer^- cell strains rapidly relax their DNA supercoils (as assayed by nucleoid sedimentation techniques). After several hours, Mer^+ cells are able to regenerate DNA supercoiling, presumably reflecting DNA repair, because Mer^- cell strains do not regenerate DNA supercoiling even by 24 hours after MNNG treatment. One exceptional Mer^- strain, A1336, prepared from an ovarian cancer, unlike any other strain studied so far, failed to relax its DNA supercoils after MNNG treatment. This likely means that the DNA supercoil relaxation observed in every other strain in response to MNNG treatment is under cellular control and is not due simply to "spontaneous" (non-enzymatic) breakage of MNNG-treated cellular DNA.

Extracts of Mer^- and Mer^+ cells were assayed for ability to alter O^6 -methylguanine. Extracts from seven 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 and human tissue. The activity from one of the Mer^+ strains was partially purified by DNA-cellulose chromatography. By contrast, none of the extracts of seven Mer^- cell strains repaired O^6 -methylguanine, an indication that the fundamental difference between Mer^+ and Mer^- strains is likely a repair protein.

In order to select a Mer^- strain suitable for cloning the *Mer* gene, human tumor cell strains are being used as recipients for the *E. coli*-SV40 derived plasmid, pSV2gpt, which confers upon the cells the ability to utilize xanthine as a purine source. Initial studies show a wide variability (about 100-fold) among the cell strains in their efficiency in serving as recipients for such transfer.

We followed up the finding that SV40 virus in 7 of 11 SV40 transformed human fibroblast cell strains produced cultures having the Mer^- phenotype. Rous sarcoma virus (RSV) and Kirsten mouse sarcoma virus (KiMSV) do not transform human fibroblasts in a stable fashion. Dr. J. Rhim (LCMB, DCCP, NCI) (Project No. Z01 CP 05060-03 LCMB) has found that RSV and KiMSV can stably produce foci in cells grown out from a flat colony of a human osteosarcoma cell strain, TE85. It was shown that TE85 and KiMSV-transformed TE85 were Mer^+ , but that the RSV-transformed TE85 strain was Mer^- . Thus the production of the Mer^- phenotype by virus is a property not only of SV40 (and likely EB) virus, but also of RNA tumor viruses.

3. Studies of inhibition of DNA repair and DNA synthesis by DNA-topoisomerase and DNA polymerase inhibitors: There is evidence that during recovery from DNA damaging agents cells repair their DNA using various activities concerned with DNA metabolism. Among these are both topoisomerases, proteins that generate and/or relax supercoiling in DNA, and DNA polymerases, proteins that catalyse the synthesis of the DNA itself. We have studied the action of the bacterial topoisomerase inhibitors, novobiocin and nalidixic acid, and that of the inhibitors of the mammalian alpha- and beta-type DNA polymerases on repair processes in cells treated with ultraviolet light or MNNG, using nucleoid sedimentation as the primary assay. The inhibitors used for alpha-type polymerases included cytosine arabinoside (ara C) and aphidicolin; for beta-type polymerases, dideoxythymidine (ddTDR).

a) Response to MNNG damage: Used individually, novobiocin, ara C, aphidicolin, and ddTDR were found not to inhibit the initial step of relaxation of nucleoid supercoiled structure, but rather to slow the later step, carried out by Mer⁺ but not Mer⁻ strains, that results in the restoration of supercoiled nucleoid DNA structure. In agreement with the idea that both alpha- and beta-type DNA polymerases function during repair, combined ara C (or aphidicolin) plus ddTDR treatment resulted in more inhibition than either alone, even used alone at a 5 or 10 times greater dose than in the combination. In one Mer⁻ strain, A1336, which is unable to relax its DNA supercoiled structure after MNNG-treatment, ddTDR was noted to induce such relaxation (as did novobiocin) possibly implicating topoisomerase or beta polymerase functions in the relaxation step. Further, A1336 was exceptional in that it was the only strain studied which was refractory to ddTDR-produced inhibition of DNA synthesis, possibly indicating in this cell strain the lack of or an altered beta-type polymerase activity.

b) Response to ultraviolet light: Results of ara C (or aphidicolin) and ddTDR inhibition of the normal UV-repair process indicated again that both polymerase types alpha and beta can carry out repair of UV-damage DNA, and by blocking both, restoration of nucleoid supercoiling is almost totally abolished.

Cells deficient in the repair of UV-damage (from patients with xeroderma pigmentosum) which, unlike normal cells do not relax DNA supercoiling after UV, were analyzed for the induction by novobiocin of such relaxation. Novobiocin treatment of ultraviolet-irradiated XP cells did induce relaxation, paralleling the effects of novobiocin on sedimentation of nucleoids from MNNG-treated repair-deficient A1336 cells. Thus, after either UV- or MNNG-treatment of cells capable of normal repair, nucleoid relaxation may involve similar mechanisms.

c) Virus studies of inhibition of DNA repair. In order to assess the effects of polymerase inhibitors on biologically effective DNA repair, aphidicolin, ara C, and ddTDR were used to attempt to suppress repair of UV-damaged adenovirus 5. Only aphidicolin was able to suppress repair to an experimentally detectible extent. It was found that hydroxyurea (previously found in this project to be an inhibitor of UV-repair) and aphidicolin together blocked repair of UV-damaged viruses more than either alone. Hydroxyurea presumably works this way by reducing the dCTP pool, with which aphidicolin competes for alpha-type polymerases.

4. Ethidium bromide titration studies of steps in DNA-repair. In order to assess the nature of the post-damage unwinding events more precisely, nucleoids prepared from MNNG- or UV-treated cells undergoing incubation to allow possible repair were sedimented in gradients containing various concentrations of ethidium bromide. The titration curves generated (as described in Methods Employed) with nucleoids from MNNG-treated cells, Mer⁺ and Mer⁻ cells, were observed first to unwind their DNA, then to introduce strand breaks into it. A1336 cells, on the contrary, responded to MNNG-damage by producing nucleoids whose pattern of supercoiling was clearly altered, but in which no strand breaks could be detected.

Studies with nucleoids from cells irradiated with ultraviolet light showed a similar response: the data indicated that UV-irradiated cells proficient in UV-repair first unwound DNA supercoils, then produced strand breaks. UV-irradiated repair-deficient cells (again, from patients with xeroderma pigmentosum) showed no strand breakage, but a definite alteration in the pattern of DNA supercoiling.

5. Studies of post-treatment behavior of L1210 cells.(in collaboration with K. Kohn, Y. Pommier, and L. Zwelling, LMP, DCT, Project No. Z01 CM-06150-01 LMPH and Z01 CP 06151-01 LMPH). Alkaline elution studies indicated two components in the repair of DNA after X-ray damage: one with a half-time of about one-half hour and delayed by an inhibitor of poly-ADPribose polymerase; the other with a half time of 2.5-3 hours and blocked by novobiocin. This slower step was found to correspond in time to the reconstruction of supercoiled nucleoids; and further, such reconstruction was blocked by novobiocin.

After m-MSA [4'-(9-acridinylamino)methane sulfonyl-m-anisidine] treatment, DNA breaks are observed by both the alkaline elution assay and the nucleoid sedimentation assay, but only after protease treatment of the DNA. The results indicate that a protein may protect the DNA double-helix after damage or that a repair-related protein becomes strongly associated with DNA.

Significance to Biomedical Research and the Program of the Institute: The results of this project suggest 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 human tumors and to be relatively ineffective against others, also indicating the possibility that the molecular basis for the success of alkylation chemotherapy depends upon tumors with heterogeneous properties. 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 treated more successfully 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 genetic 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: To pursue the goals outlined above in Objectives and to publish the results.

Publications:

Day, Rufus S., III.: Adenovirus: A probe for human cells deficient in DNA repair. Bioscience 31: 807-813, 1981.

Day, Rufus S., III, and Ziolkowski, C.H.J.: Differential effects of hydroxyurea on the survival of UV- and MNNG-treated adenovirus 5. Mutat. Res. (In press).

Day, Rufus S., III, and Ziolkowski, C.H.J.: Hydroxyurea inhibits the repair by human cell strains of UV-damaged adenovirus 5. Mutat. Res. (In press).

Day, R.S., III, and Ziolkowski, C.H.J.: Induced reversion using human adenovirus. In Kilbey, et al. (Eds.): Handbook of Mutagenicity Test Procedures, Vol. 2. Amsterdam, Elsevier/North-Holland Biomedical Press (In press).

Day, Rufus S., III and Ziolkowski, C.H.J.: UV-induced reversion of adenovirus 5ts2 infecting human cells. Photochem. Photobiol. 34: 403-406, 1981.

Day, Rufus S., III, Ziolkowski, C. and DiMattina, M.: Studies on fibroblasts from persons having Cockayne's Syndrome using UV-irradiated adenovirus 5. Photochem. Photobiol. 34: 603-607, 1981.

Mattern, M.R.: Differences between diploid and transformed human cells in the incorporation of thymidine and 5-bromodeoxyuridine into DNA. In Katz, R. and Cox, R.P. (Eds.): Intact Mammalian Cell Lines in the Study of Inborn Metabolic Diseases. Proceedings of a workshop on intact mammalian cells in the study of metabolic diseases. Bethesda, Maryland, 1980 Washington, D.C. Government Printing Office (In press).

Mattern, M.R., Paone, R.F., and Day, R.S., III: Eukaryotic DNA repair is blocked at different steps by inhibitors of DNA topoisomerases and of DNA polymerases alpha and beta. Biochim. Biophys. Acta 697: 6-13, 1982.

Mattern, M.R., Paone, R.F., and Day, R.S., III.: Human tumour strains defective in the repair of alkylated DNA fail to regenerate rapidly sedimenting nucleoids after N-methyl-N'-nitro-N-nitrosoguanidine treatment. Carcinogenesis 2: 1215-1218, 1981.

Slor, H., Mizusawa, H., Neihart, N., Kakefuda, T., Day, R.S., III, and Bustin, M.: Immunochemical visualization of the binding of a chemical carcinogen to the genome. Cancer Res. 41: 3111-3117, 1981.

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., Rosenstein, B.S., and Setlow, R.B.: Excision repair and patch size in UV-irradiated bacteriophage T4. J. Virol. 40: 465-471, 1981.

Zwelling, L.A., and Mattern, M.A.: Intercalator DNA breaks and cytotoxicity are indistinguishable in normal, xeroderma pigmentosum, and ataxia telangiectasia fibroblasts. Mutat. Res. (In press).

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Role of DNA Damage and Repair in In Vitro Chemical Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ko-Yu Lo	Guest Researcher	LMC	NCI
OTHERS:	Takeo Kakunaga	Chief, 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:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been for a long time an unsolved question of how the ultimate form of benzo(a)pyrene (BP), benzo(a)pyrene diol epoxide (BPDE-I), reaches nuclear DNA in spite of its instability in aqueous solution. By using an exogenous plasmid DNA method to assay the binding activity of BPDE-I to DNA it was found that the microsomal fraction of the cell homogenate has the ability to stabilize BPDE-I in aqueous solution. However, the inability of partially purified P-450 to stabilize BPDE-I indicates the importance of a lipid moiety in protecting BPDE-I from hydrolysis. Translocation of BPDE-I from microsome to nuclei (demonstrated in human cell culture (KD) and rat hepatocytes) indicates that an active carcinogen metabolite can be transferred from its activation site to the target molecules, a possible mechanism of cytoplasmic activation of carcinogen followed by interaction with nuclear DNA. A portion of BPDE-I remained active in cells by a cellular stabilizing mechanism. Thus, secondary BPDE-I DNA adducts were formed upon cell lysis during the isolation of DNA. This introduced experimental artifacts concerning the amount of carcinogen actually bound to DNA and in turn changes the repair rate of the cells.

Project Description

Objectives: The objective of this project is to study the biochemical mechanism of induction of transformation *in vitro* by chemical carcinogens. Current interests are 1) localization of the active metabolite (BPDE-I) of benzo(a)pyrene (BP) in subcellular fractions of cell homogenate using a novel exogenous DNA method developed by us; 2) mediation of BPDE-I by cellular components to the target nuclear DNA; and 3) fate of BPDE-I in cells and clarification of its biological importance with special attention to DNA damage and repair.

Methods Employed: Cell culture, radioactive tracer, gel electrophoresis; subcellular fractions were prepared by differential centrifugation. Rat liver nuclei were prepared by the method of Kasper (*Method Enzymol.* 31: 279, 1974). KD nuclei were isolated by swelling cells in hypotonic buffer containing 0.2% NP-40, followed by homogenization and centrifugation. DNA was isolated by phenol extraction, ethanol precipitation, and purified through CsCl-ethidium bromide centrifugation.

Major Findings: Discovery that BPDE-I remains active in cells. When human cells (KD & XP) were treated with [³H] BPDE-I (0.33-3.3 μ M) and then post-incubated with carcinogen-free depleted medium, the removal of BPDE-DNA adducts showed a biphasic kinetics--a fast repair phase followed by a slower repair phase. Since the repair-deficient XP and normal cells had similar types of removal kinetics, there is a possibility that carcinogen-DNA adducts measured in the fast removal phase may contain some adducts produced during the isolation of DNA under these experimental conditions. In order to (a) demonstrate that the binding of carcinogen could occur if any of the active metabolites are present in cells upon cell lysis and (b) distinguish these carcinogen adducts from the ones previously formed in cells during the carcinogen treatment period, exogenous plasmid PBR322 DNA was introduced into the reaction mixture to assay for the presence of BPDE-I-DNA adducts after cell lysis. The plasmid DNA can be separated from the nuclear DNA by banding on a CsCl-ethidium bromide density gradient. It was found that the BPDE-I binding curve of plasmid DNA was parallel to the binding of nuclear DNA. The association of ³H-labeled radioactivity with the plasmid DNA samples therefore indicates the presence of active BPDE-I in cells and that BPDE-I-DNA adducts indeed formed during the isolation of DNA.

Localization of Active Carcinogen Metabolites in Subcellular Fractions

Results above indicated the stabilization of BPDE-I by some cellular components. The ability of BPDE-I to bind to exogenous DNA was measured in the presence of subcellular fractions. It was found that the microsomal fraction was able to enhance the binding of BPDE-I to DNA. BPDE-I preincubated with microsomes, followed by assaying the binding activity of BPDE-I to DNA, indicated that BPDE-I is stable in microsomes at 37° for at least 2 hours and the activity increases with increasing microsome concentrations. Soluble fractions (110,000 xg supernatant) and BSA have no such ability. Partially purified P-450 and microsomes pre-digested with lipase lose the ability to protect BPDE-I from hydrolysis which suggests the lipid moiety of microsomes may play an important role in stabilization of BPDE-I.

Translocation of BPDE-I from Microsome to Nucleus

To ascertain that a carcinogen metabolite can be transferred from its activation site to the target molecule DNA, carcinogen-microsome complexes were prepared by mixing the microsomes with BPDE-I, then centrifuging at 110,000 xg to collect the microsomes. The BPDE-I-containing microsomes were then incubated with nuclei. At the end of incubation, nuclei were collected, washed and DNA was isolated. It was found that 35% of the radioactivity in the BPDE-I-microsome complex was transferred to the nuclei. The amount of BPDE-I bound to DNA in these samples was 120% of the control (nuclei reacted with BPDE-I directly) in the KD system and 70% in the rat hepatocyte system.

Significance to Biomedical Research and the Program of the Institute: 1) The discovery that the short-lived BPDE-I is stable in cells and that carcinogen-DNA adducts could be formed during the isolation of DNA indicates the existence of cellular stabilizing mechanisms for unstable carcinogens. It also suggests that experimental artifacts could be introduced in determining the amount of carcinogen actually bound to DNA, which, in turn, changes the apparent repair rate of the cells. Therefore, caution should be taken in the interpretation of the relation of this experimental data to the mutagenic and carcinogenic processes.

2) The translocation of active carcinogen metabolites from microsome to nucleus demonstrated in KD cells and rat hepatocytes suggests a possible mechanism of cytoplasmic activation of carcinogen followed by reaction with target nuclear DNA and of mammalian cell mutagenesis by cocultivation of cells with rat hepatocytes.

Proposed Course: To characterize and identify the molecular nature of the stabilizer or carrier of BPDE-I and to develop an optimum method for measuring DNA adducts and their repair without any secondary modification and artifact.

Publications:

Lo, K.Y. and Kakunaga, T.: Similarities in the formation and removal of covalent adducts in benzo(a)pyrene-treated BALB/3T3 variant cells with different induced transformation frequencies. Cancer Res. (In press).

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies to Carcinogen Metabolizing Enzymes of Humans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.S. Park	Visiting Associate	LMC NCI
OTHER:	H.V. Gelboin	Chief	LMC NCI
	R. Robinson	Biologist	LMC NCI

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:

1.75

PROFESSIONAL:

0.5

OTHER:

1.25

CHECK APPROPRIATE BOX(ES)

- (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
- (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Benzo(a)pyrene (BP) is metabolized either to nontoxic water soluble conjugates or to the ultimate carcinogen, BP-7,8-diol-9,10-epoxide. The direction of BP metabolism might be dependent on the presence of specific predominant forms of enzymes involved in the BP metabolism of individuals exposed to chemical carcinogens. Monoclonal antibodies (MABs) are specific to antigenic determinants and are useful tools to test and locate specific enzymes. Cytochrome P-450 and epoxide hydrolase (EH) are key components of the system responsible for the metabolism of BP. In order to prepare MAB probes to these enzymes Balb/c female mice were immunized with pure preparations of cytochrome P-450 and human epoxide hydrolase. The primed spleen cells were fused by polyethylene glycol with myeloma cells and hybridomas were screened for antibody produced against cytochrome P-450 and EH. Four hybridomas produced MABs which bind to human liver cytochrome P-450 but do not cause precipitation. Against EH, four hybridomas produced MABs which bound and precipitated only the form which was used as antigen but not other forms. Effects of the MABs on enzyme activities and the cellular location of the antigens are yet to be clarified.

Project Description

Objectives: Activation of benzo(a)pyrene is initiated by mixed function oxidases and, followed by EH, forms carcinogenic products as well as nontoxic water soluble conjugates. Several forms of the P-450s have been reported and 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 environments. Microsomal preparations of placenta from women who smoke heavily possess high levels of mixed function oxidases. Human monocytes, lymphocytes, and lung cell lines are inducible by benzo(a)anthracene. The objective of this work is to prepare MABs to enzymes involved in BP metabolism and to use these MABs as tools to study the multiplicity of enzymes as well as to identify the predominant forms inducible by carcinogens.

Methods Employed: Balb/c female mice were immunized with purified or partially purified cytochrome P-450 derived from the microsomes of human liver and placenta of women who smoke heavily. 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 the respective cytochrome P-450. Ouchterlony double immunodiffusion analysis was adapted for precipitin reactions. The effect of MABs on enzyme activities was measured by fluorometry and HPLC.

Major Findings: Four MABs were obtained from hybrids made by the fusion of myeloma with spleen cells of mice immunized with human liver cytochrome P-450. They were all immunoglobulin subclass IgG1. Mouse serum to the cytochrome P-450 precipitated the antigen but the MABs did not. Direct inhibition of aryl hydrocarbon hydroxylase activity was not observed but activity could be precipitated from solution by precipitating the MAB enzyme complex from solution with protein-A bound to Sepharose 4B. Four MABs were obtained against EH. These MABs bound and precipitated that form of EH used as an antigen, but not the other forms. The effect of these MABs on EH activity is yet to be determined.

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 ultimate carcinogenic metabolite of BP, BP-7,8-diol-9,10-epoxide. Both of these enzymes are present in multiple forms. Preparation of MABs to a specific form of these enzymes is essential for the study of the activation and regulation of BP metabolism. Provided with a series of monoclonal antibodies to enzymes in BP metabolism, not only can we better understand chemical carcinogenesis, but we will be able to analyze the differences in individual susceptibility to cancer and develop techniques for screening and preventing the occurrence of human cancer among populations exposed to environmental carcinogens.

Proposed Course: 1) MABs against all of the different forms of cytochrome P-450 and EH will be prepared. 2) An assay system is under development for MABs which do not show direct inhibition of enzyme activities. 3) The cross reactivity of MABs to human enzymes with enzymes from different animals will be tested.

Publications:

None

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PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05089-04 LMC

PERIOD COVERED
October 1, 1981 to January 1, 1982

TITLE OF PROJECT (80 characters or less)

Effect of Epoxide Hydrolase in DNA Binding and BP Metabolism by P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: E.M. Gozukara Visiting Scientist LMC NCI
OTHER: H.V. Gelboin Chief LMC NCI

COOPERATING UNITS (if any)
F.P. Guengerich, Associate Professor of Biochemistry, Vanderbilt University, Knoxville, Tennessee

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TOTAL MANYEARS: 1.2 PROFESSIONAL: 1.0 OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The effects of human liver epoxide hydrolase were examined by using 3H(-)-7, 8-diol BP as a substrate to generate diol epoxide I and II in the incubation medium, with uninduced and BA-preinduced human monocytes, lymphocytes, Fischer rat liver (TRL-2) cells, and Buffalo rat liver (BRL) cells. The binding of reactive metabolites to added and intact cellular DNA was determined. The epoxide hydrolase was added either to incubation mixtures or to tissue culture media and inhibition of DNA binding was observed.

In the second part of the project, major forms of cytochrome P-450 were purified from 3-methylcholanthrene (3MC), phenobarbital (PB), and Beta-naphtho-flavone (BNF)-treated rat liver microsomes. A purified, reconstituted microsomal mixed function oxidase (MFO) system, containing different forms of cytochrome P-450, NADPH cytochrome c (P-450), reductase and dilauroylglyceryl 3-phosphocholine was used to analyze benzo(a)pyrene metabolism. Each cytochrome P-450 exhibited a unique pattern of metabolism.

Project Description

Objectives: To determine the metabolism of $^3\text{H}(-)\text{t-7}$, 8-diol BP and covalent binding of reactive metabolites to added and intact cellular DNA in tissue culture media. To demonstrate the unique positional specificities of BP metabolism by different forms of cytochrome P-450 which may regulate the balance between activation and detoxification pathways of polycyclic aromatic hydrocarbons (PAH).

Methods Employed: Fluorospectrophotometry, high pressure liquid chromatography, tissue culture techniques, slab gel electrophoresis and autoradiography.

Major Findings: Human monocytes, lymphocytes, Fischer rat liver (TRL-2) cells, and Buffalo rat liver (BRL) cells catalyze binding of $^3\text{H}(-)\text{t-7}$, 8-diol BP to added and intact cellular DNA in tissue culture media. Cells that are preinduced by benzanthracene (BA) exhibit greater levels of DNA binding than do uninduced cells. We reported that addition of $^3\text{H}(-)\text{t-7}$, 8-diol BP to tissue culture media resulted in the binding of metabolites of this compound to intact cellular DNA. In all cases the addition of epoxide hydrolase to the incubation or tissue culture media reduced the amount of reactive metabolites binding to DNA.

Highly purified cytochrome P-450s from the liver of rats treated with either 3-methylcholanthrene (3MC), phenobarbital (PB) or β -naphthoflavone (BNF) were used in a reconstituted system to analyze BP metabolism by high pressure liquid chromatography. In this study, twelve metabolites of BP were separated. Six unknown metabolites were also observed. BNF and 3MC-type cytochrome P-450 shows that in both cases 7, 8-dihydrodiol is a major diol, 3-OH-BP and 9-OH-BP are major phenols and 1, 6-, and 3, 6-quinones are major quinones. This result suggests that 3MC and BNF induced largely the same forms of cytochrome P-450. However, PB-type cytochrome P-450 is of a different form. The differences in metabolism are striking. Thus, the P-450-3MC and P-450-BNF show a large preference for metabolism at the 1,3-position, the 7,8-position, and the 6-position, whereas the P-450 PB prefers metabolism at the 4,5-position. All forms of P-450 show similar preference for the 9,10-position.

Proposed Course: This project has been halted temporarily and will resume when we have successfully prepared monoclonal antibodies to the different P-450s.

Publications:

Gozukara, E.M., Belvedere, G., Robinson, R.C., Deutsch, J., Coon, M.J., Guengerich, F.P. and Gelboin, H.V.: The effect of epoxide hydrolase on benzo(a)pyrene diol epoxide hydrolysis and binding to DNA and mixed function oxidase proteins. Molecular Pharmacol. 19: 153-161, 1981.

Gozukara, E.M., Guengerich, F.P., Miller, H. and Gelboin, H.V.: Different patterns of benzo(a)pyrene metabolism of purified cytochrome P-450s from methylcholanthrene, β -naphthoflavone phenobarbital and treated rats. Carcinogenesis 3: 129-133, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05109-03 LMC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Interaction of Chemical Carcinogens with the Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Bustin	Visiting Scientist	LMC NCI
OTHERS:	P.D. Kurth	Senior Staff Fellow	LMC NCI
	M.M. Seidman	Senior Staff Fellow	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:

1.4

PROFESSIONAL:

1.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The influence of the structure of the chromatin fiber on the binding of the chemical carcinogen benzo(a)pyrene diol epoxide-1 (BPDE-1) to the genome was studied. Antibodies specific to DNA modified by BPDE-1 were elicited and used to visualize the binding sites of the carcinogen to DNA in intact cells and in polytene chromosomes by immunofluorescence and in SV40 minichromosomes by immunoreplica techniques. The results indicate that 1) in polytene chromosomes an apparent "hot spot" for antibody binding corresponds with certain transcriptionally active loci and 2) in the SV40 minichromosomes the non-nucleosomal origin of replication shows a 1.4 increase in BPDE-1 binding as compared to the rest of the chromatin. It is concluded that the packing of the cellular DNA into nucleosomal conformation does not significantly affect the binding of BPDE-1 to DNA.

Project Description

Objectives: To study the mechanism of the interaction of chemical carcinogens with the genome. To understand the influence of the structure and packing of the chromatin fiber on the binding of a carcinogen to its target.

Background and Research Strategy: The interaction of a carcinogen with the genome of a target cell seems to be the 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 and is used 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.

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?

The third approach involves the use of antibodies specific for BPDE-1 DNA to study various aspects of damage to the genome induced by the carcinogen and its subsequent repair.

Methods Employed: An encapsidation mutant growing in permissive cells is used as the target for the carcinogen. The carcinogen is either benzo(a)pyrene or its metabolite, benzo(a)pyrene 7, 8-diol-9, 10-epoxide. Detection of the binding of the carcinogen to DNA is accomplished by two methods. The first

is to use benzo(a)pyrene as a depurinating reagent and use accepted DNA sequence techniques to pinpoint the location of the damaged base. The second involves transfer of the modified DNA fragment to DBM paper and visualize the location of the modified DNA fragment by accepted immunochemical techniques.

For the polytene chromosome system, salivary glands are obtained from the fourth instar of Chironomus thummi. Polytene chromosome squashes are prepared by conventional techniques. The squashes are reacted with ^3H -diol epoxide and the location of bound carcinogen visualized by autoradiography. The grain density in the various regions of the polytene chromosomes is determined semiautomatically using an Artek Model 880 micro/macro counting system.

For determination of the *in vivo* binding sites, larvae are grown in petri dishes in 10% DMSO and about 1 mCi ^3H -benzopyrene. After incubation for various times the larvae are washed and squashes of salivary glands are processed for autoradiographs.

The presence of a mixed-function oxidase system in Chironomus is detected by difference spectroscopy and by fluorometric assay for aryl hydrocarbon hydroxylase activity.

The ability of Chironomus to metabolize benzo(a)pyrene is determined by high performance liquid chromatography using a Whatman Partisil ODS-2 column with a linear 60-100% methanol-water gradient.

Major Findings: 1) Polyclonal antibodies against benzo(a)pyrene diol epoxide modified DNA react specifically with cells treated with benzo(a)pyrene diol epoxide. The binding can be visualized by immunofluorescence. Within a population of cells all the cells are modified to the same degree. The various cells, however, differ in their ability to remove the carcinogen bound to the genome. The accessibility of the repair enzyme to the modified DNA may affect the rate at which the adduct is removed from the DNA. 2) Spreads of polytene chromosomes prepared from Chironomus thummi were reacted with ^3H -BPDE-1. The location of the bound molecules were visualized by autoradiography. A slight preference for carcinogen binding to the interbands of these chromosomes was observed. The binding of the carcinogen to DNA and RNA was distinguished from the binding to protein by immunofluorescent studies using anti-BPDE-1 antibodies. Treatment of the chromosomes with RNAase prior to antibody addition effectively removes RNA thereby allowing visualization of the loci at which the adduct is bound to DNA. A "hot spot" for carcinogen binding to the DNA in chromosome III of the larvae was detected. This region is not preferentially enriched in AT or CG regions as determined by counterstaining with quinacrine or mithramycin. Uridine incorporation studies suggest that transcribable regions correspond to the regions were anti-BPDE-1 preferentially bound. The results indicate that the transcribable regions are "hot spots" for carcinogen binding. However, it is still possible that in these regions the adducts are more accessible to the antibodies. Chironomus larvae metabolize benzo(a)pyrene and have a constitutively high level of aryl hydrocarbon hydroxylase. Examination of the polytene chromosomes from larvae which have been fed benzo(a)pyrene will reveal whether there are preferential interaction sites in DNA under *in vivo* conditions. 3) An immunochemical method which allows the localization of a single BPDE-1

adduct per SV40 genome has been used to study the binding of BPDE-1 to nucleosomal DNA and to the coding and noncoding strands of SV40 DNA. No significant difference in the ability of the various strands or the various nucleosomal regions of SV40 to bind BPDE-1 was detected. In part of the SV40 molecules the origin of replication is in a nonnucleosomal conformation. The binding of BPDE-1 to this region, compared to the rest of the molecule, was enhanced slightly.

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 to 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.

Publications:

Slor, H., Mizusawa, H., Neihart, N., Kakefuda, T., Day, R.S. and Bustin, M.: Immunochemical visualization of binding of the chemical carcinogen benzo(a)pyrene diol epoxide to the genome. Cancer Res. 41: 3111-3117, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05125-02 LMC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Characterization of Monoclonal Antibodies to Rat Liver Cytochrome P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.S. Park	Visiting Associate	LMC NCI
OTHER:	H.V. Gelboin	Chief	LMC NCI
	R. Robinson	Biologist	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:

1.75

PROFESSIONAL:

0.5

OTHER:

1X25

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cytochrome P-450 plays key roles in the metabolism, activation and detoxification of drugs, endogenous steroids, xenobiotics and environmental carcinogens. Cytochrome P-450 isolated from the liver of rats treated with phenobarbital (PB-P-450) is distinct from the cytochrome P-450 isolated from the liver of rats treated with 3-methylcholanthrene (MC-P-450). Six monoclonal antibodies (MABs) to PB-P-450 and T2 to MC-P-450 were obtained from hybrids made by the fusion of myeloma cells with spleen cells derived from mice immunized with PB- or MC-P-450. These MABs are specific for their respective antigens by radio-immunoassay and double immunodiffusion assays and in inhibition of enzyme activity. These MABs have proved to be useful for identification of specific forms of cytochrome P-450 among the multiple forms present in rat microsomes and for investigation of individual differences in susceptibility to chemical carcinogens.

Project Description

Objectives: Benzo(a)pyrene (BP) is activated by aryl hydrocarbon hydroxylase (AHH) and epoxide hydratase, both of which exist in multiple forms. Metabolism of BP by these enzymes leads in two directions: to the formation of nontoxic, water soluble, excretable conjugates and/or to the formation of active carcinogenic forms, primarily BP-7,8-diol-9,10-epoxides. Any exposure of individuals to the environment of chemical carcinogens might shift the balance of the metabolism to produce different proportions of those metabolite products. Rat liver microsomes are highly inducible for these carcinogen metabolizing enzymes. We use these cross-reactive MABs to identify and characterize human carcinogen metabolizing enzymes.

Methods Employed: Balb/c female mice were immunized with purified cytochrome P-450 of rats which were treated with phenobarbital, 3-methylcholanthrene or with 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 monoclonal antibodies to P-450. The effect of monoclonal antibodies on P-450 enzyme activity was measured both fluorometrically and by HPLC.

Major Findings: 1) Six hybridomas producing MABs were obtained by the fusion of myeloma and spleen cells of mice immunized with PB-P-450. Two of the six hybrids were IgG1 and four were IGM producers. All the MABs bound to PB-P-450 but only one was inhibitory to the AHH activity of purified PB-P-450 (94%) and that of PB-microsomes (50%), while not inhibiting purified MC-P-450 or MC-microsomes. 2) Twelve MABs producing hybridomas were obtained by the fusion of myeloma and spleen cells of mice immunized with MC-P-450. Three of the twelve were very active and inhibited 95% of AHH activity of purified MC-P-450 but not the activity of PB-P-450. These MABs also inhibited 80% of the microsomal activity of MC treated rats.

Significance to Biomedical Research and the Program of the Institute: The various cytochrome P-450s display stereoselectivity for both substrate and product formation. Therefore, the balance of BP metabolism between detoxification and carcinogen formation is dependent on the presence of specific forms of the enzymes of individuals after exposure to different environments. The MABs prepared to PB-P-450 are specific to PB-P-450. However, the MABs prepared to MC-P-450 are active toward both MC-P-450 and β -naphthoflavone-induced cytochrome P-450. Therefore, a typical cytochrome P-450 could be induced by several inducers with common properties. This technique is a powerful new tool to study the genetics of these crucial enzymes of carcinogen metabolism.

Proposed Course: 1) There appear to be minor forms of cytochrome P-450 in addition to the predominant forms found after induction by PB or MC. 2) It was found that one MAB to MC-P-450 inhibited the AHH activity of human placental microsomes of women who smoked heavily. Testing of the cross-reactivity of these MABs with P-450s from other human, rat, or rabbit tissue will be continued. 3) MABs made to all different forms of cytochrome P-450 would be useful for phenotyping the multiple forms of the isozymes and thus screening for humans with specific patterns of enzymes that may lead to proneness to

chemical carcinogenesis. Toward this end, we will continue isolating P-450 specific MABs using a variety of P-450 preparations from rats.

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: Analysis of cytochromes regulation and function. In Sato, R. and Kato, R., Proc. Fifth International Symposium on Microsomes, Drug Oxidations, and Drug Toxicity. Tokyo, Jap. Sci. Soc. Press, 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 LM2 and cytochrome P-450 LM4. Mol. Pharmacol. 21: 248-258, 1982.

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.

Park, S.S., Persson, A., Coon, M.J., and Gelboin, H.V.: Monoclonal antibodies to rabbit liver cytochrome P-450 LM2. FEBS Lett. 116: 231-235, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05195-02 LMC
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Genetics and Regulation of Cytochrome P-450 Biosynthesis		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: J.B. Fagan Senior Staff Fellow LMC NCI OTHER: S.P.C. Cole Visiting Fellow LMC NCI H.V. Gelboin Chief 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: 1.1	PROFESSIONAL: 1.1	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Cytochrome P-450 is a central component of the aryl hydrocarbon hydroxylase (AHH) system. Regulation of AHH enzymatic activity has been carefully studied in vivo, as well as in cell culture using <u>Buffalo rat liver (BRL) cells</u> and other cell types. The accessibility of the BRL cell culture system to well-controlled experimental manipulation makes it ideal for studying the <u>regulation of biosynthesis of the cytochromes P-450 and of other AHH components</u> . There is no direct information on the <u>regulation of synthesis, processing and turnover of AHH system polypeptides</u> , nor is there direct information on the <u>regulation of rates of transcription, processing and degradation of the mRNAs for AHH-system enzymes</u> . Our objective is to use the BRL cell culture system to study regulation of the AHH system at these two levels.		

Project DescriptionObjectives:

- 1) 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.
- 2) Characterize the rates of P-450 synthesis in control and polycyclic aromatic hydrocarbon (PAH)-induced BRL cells.
- 3) Characterize the stability and half-life of P-450 polypeptides in control and PAH-induced BRL cells.
- 4) Aryl hydrocarbon hydroxylase activity in BRL cells can be induced by a number of different treatments, including culture with several 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.
- 5) 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.
- 6) Identify different P-450s in the BRL cells using antibodies to purified P-450s and monoclonal antibodies; compare results with those obtained in other systems.
- 7) Study processing of newly synthesized P-450 peptides by proteolysis, glycosylation and heme insertion.
- 8) Objectives 2 through 7 will also be carried out for the regulation of other enzymatic components of the AHH system including epoxide hydratase (EH).
- 9) 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 Z01 CP 05196-02 LMC). Amounts, sizes, rates of transcription and degradation, and processing of P-450 mRNAs will be examined and compared quantitatively.

Methods Employed: Standard cell culture, karyotyping and cloning procedures are being used with BRL cells. Rates of peptide synthesis, stability, turnover and degradation are measured by labeling cells in culture with ³⁵S-methionine (either steady state or pulse-chase labeling). This is followed by immunoprecipitation of solubilized, ³⁵S-labeled peptides with various monoclonal antibodies described in annual report Z01 CP 05125-02 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 Z01-CP-05196-02-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 benzoanthracene (BA), 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 ³⁵S-labeled peptides immunoprecipitated with MC-P-450 IgG.

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 1 has been attained and work is in progress and some preliminary goals have been attained for objectives 2 through 7. In this upcoming research period, these objectives, as well as the other objectives, will be pursued.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05196-02 LMC
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cloning, Structure, and Regulation of the Genes for the Cytochromes P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.B. Fagan	Senior Staff Fellow	LMC NCI
OTHERS:	H.V. Gelboin	Chief	LMC NCI
	E. Gozukara	Visiting Scientist	LMC NCI
	S.P. Cole	Visiting Fellow	LMC NCI
	J. Pastewka	Chemist	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:

2.8

PROFESSIONAL:

1.8

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cytochromes P-450 are central to the metabolism of xenobiotics and drugs and to the activation and detoxification of carcinogens. Whether a potentially carcinogenic molecule is metabolized to an active carcinogen or to an excretable form may depend on the activity and substrate specificity of the forms of the P-450s which predominate in the target tissue. As a result of this, susceptibility to cancer induction may well depend on the regulation of expression of the genes for specific cytochromes P-450. We are using recombinant DNA techniques and other molecular biological methodologies to study the structure and the regulation of the genes for the P-450s. We have constructed cdNA clones complementary to methylcholanthrene(MC)-induced P-450 mRNA. We have used these clones to quantitate MC-P-450 mRNA levels in control and MC-induced rat liver and to isolate a complete, native MC-P-450 gene. The structure of this gene is now being analyzed and the regulatory regions of this gene will be identified and characterized both structurally and functionally. This gene will also be used to isolate related P-450 genes.

Project DescriptionObjectives:

- 1) Construct, identify and characterize clones carrying cDNA complementary to the mRNAs for the cytochromes P-450 induced by methylcholanthrene, phenobarbital and other inducers.
- 2) Isolate and characterize cloned fragments of the rat genome containing the native genes for inducible cytochromes P-450.
- 3) Study regulation of P-450 gene expression at the mRNA level using cloned P-450 cDNA and gene sequences as hybridization probes. Specific objectives:
 - a) quantitation of the levels of P-450 mRNAs under various induction conditions,
 - b) quantitation of transcription rates of P-450 mRNAs, c) quantitation of degradation rates of P-450 mRNAs and d) characterization of rates and routes of P-450 mRNA processing.
- 4) Analyze the structure of the P-450 genes. Specific objectives: a) detailed characterization of the regulatory sequences associated with the P-450 structural genes by DNA sequence analysis of the cloned P-450 genes, b) electron microscopic analysis of heteroduplexes between P-450 mRNA and cloned P-450 genes to assess splicing and other structural characteristics of the P-450 mRNAs and genes, c) comparison of the regulatory sequences associated with different P-450s which are induced by different compounds and d) comparison of the structural genes for P-450s induced by different compounds.
- 5) Correlate regulatory data from objective 3 with structural data from objective 4 for the purpose of identifying relationships between gene structure and regulation.
- 6) 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 (as previously reported in Fagan *et al.*, *J. Biol. Chem.* 256: 520-525, 1981 and Fagan *et al.*, *Nucleic Acids Res* 6: 3471-3480, 1979) 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 being synthesized and cloned in the plasmid pBR322 by modifications of previously reported methods (see reference above). Clones of the complete P-450 genes are being isolated from libraries of rat genomic DNA ligated into phage lambda.

Clones are identified 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 heteroduplex mapping using the electron microscope, hybridization-selected translation and immunoprecipitation, restriction endonuclease analysis, DNA sequencing, Southern blot hybridization, solution hybridization, and in vitro transcription analysis.

Major Findings: 1) We have cloned cDNA complementary to MC-P-450 mRNA and characterized one clone in detail. It contains a 600 base-pair insert of P-450 cDNA and, by Northern blot analysis and hybridization-selected translation, it is complementary to a MC-P-450 mRNA which is 2000 nucleotides long and codes for a 51.5 kilodalton peptide. This peptide is an MC-P-450 peptide since it was recognized by a monoclonal antibody to MC-P-450. 2) We have used this cloned cDNA to quantitate MC-P-450 mRNA levels in control and MC-induced liver, showing that the level of this mRNA increases 4-6 fold by 24 hrs. after MC injection. 3) By two independent methods we have determined that there are at least two different sizes of MC-inducible mRNA which code for peptides immunoprecipitable with IgG highly specific for MC-P-450. These mRNAs are 2000 ± 110 and 2700 ± 110 nucleotides long. The smaller mRNA codes for a 51.5 kilodalton peptide, while the larger seems to be a mixture of two mRNAs that are partially resolvable under some conditions and which code for peptides of 47.0 and 54.0 kilodaltons. 4) We have determined that PB-P-450 mRNA is 2000 ± 110 nucleotides in length. 5) We have isolated a native MC-P-450 gene in its totality. This gene is about 6800 nucleotides long, has 7 intervening sequences and hybridizes, even under low stringency hybridization conditions, to mRNA of one size, 2000 nucleotides. 6) We have identified two mRNAs which were induced by MC but are not cytochrome P-450s. We have isolated a cDNA clone for one of these mRNAs.

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, the influence of this system on drug action and other questions in carcinogenesis, toxicology and pharmacology. Furthermore, since the P-450 system is inducible and is highly accessible to experimental manipulation both in vivo and in cell culture, this system is also interesting on a more fundamental level as a model 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 regulation.

Proposed Course: At present we have cDNA clones for one MC-P-450 and have isolated the complete, native gene for that P-450. We are constructing recombinants with cDNA sequences complementary to other MC- and PB-induced P-450s. As these become available, the native genes for these P-450s will be isolated and characterized structurally and functionally. These genes will then be compared. Presently, the structure of the one MC-P-450 gene that we have isolated is under careful investigation. This work will be continued during the upcoming research period. Long-term objectives include functional characterization of the promoter and other regulatory regions of this and other P-450 genes using in vitro transcription and/or DNA-mediated gene transfer systems. Regulation of P-450 gene expression is presently under investigation in cell culture (See Z01 CP 05195-02 LMC) and in vivo as well. In vivo, we have isolated RNA from rat liver and used in vitro translation and immunoprecipitation with P-450 specific monoclonal and aclonal antibodies to determine the multiplicity and the relative amounts of P-450 mRNAs present in the livers of rats of both sexes treated with various inducers and present as a function of time following the administration of inducers. These studies, which provide approximate information concerning the levels of a number of P-450 mRNAs, will be followed by Northern-blot and dot-blot hybridization analysis of the same RNA preparations. These studies will provide very precise information concerning the levels of specific individual P-450 mRNAs.

Publications:

Fagan, J.B., Park, S.S., Guengerich, F. and Gelboin, H.V.: DNA cloning and monoclonal antibodies: Analysis of cytochrome P-450 regulation. Sato, R. and Kato, R., Proc. Fifth International Symposium on Microsomes and Drug Oxidases. Tokyo, Jap. Sci. Soc. Press, 1982.

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. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05200-02 LMC												
PERIOD COVERED October 1, 1981 to September 30, 1982														
TITLE OF PROJECT (80 characters or less) Molecular Mechanism of Mutation Generated by Environmental Carcinogens														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table border="0" style="width: 100%;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 40%;">T. Kakefuda</td> <td style="width: 30%;">Medical Officer</td> <td style="width: 10%;">LMC NCI</td> </tr> <tr> <td>OTHER:</td> <td>H. Mizusawa</td> <td>Visiting Associate</td> <td>LMC NCI</td> </tr> <tr> <td></td> <td>C.H.R. Lee</td> <td>Staff Fellow</td> <td>LMC NCI</td> </tr> </table>			PI:	T. Kakefuda	Medical Officer	LMC NCI	OTHER:	H. Mizusawa	Visiting Associate	LMC NCI		C.H.R. Lee	Staff Fellow	LMC NCI
PI:	T. Kakefuda	Medical Officer	LMC NCI											
OTHER:	H. Mizusawa	Visiting Associate	LMC NCI											
	C.H.R. Lee	Staff Fellow	LMC NCI											
COOPERATING UNITS (if any) M. Rosenberg, LB, NCI, NIH; K. McKenney, LB, NCI, NIH; H. Shimatake, LB, NCI, NIH, Y. Ito, LMM, NIAID, NIH; Y. Hamagishi, LMM, NIAID, NIH														
LAB/BRANCH Laboratory of Molecular Carcinogenesis														
SECTION Nucleic Acids Section														
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords) The topic of the present study is the establishment of <u>plasmid-mediated, mutation assay systems</u> which allow us to determine the primary and higher level structural changes in DNA associated with mutation. These changes are generated by chemical and physical effects on DNA in vitro and in prokaryotic and eukaryotic cells. Pretreatment of plasmid DNA with carcinogens prior to transfection into E. coli, or eukaryotic cells permits us to <u>assay mutation with no toxic effect on the host cells</u> . The plasmid used for the study consisted of a combination of at least two well-defined genes, one of which is used for transformation selection and the other for <u>mutation assay</u> . Mutated plasmid DNA, isolated and cloned individually in large quantities, is investigated for determination of the <u>chemical nature of DNA modification</u> (such as sequence alteration). The altered genetic functions of mutated plasmids were tested by repeated transfection of the DNA and by subcloning of specific segments of DNA in other normal vector DNA.														

Project Description

Objectives: 1) To observe the molecular mechanism of mutations caused by chemical and physical carcinogens, 2) to observe genetic factors of the cell involved in mutagenesis, and 3) to observe the structure/function relationship of mutation at a DNA sequence level.

Methods Employed: 1) The benzo(a)pyrene (BP) metabolite, t-7,8-dihydrodiol-9,10-epoxide (BPDE-1) and the closed circular DNA of plasmid pK0 482 were incubated for DNA modification. The rate of modification was measured by the radioactivity and UV absorption spectrum. Ampicillin resistance (amp-r) from the β -lactamase gene and the galactose kinase (gal-K) genes in pK0 482 plasmid were used for transformation and mutation assays, respectively, in amp-sensitive and gal k- strains of *E. coli*. 2) The pKG 1820 plasmid consisting of the transcriptional terminator sequence between the promoter and gal K structural gene was used for selecting deprived terminator function by mutation at or upstream of the terminator. 3) *E. coli* strains of AB1886 (uvrA-), AB2463 (recA-) and AB1157 (wild type) were transfected with carcinogen-modified DNA or normal plasmid DNA followed by exposure to carcinogens. 4) Mutagenized plasmid DNA was isolated and analyzed for DNA sequence alteration and other changes. 5) A small fragment of DNA which contained the altered sequence was isolated by restriction enzymes and inserted into the same region of normal plasmid DNA to confirm that the mutation was located in the segment of genome. 6) Plasmid DNA consisting of genomes of amp-r, gal-K, polyoma middle T-antigen (MT), and two replication origins of pBR322 and polyoma virus were constructed. The generation of mutations in eukaryotic cells by chemical carcinogens was assayed as in *E. coli*. The expression of T-antigen in *E. coli* and eukaryotic cells was assayed by a specific antigen and its kinase activity.

Major Findings: In AB1157 (wild type) and AB2463 (recA-) strains, the transformation efficiency was about 37% with approximately three molecules of BPDE-1 covalently bound to a single pK0 482DNA (4.5K base pairs). In AB1886 (uvrA-), the same level of transformation efficiency was obtained by only one molecule of BPDE-1 per molecule of pK0482DNA. The mutation frequency in the uvrA- strain was significantly higher than the wild type suggesting that the DNA repair mechanism was lacking or deficient in the uvrA- strain, resulting in a higher rate of mutation. The mutation frequency (2%) observed in uvrA- strain was extremely high compared with other conventional mutation assays because of the nontoxicity on the host cells. Other preliminary experiments with tryptophan pyrrolisates (TRP1 and 2) plus a rat liver microsome fraction (S-9) also indicated a high level of mutation. In the pKG 1820 plasmid system, colonies which showed expression of gal K gene as the result of a defective terminator caused by mutation were picked. The mutated plasmid DNAs were isolated by single colony isolation and the DNA sequence determined by Maxam and Gilbert's method. The terminator region showed either insertion of an A:T pair in the cluster of (A:T)₆ or deletion of the G:C pair from the cluster of (G:C)₃. All of these were frame-shift mutations and occurred either in the terminator sequence or upstream to it. Therefore, the cluster regions are considered to be "hot spots" in terms of mutagenesis by BPDE-1. Such insertion or deletion of a base pair has never been observed in control DNA. Translocation of a large segment

of DNA at or near the terminator segment was also observed. A recombinant DNA consisting of the genes of MT, a promoter originally derived from lambda phage, the amp-r gene, and replication origins was constructed. MT was expressed in *E. coli*. Transformation and mutation assays in *E. coli*, as well as in eukaryotic cells, are in progress. Studies on the possibility of protein kinase activity, the antibody reaction of the gene product, and the DNA sequence change of the MT gene are in progress.

Significance to Biomedical Research and the Program of the Institute: The result of this project indicated that the plasmid-mediated mutation assay is an extremely sensitive and accurate method. This method can be applied for relatively weak mutagens (undetectable by other conventional methods) or highly toxic agents. DNA sequence changes can be observed by selecting mutant colonies and isolating and propagating mutated DNA in a large quantity. It is also useful to investigate the effect of the DNA repair mechanism by the use of cell strains with different genetic backgrounds for DNA repair or manipulating the host cells (by UV or chemicals) prior to plasmid DNA transfection. Recombinant DNA consisting of the MT gene will provide a useful tool to investigate the molecular mechanisms of mutation in eukaryotic cells. Mutation could be studied by observing the structure/function relationship at single gene and DNA sequence level.

Proposed Course: The plasmid-mediated mutation assay developed in this laboratory will continue to be applied for a number of other environmental carcinogens. New recombinant DNA consisting of the structural genes for metallothioneine polyoma virus middle T-antigen, and of cAMP receptor protein will be constructed. Using the shuttle vector system, which has recently been produced in our laboratory, modification of gene expression by chemical carcinogen and DNA sequence analysis will be carried out.

Publications:

Mizusawa, H., Lee, C-H, Kakefuda, T.: Alteration of plasmid DNA-mediated transformation and mutation induced by covalent binding of benzo(a)pyrene-7,8-dihydrodiol-9,10-oxide in *Escherichia coli*. Mutat. Res. 82: 47-57, 1981

Mizusawa, H., Lee, C-H., Kakefuda, T, McKenney, K., Shimatake, H. and Rosenberg, M.: Base insertion and deletion mutations induced in an *E. coli* plasmid by benzo(a)pyrene-7,8-dihydrodiol-9,10-oxide. Proc. Nat. Acad. Sci. USA 78: 6817-6820, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05201-02 LMC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Mechanisms of Carcinogen-DNA Interaction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: T. Kakefuda Medical Officer LMC NCI
OTHER: C.H.R. Lee Senior Staff Fellow LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The topic of the present study is the accumulation of new information regarding the structural modification of DNA associated with carcinogen interaction. A few model systems have been established to analyze the conformational changes in double-stranded circular DNA (pBR322 plasmid and SV40 virus) resulting from covalent and noncovalent binding of benzo(a)pyrene metabolites and mutagenic amino acid pyrolysates, dehydration by organic solvents, temperature change, polyamines and salts. The results indicate that the helical structure of DNA changes specifically under different physico-chemical conditions. Perturbation of the water activity around DNA and the consequent structural transformation may change the binding efficiency as well as binding specificity of the carcinogens. Such microenvironmental changes are expected to occur physiologically in living cells. Alteration of target sites is dependent not only on the chemistry of carcinogens but is also influenced by DNA structure.

Project Description

Objectives: 1) To observe different forms of DNA structural modifications by carcinogen interactions; 2) to observe the dynamic changes of the DNA helix resulting from the physical and chemical microenvironmental changes surrounding the helix; 3) to observe changes in target sites of carcinogen binding in DNA, 4) to make antibodies against carcinogen-DNA adducts and to determine the geometry of DNA modification on isolated DNA, as well as in the cells; and 5) to make available information obtained from these experiments for the understanding of the molecular mechanism of mutation and cancer in living organisms.

Methods Employed: 1) Closed circular DNAs (pBR322 or SV40) are linearized by endonuclease Eco RI. Purified DNAs were circularized by T4 ligase in the presence of different kinds of organic solvents, salts, polyamines, putrescine, and cadaverine at various temperatures. DNAs having different superhelical density, thus produced, are analyzed by two-dimensional agarose gel electrophoresis, adding ethidium bromide only in the second electrophoresis. 2) A similar experiment using a DNA-relaxing enzyme was carried out in the presence of a variety of benzo(a)pyrene (BP) metabolites, mutagenic amino acid pyrolysates (TRP-1, TRP-2, Glu-1, Glu-2 and IQ etc.), acetylamino fluorene derivatives and other muta-carcinogenic compounds to observe the noncovalent binding with DNA. 3) Equilibrium dialysis, UV absorption spectroscopy and fluorescence quenching were carried out to find DNA carcinogen interactions. 4) Antisera specific to DNA modified with benzo(a)pyrene metabolite BP diol-epoxide (BPDE) was produced and reacted with either DNA or cells treated with the same compound. The binding sites were observed by immunoelectron microscopy and immunofluorescence microscopy. 5) DNA was base-labeled with radioactive dNTP using the nick translation technique. The ultimate form of BP metabolite, diolepoxide I and the radiolabeled DNA were incubated under different experimental conditions. The purified DNA was then hydrolyzed with pancreatic DNase, BP-DNA adducts were isolated through Sephadex LH-20 column chromatography. The late eluants were further analyzed with HPLC chromatography to quantitate and identify different forms of the adducts.

Major Findings: 1) The two-dimensional gel electrophoresis of DNA ligated in the presence of organic solvents or at different temperatures showed that a) the DNA unwinds upon dehydration by organic solvents or elevation of temperature, b) the conformation change is a continuous process; c) unwinding of one linking number of a DNA helix is 12.2 ± 0.4 kcal/mole independent of both DNA size and its conformation. 2) Covalent binding to DNA of less than 10 molecules of BPDE per SV40 DNA (5.3Kb) did not significantly alter the superhelical density; binding occurred almost exclusively with the deoxyguanosine residue of DNA. An increased amount of covalent binding caused a cumulative microenvironmental change, particularly in the minor groove of the helix, which resulted in dehydration and destabilization of DNA. Such unstable DNA opens new target sites which are normally unreactive or poorly reactive with specific types of carcinogens. 3) Antisera against BPDE-DNA adducts reacted with double-stranded BPDE-modified-DNA. The reaction was greatly enhanced after denaturation by heat or organic solvent (dehydration which led to unwinding of the double helical DNA). Antisera did not react with unmodified DNA, free benzo(a)pyrene, or proteins modified by BPDE. Indirect immunofluorescence studies indicated that the antisera bound specifically to the nuclei of the cells which are previously treated with BPDE.

The intensity of fluorescence was proportional to the dose of BPDE used to treat the cells. An immunoelectron microscopic observation of DNA which reacted with ferritin-conjugated IgG confirmed the observation described above. 4) Those BP metabolites which are closely related in chemical structure and/or metabolic pathway (such as t-7,8-dihydrodiol BP or tetrol BP) and the potent mutagenic amino acid pyrolysates demonstrated marked affinity to double-stranded DNA as observed by the DNA-relaxing enzyme study, equilibrium dialysis, and fluorescence quenching. The noncovalent affinity of these compounds showed a remarkable parallel relation to the mutagenic potential. The compounds that are non-mutagenic after metabolic activation showed no or only weak affinity with DNA.

Significance to Biomedical Research and the Program of the Institute: The structure of DNA changes dramatically during transcription, replication and high level organization in the cell. Winding and unwinding of the double helix occurs when DNA is in a physiologically and genetically active state. The structure of DNA also changes passively under the effects of drugs, physical and chemical microenvironmental changes and partial damage by carcinogens, UV and other nucleophiles. Several different forms of DNA, such as A, B, C and Z forms have been known to occur under specific conditions. The chemical reactivity of mutagens and carcinogens has been studied in the past with particular emphasis on the chemical structure of the drugs and their metabolites, almost totally ignoring the primary and high order structure of DNA. Our study indicated that carcinogen-DNA interaction is greatly affected by the DNA conformation, which is subject to drastic changes under different microenvironmental conditions. The model system we established provides important information on the dynamic changes of DNA structure. HPLC analysis of base-labeled DNA which had been reacted with chemical carcinogens allowed us to study the shift in target sites in DNA under different experimental conditions. The noncovalent interaction between potentially potent carcinogens (or their precursor molecules) and DNA is an indication of the fact that noncovalent affinity may be an important initial step for mutagenesis and carcinogenesis.

Proposed Course: Studies on covalent binding of carcinogens under different experimental conditions, combined with HPLC analysis of the DNA adducts, will be continued. DNA base sequences with a cluster of identical runs of nucleotides are found to be so-called "hot-spots" in terms of mutation generated by BPDE as indicated in our previous study. Experiments will be designed to study the DNA sequence specificity involved in chemical carcinogenesis.

Publications:

Lee, C-H., Mizusawa, H. and Kakefuda, T.: Unwinding of double-stranded DNA helix by dehydration. Proc. Natl. Acad. Sci. USA 78: 2838-2842, 1981.

Slor, H., Mizusawa, H., Neihart, N., Kakefuda, T., Day, R.S. III and Bustin, M.: Immunochemical visualization of binding of the chemical carcinogen benzo(a)pyrene diol-epoxide I to the genome. Cancer Res. 41: 3111-3117, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05204-02 LMC
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PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
DNA Sequence Alterations in vivo Following DNA Modification by Carcinogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael Seidman	Senior Staff Fellow	LMC NCI
OTHERS:	Sekhar Chakrabarti	Visiting Fellow	LMC NCI
	Hiroshi Mizusawa	Visiting Associate	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: 1.75	PROFESSIONAL: 1.75	OTHER: 0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 in 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 is being sequenced.

Project Description

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 have been 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 survive on ampicillin medium are counted and the number of mutants at the appropriate locus are determined. These data are plotted as a function of the amount of BPDE on the plasmid. Selected mutants are being sequenced.

Major Findings: 1) Mutagenesis in this system is targeted. Mutations arise in the targeted regions, not in other, nondamaged regions of the plasmids. 2) After SOS induction by UV light, of recipient bacteria, functions for survival (error-free repair) and mutagenesis are induced above constitutive levels. The repair activity encoded by the UVR A gene rises and falls in the first 30 minutes after induction, while the mutagenesis activity is fully induced only after 60 minutes after induction. Thus, the two functions can 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: The mutants generated in these experiments are being sequenced. The relationship between replication and carcinogen-induced mutagenesis is being studied.

Publications: None

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Naturally Occurring Inhibitors of Different Forms of Cytochrome P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H.V. Gelboin	Chief	LMC	NCI
OTHER:	R. Robinson	Biologist	LMC	NCI
	T. Fujino	Expert	LMC	NCI

COOPERATING UNITS (if any)

T. Sugimura, National Cancer Research Institute, Tokyo, Japan

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.9

PROFESSIONAL:

0.2

OTHER:

0.7

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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. The form of AHH that is induced by polycyclic hydrocarbons is strongly inhibited by 7,8-benzoflavone, while this compound inhibits weakly or stimulates the form of AHH found in untreated or phenobarbital (PB)-treated rats. In contrast, 1-maackiain acetate greatly inhibits the AHH from untreated or PB-treated rats and inhibits weakly or stimulates the form of AHH induced by polycyclic hydrocarbons. Therefore, 7,8-benzoflavone and 1-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 methylcholanthrene and PB-induced P-450s.

Project Description

Objectives: To investigate naturally occurring inhibitors which are present in our environment and to characterize their effects on mixed function oxidases. To use these inhibitors to characterize the mixed function oxidases of microsomes obtained from 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 non-treated, PB- and MC-treated rat, and from human placenta; and 3) AHH 7-ethoxycoumarin deethylase and benzphetamine hydroxylase are determined for each of these preparations with or without the addition of inhibitors. Analysis of benzo(a)pyrene metabolism is performed with the aid of high pressure liquid chromatography.

Major Findings: 7,8-Benzoflavone, 1-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. 1-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 1-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, 1-maackiain acetate strongly inhibits the AHH of normal human liver and has little effect on the cigarette smoke-induced AHH of human placenta. Intermediate compounds in the synthesis of 1-maackiain acetate have strong inhibitory effects on nontreated and PB-treated microsomes. This study will enable us to understand the structural relationship between the structure and the inhibitory activity of these chemicals.

Significance to Biomedical Research and the Program of the Institute: 1-Maackiain acetate, as well as other flavones, and 7,8-benzoflavone may be useful inhibitors of specific forms of AHH in human as well as animal tissues. The unusual specificity of 1-maackiain acetate as an inhibitor may make it a valuable tool for probing the characteristics of cytochrome P-450 and the active site of the different forms of cytochrome P-450 and for assessing the factors that determine the routes of polyaromatic hydrocarbon metabolism. This inhibitor may help in clarifying the relationship between drug and carcinogen activation and detoxification. It is possible that 1-maackiain acetate and 7,8-benzoflavone 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 carcinogenesis. To investigate other classes and related flavonoids as modulators of carcinogenesis. To investigate the structural features of these flavones in the inhibition of enzyme activity.

Publications:

Gelboin, H.V., West, D., Gozukara, E., Natori, S., Nagao, N., and Sugimura, T.:
1-Maackiain Acetate specifically inhibits different forms of aryl hydro-
carbon hydroxylase in rat and man. Nature 291: 659-661, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05208-02 LMC
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PERIOD COVERED
September 1, 1981 to October 30, 1982

TITLE OF PROJECT (80 characters or less)

Typing of Cytochrome P-450 in Human Tissues Using Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: T. Fujino	Expert	LMC NCI
OTHER: H.V. Gelboin	Chief	LMC NCI
R. Robinson	Biologist	LMC NCI
S.S. Park	Visiting Associate	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: 1.4	PROFESSIONAL: 0.8	OTHER: 0.6
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The diversity of the cytochrome P-450 in human tissues, i.e. placenta, monocytes and lymphocytes was investigated using monoclonal antibodies to 3-methylcholanthrene (MC) or phenobarbital (PB)-induced rat liver cytochrome P-450. Monoclonal antibodies to rat liver 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. Monoclonal antibodies may be applied usefully in analyzing the multiplicity, distribution and function of the cytochromes P-450 in human tissues.

Project Description

Objectives: Differences in the profile of P-450s present in different tissues may account for differences in metabolism which result in either the carcinogenic activation or the detoxification of drugs, mutagens, and potential carcinogens. We are using P-450-specific monoclonal antibodies to investigate the diversity and multiplicity of P-450s. We are using these monoclonal antibodies to 1) develop a phenotypic description of the number and quantity of P-450s in tissues and individuals, 2) to determine their role in the detoxification or activation of specific xenobiotics, and 3) to determine their role in individual variation in drug and carcinogen responsiveness.

Methods 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 placenta from women who smoke. AHH and 7-ethoxycoumarin deethylase activities were measured by a fluorospectrophotometric 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 detect common antigenic sites present in human placenta and lymphocytes. 3) The AHH activity of human placenta was inhibited 90%, while the 7-ethoxycoumarin deethylase activity was inhibited from 0-70% in different individuals. The inhibition of 7-ethoxycoumarin deethylase varied widely among individuals. 4) Human monocytes and liver AHH and 7-ethoxycoumarin deethylase activities are carried out by P-450s which are different from the monoclonal antibody-sensitive P-450 in placenta and lymphocytes.

Significance to Biochemical Research and the 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 carcinogen will be useful for the detection and prevention of chemical carcinogenesis.

Proposed Course: Monoclonal antibodies to human cytochrome P-450 will be explored further. These monoclonal antibodies might attack different antigenic determinants of cytochrome P-450 which have not yet been identified by other methods. The diversity and multiplicity of the cytochromes P-450 and their catalytic specificities will be studied further using monoclonal antibodies and HPLC analysis of polycyclic hydrocarbon metabolism. Monoclonal antibodies which should be specific for other antigenic sites on the cytochrome P-450 molecule are now in preparation. These, as well as the monoclonal antibodies now on hand, will be used in these studies.

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 (In press).

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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U. S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05236-01 LMC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

5-Methyl Cytosine-Containing Sequences in the Chicken Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Francine C. Eden Senior Staff Fellow LMC NCI
OTHER: Veronica O'Neill Visiting Fellow LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

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Nucleic Acids Section

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TOTAL MANYEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0.0

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(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The DNA of higher animals carries a minor fraction of its cytosine in the modified form 5-methyl cytosine. There is reason to think that this modification of the genetic material itself, an activity that nature has usually avoided, must have a specific and important purpose when and where it does occur. Our goal is to relate cytosine modification to two important processes governing how the genome is used: 1) transcription and 2) gene rearrangement and/or amplification. 5-methyl cytosine-containing sequences can be identified using restriction endonucleases which are sensitive to methylation. By this means we have already located many repeated sequences in chicken DNA which show a concerted methylation pattern among hundreds or thousands of copies. Methylated and unmethylated prototype sequences are being isolated by molecular cloning. The cloned DNA segments are being used to measure transcription (RNA-DNA hybridization in solution, Northern blotting) and genome reorganization (Southern blotting of restricted genomic DNA). We are also sequencing cloned DNA segments to determine the context in which 5-methyl cytosine occurs.

Project Description

Objectives: To understand the functional meaning of the occurrence of 5-methyl cytosine in the chicken genome.

General Strategy:

1. Determine the distribution of 5-methyl cytosine in the chicken genome, especially within the repeated DNA component.
2. Clone representative repeated DNA sequences of the methylated and the unmethylated type.
3. Use the cloned probes to explore:
 - a) Changes in methylation. Test DNA from many sources including embryonic cells from different developmental stages, different adult tissues, normal and transformed cells in culture.
 - b) Transcription from methylated and unmethylated regions of the genome.
 - c) Genome reorganization in the vicinity of methylated bases.
 - d) The DNA sequence context in which methylation occurs.

Methods Employed: 5-methyl cytosine in genomic DNA is detected using restriction endonucleases sensitive to methylation. Especially useful in this are MspI (cleaves CCGG or CmeCGG but not meCCGG), HpaII (cleaves CCGG) and Nci I (cleaves CCXGG where X= C or G). In combination with other restriction enzymes, these allow reasonably precise localization of 5-methyl cytosine-containing sequences. The experiments involve either two-dimensional restriction analysis of total DNA using the procedure of Smith and Thomas (*Gene* 13: 395-48, 1981) or Southern blotting of restricted total DNA and hybridization to cloned probes.

Chicken DNA fragments are cloned either in PBR322 or in bacteriophage lambda. All cloning is performed according to NIH guidelines governing recombinant DNA research.

Changes in methylation are measured by isolating DNA from different cell types and digesting it with HpaII, MspI, or Nci I. The DNA fragments are separated in agarose gels, transferred to nitrocellulose, and hybridized to cloned probes labeled with ³²P by nick translation.

Transcription of methylated and unmethylated DNA sequences is measured using RNA isolated from a variety of cell types. RNA is first enriched for poly-A containing species by oligo dT-cellulose chromatography, then hybridized to cloned DNA segments in solution allowing titration of the quantity of RNA transcripts. Sequences transcribed in sufficient abundance are further characterized by separation of RNA in agarose gels, transfer to diazotized paper, and hybridization to cloned probes labeled by nick translation.

Genome reorganization and/or amplification is explored by Southern blotting of restricted total DNA from a variety of sources and hybridization to cloned probes representing methylated or unmethylated repeated DNA sequences. Rearrangement is

detected as a change in the sized of restriction fragments, and amplification is detected by a change in the intensity of hybridization to specific genomic DNA fragments. Sequencing of cloned DNA fragments is performed by the method of Maxam and Gilbert.

Major Findings: The chicken genome contains a substantial portion of its repeated DNA organized as very long clusters of repeated sequences. These clusters contain sequences that are heavily methylated in a variety of cell types. Cloning and characterization of methylated sequences has already been completed and is the subject of earlier publications (Eden, F.C., Musti, A.M., and Sobieski, D.A., J. Mol. Biol. 148: 129-151, 1981; Musti, A.M., Sobieski, D.A., Chen, B.B., and Eden, F.C., Biochem. 20: 2989-2999, 1981; Sobieski, DA. and Eden, F.C., Nuc. Acids Res. 9: 6001-6015, 1981). During the period covered by this report the methylation survey has been greatly broadened using two-dimensional restriction analysis of total DNA. Total DNA is digested with BamHI and fractionated preparatively using an agarose gel in the bull's eye electrophoresis apparatus. This separates the genomic fragments into 30-40 fractions according to length after BamHI digestion. Individual fractions are end-labeled using 32P-ATP and T4 polynucleotide kinase. Paired digestions of the labeled DNAs with MspI and HpaII follow. The double-digested DNA samples are separated in long acrylamide gels, dried, and autoradiographed. Repeated DNA sequences in the genome stand out against the background smear because of their high copy number. Comparison of MspI and HpaII lanes allows assessment of their extent of methylation. We have identified more than 50 different repeated DNA sequences that vary from unmethylated to completely methylated in DNA from adult liver. We are now in position to a) clone them and b) perform all of the types of analyses listed above. We will concentrate on cloning unmethylated repeated sequences first, providing the companion probes for the highly methylated sequence probes cloned previously. There has been substantial progress in the sequencing of DNA fragments representing methylated repeated sequences in the chicken genome. In all, more than 1200 base pairs have been sequenced. The methylated regions are high in G+C content--60% vs. 40% for total chicken DNA. With one exception, they are not internally repetitive (the exception to be discussed below). Computer analysis failed to detect any consensus sequence associated with methylation. Thus 5-methyl cytosine can occur in many different sequence contexts in the genome. We are soon to be in position to sequence our newly identified repeated sequences of the unmethylated variety which will extend this description further.

Within long methylated regions of the chicken genome, there are specific undermethylated areas (Eden et al., JMB op. cit.). We have sequenced this undermethylated area and find it to consist of what appears to be an insertion; that is, a different repeated sequence. The insertion is composed of a tandemly repeating DNA sequence. There are 5.2 contiguous units of a basic 38 base pair repeat. They vary little from the consensus sequence GTCCCTCCCCGGCTCATTGTCAGCGGCATCTCT, with only four variable positions and only two alternative bases found at each variable position. The tandemly repeating sequence is flanked by unrelated DNA sequences (several hundred nucleotides to each side have been sequenced). However, immediately adjacent to the tandem repeat there are short direct repeats of the sequence GCTCCCGTGGC. This is a diagnostic feature usually indicative of a transposition event; it strongly suggests that the tandemly repeating region is a fragment of a satellite-like component which was inserted into its present location by transposition; the direct repeats would constitute a target-site

duplication. It is very interesting that this insertion forms an interruption in a very concerted methylation pattern. The tandemly repeated region is being excised from the parent clone and recloned to provide a specific probe for it. Its methylation status, when it is not associated with other methylated repeats, will then be explored.

Significance to Biomedical Research and the Program of the Institute: Our present understanding of the molecular basis of cancer indicates that the alteration of genes and/or the altered expression of genes plays a key role. Thus it is very important to understand the normal processes whereby gene alteration and expression are controlled. The modification of bases in DNA could influence these processes very directly and our work is aimed at establishing this.

Proposed Course: During the next year we plan to complete the cloning of methylated and unmethylated sequences, to sequence unmethylated prototypes, to explore changes in methylation focusing on normal and transformed cells in culture, and to begin measurements of transcription from methylated and unmethylated genomic regions. The genome organization of the interesting tandemly repeated sequence will be explored.

Publications:

Sobieski, D.A. and Eden, F.C.: Clustering and methylation of repeated DNA: Persistence in avian development and evolution. Nucleic Acids Res. 9: 6001-6015, 1981.

PERIOD COVERED

March 17, 1982 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Oncogenes of Chemical Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F.C. Eden	Senior Staff Fellow	LMC NCI
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	A.V. Furano	Staff Physican-Research	LBP A
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COOPERATING UNITS (if any)

None

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Laboratory of Molecular Carcinogenesis

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TOTAL MANYEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Gene transfer experiments can be used to identify and characterize oncogenes. We are developing a rat donor/mouse recipient gene transfer system with broad utility for studying oncogenes from cells transformed by chemical carcinogens. The first requirement is a probe (cloned DNA fragment) that will hybridize with any rat DNA segment of reasonable length and will not hybridize to mouse DNA. We have developed such a probe and are presently confirming its utility in model experiments with donor DNA from virally transformed rat cells. This probe is a composite of different repeated sequences. All of them are interspersed in the rat genome and each of them hybridizes to a substantial fraction of recombinant phages in a rat library. Some of them are transcribed in a tissue-specific manner. Thus, when oncogenes are detected in transformed cells, the composite probe can be disassembled into components to determine which family of repeated sequences has a member contiguous with each oncogene. If transcription of adjacent genomic segments is required for expression of oncogenes, this will be apparent. Thus, we have developed a probe that can detect oncogenes and provide information about them in a single step.

Project Description

Objectives: 1) To detect, isolate, and characterize oncogenes from cells transformed by chemical carcinogens and 2) to determine which parameters influence oncogene selection and/or activation. The variables to be tested include the type of carcinogen, the protocol of administration (to whole animals vs. cultured cells), the tissue of origin of the transformed cells, and the effect of tumor promoters.

Methods Employed: 1) Development of a Rat Repeated Sequence Probe For Use in Rat Donor/Mouse Recipient Gene Transfer Experiments. Many different rat-repeated sequences have been cloned in PBR322 by Anthony Furano and co-workers (Project No. Z01 AM 23580-19LBP). Many of these were made available to us in the form of plasmid-containing bacterial colonies. The cells were propagated and plasmid DNA isolated by the cleared lysate procedure and CsCl ethidium bromide gradient centrifugation. Each recombinant plasmid was labeled with ³²P by nick translation and used to screen recombinant DNA libraries in bacteriophage lambda. We used a rat HaeIII partial library, a rat EcoRI partial library (gifts of T. Sargent) and a HaeIII partial library of mouse DNA. The frequency of positive plaques was scored and replicate lifts from the same plate were used to determine the overlap of the probes in each library.

2) Detection of Chemical Oncogenes. Donor DNA will be prepared from chemically transformed rat cells by the method of Perucho, et al. (Cell 27: 467-476, 1981). NIH 3T3 cells will be used as recipients. DNA transformation will be performed by the CaPO4 precipitation method. Cells from positive foci will be selected, propagated, and used for DNA isolation. This DNA will be analyzed for the presence of rat DNA sequences by hybridization of Southern blots with the rat repeated DNA probe described above. If this test is positive the DNA will be used as donor in a second round of gene transfer. These positive foci will again be selected and propagated. The DNA from them should contain an identifiable oncogene (that is, a rat DNA segment common to many different transformed foci).

3) Preliminary Characterization of Chemical Oncogenes. At the second round of gene transfer, positive donor DNAs will be compared by developing a restriction resistance profile of each of them. This will tell us if we are dealing with the same or different genes in the many samples. The composite repeated sequence probe will be disassembled into components to determine which families of rat repeated sequences are adjacent to the oncogenes.

4) Cloning of Oncogenes. A library of transformed cell DNA will be constructed in phage lambda using partial digestion with AluI and HaeIII to fragment the DNA. The library will be screened with the appropriate rat repeated sequence probe (determined in step 3 above). The positive phages will be purified and propagated. The recombinant DNA will be analyzed by restriction analysis and a single copy portion will be located. This will be subcloned in PBR322 and used to screen a library of normal rat DNA. The normal gene counterpart to the oncogene will thus be identified and isolated. The normal and transforming genes will be compared by restriction analysis and DNA sequencing. The alteration leading to cancer will be identified by this means.

Major Findings: During the short period covered by this report, only the first step, development of a rat-repeated sequence probe, has been accomplished. Most of the gene transfer experiments detecting oncogenes, which have been reported in the literature, use human donor DNA and the very ubiquitous human repeated sequence called "Alu" to detect human DNA in the transformants. Rodent genomes also contain Alu-like sequences having homology to the human sequence, but the rat and mouse Alu families are too closely related to distinguish rat from mouse DNA by hybridization. We have found a group of other repeated sequences in rat DNA which are less ubiquitous than the Alu family but which do not hybridize to mouse DNA. By using the whole group together as a screening probe, we now detect nearly any rat DNA segment in a mouse cell. This opens the door to gene transfer experiments of all types and allows us to tap the extensive repertoire of chemically transformed rat cells as donors.

Information about the repeated sequence component of the rat genome is a by-product of probe development. The table below summarizes information about 11 different families of repeated DNA in the rat. We note that three of the probes (2H1, 2A12, and 2G2) hybridize to an overlapping set of phages in the rat library. This means that these fragments are either parts of a longer repeated sequence or are near neighbors in the genome much more often than chance would predict. Interestingly, the three probes differ markedly in the abundance of transcripts from them in different cell types (A. Furano, unpublished) and one of the probes (2H1) does hybridize to mouse DNA at lower criterion while the other two do not, so their evolutionary conservation also differs.

With respect to the other probes, we note that there is no obvious relationship between copy number and dispersal in the genome; some probes with thousands of copies hybridized to less than 0.1% of the plaques in the rat Eco library, indicating that they have a clustered rather than a dispersed organization.

Significance to Biomedical Research and to the Program of the Institute: Gene transfer opens the door to understanding the molecular basis of cancer in this decade. Our work is directly contributing to this breakthrough. By focusing on chemically transformed rodent cells, we will provide information complementary to, but not overlapping with, the work of others on human transformed cells. The rodent system contrasts with the human one in that it is an experimental system where significant parameters can be systematically varied. Thus we anticipate that a reasonably small number of experiments will have high information content and will contribute substantially to our general understanding of cancer.

Proposed Course:

The proposed course of this research has already been described under methods. During the next year we expect to:

- 1) Obtain a positive gene transfer in using donor DNA from a virally transformed rat cell line and determine which family of rat repeated DNA sequences is adjacent to the oncogene.

- 2) Isolate donor DNA from a variety of chemically transformed rat cell lines, obtain successful gene transfers into NIH 3T3 cells, and accomplish preliminary characterization of the relevant oncogenes. We will have some idea of the diversity of potential chemical oncogenes at that point and it will influence the future course significantly. We will also compare the adjacent repeated DNA sequences among the various oncogenes in chemically transformed lines and between chemically and virally transformed lines. The influence of transcriptionally active (or inactive) adjacent genomic segments on oncogene expression will be apparent from these comparisons.

Publications:

None

Table 1

Characteristics of Cloned Rat Repeated DNA Sequences

Probe	Relative Copy Number in Rat Genome	Hybridization to Mouse DNA	Frequency of Positive Plaques	
			Rat Hae Library	Rat Eco Library
PR3	moderate	-	NT ¹	0.005
PR6	moderate	-	NT	<0.001
1E3	high	+	NT	0.05
4D4	low	-	NT	<0.001
2H1	high	+	NT	0.14
1A6	moderate	-	NT	NT
2A12	high	-	0.16	0.15
3B5	high	-	0.55	0.80
4D12	high	-	0.72	0.63
5E6	high	-	0.81	0.71
2G2	high	-	0.21	0.23

¹Not tested

Z01 CP 05240-01 LMC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Rearrangement of a Plasmid Sequence in Normal and Tumorigenic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael Seidman	Senior Staff Fellow	LMC NCI
OTHERS:	Abdur Razzaque	Visiting Fellow	LMC NCI
	Hiroshi Mizusawa	Visiting Associate	LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less, underline key words)

A system for studying gene stability and rearrangement in normal and tumorigenic cells has been devised. A plasmid shuttle vector has been constructed. 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 will replicate 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 is introduced into a bacterial host which permits the detection of the presence or absence of a functional galactokinase gene. With this assay it is possible to quantitatively assess the stability of the plasmid in the mammalian cells. In our first experiments we have used a permanent cell line of African green monkey kidney cells as the mammalian host and find that about 1% of the replicated plasmids have lost a functional galactokinase gene. Analysis of the defective plasmids indicate that molecules both larger and smaller than the starting material appear. Experiments designed to assay the plasmid stability in tumorigenic cells are in progress.

Project Description

Objectives: To study DNA sequence rearrangement in tumorigenic cells and to identify DNA sequences and gene functions involved in genome rearrangement.

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 an instability frequency for the galactokinase gene in mammalian cells calculated.

Major Finding: The instability frequency for the plasmid in a permanent cell line of African green monkey kidney cells is about 1.2%. The same assay applied to primary cells of the same source yields a value of 0.25%. Analysis of the defective plasmids indicates that there is a class of molecules that are as much as 4kb larger than the original plasmid. Other defectives have deletions which range from less than 100 base pairs to a few thousand base pairs.

Significance to Biomedical Research and the Program of the Institute: Genome instability is a typical feature of many tumorigenic cells. Our procedure permits a quantitative assay of different cells for activities involved in DNA sequence rearrangement. It will be possible to study the role of specific sequences on the plasmid, as well as gene functions encoded by the plasmid, in rearrangement.

Proposed Course: The defective plasmids are being characterized with particular attention being given to the possibility that cellular sequences have integrated into the vector. Tumorigenic cells are being assayed for rearrangement activity and the assay will be extended to human tumor lines.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
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U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05241-01 LMC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Role of Left-Handed Helical DNA in Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Hiroshi Hamada Visiting Associate LMC NCI
OTHER: Takeo Kakunaga Chief, Cell Genetics LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

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Cell Genetics Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.7

PROFESSIONAL:

0.5

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies have shown that purine-pyrimidine alternating sequences can form a left-handed helical conformation, called Z-DNA. If such sequences exist in natural genomes, they may have great implications in the regulation of gene expression, in the process of cell differentiation, and in carcinogenesis. We have surveyed the potential Z-DNA forming sequences in various eukaryotic genomes. First, we found a large copy number of dT-dG alternating sequence in eukaryotic genomes studied from yeast to human. In fact, two different cloned human actin genes contained (dT-dG)₂₅ and (dT-dG)₁₅. The nucleotide sequence of dT-dG alternating region and its flanking region in various genes indicated that the repeated element consists only of (dT-dG)_n. Second, dG-dC alternating sequence was found to be moderately repeated in some eukaryotic genomes, but not detected in others. These results provide the direct evidence for the general and abundant occurrence of potential Z-DNA-forming sequences in natural organisms' genomes. Experiments are now underway to test the hypothesis that Z-DNA sequences may be the regulatory elements for gene expression and the key element in carcinogenesis.

Project Description

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 Z-DNA sequences were surveyed in a number of eukaryotic genomes by the hybridization of ^{32}P -labeled poly (dT-dG).poly (dC-dA) or ^{32}P -labeled poly (dG-dC) to each cellular DNA.

The cloned human genes that hybridized to these probes were further studied by direct DNA sequencing to confirm the presence of the precise purine-pyrimidine alternating sequence.

Major Findings: 1) Discovery of a stretch of (dT-dG) alternating nucleotide sequence in human actin genes. A phage clone, Ha-25, which contains a human cardiac muscle actin gene, was obtained by the screening of a human DNA library that had been constructed by partial EcoRI digestion of DNA from a β -thalassemia patient and ligation to a charon 4A phage vector. The nucleotide sequence of the entire coding region and some introns of the cloned cardiac muscle actin gene showed the presence of a simple purine-pyrimidine alternating sequence, a 25 times repeat of TG dinucleotide (designated the Z1-element) in one of its introns. A stretch of alternating TG dinucleotides has been shown to form Z-DNA under some conditions in solution. Another phage clone, Ha-314, which contains other types of human actin genes, was isolated from a human DNA library constructed from partially EcoRI digested Hut-14 cell DNA, a chemically transformed human fibroblast cell line. This clone was also shown to contain a Z1-element by hybridization with ^{32}P -labeled poly (dT-dG).poly (dC-dA). DNA sequencing of the Z1-containing region has shown a 15 times TG precisely alternating sequence.

2. Z1-element as a highly repeated sequence in human genomes. Southern blotting analysis was used to detect highly repeated sequences in human cardiac muscle actin gene using the ^{32}P -labeled total DNA from HeLa cells as a hybridization probe. The highly repeated element was mapped in the 110 bp region where (dT-dG)₂₅ is located. In the second Southern blotting analysis the probe and sample were reversed. ^{32}P -labeled DNA fragment containing (dT-dG)₂₅ was used as a hybridization probe against an EcoRI digest of human DNA. Again the DNA fragment containing (dT-dG)₂₅ was found to contain the highly repeated element. ^{32}P -labeled poly (dT-dG).poly (dC-dA) was synthesized as the probe that specifically hybridized to (dT-dG)_n sequences. This probe specifically hybridized to DNA fragments containing (dT-dG)₂₅ when hybridized to a restriction digest of Ha-25 DNA fragment, indicating the high specificity of the probe. This probe also produced a smear hybridization in gels containing restriction-digested total human DNA, thus indicating that (dT-dG)_n is a repeated element in human genomes. The copy number of (dT-dG)_n was estimated by Dot analysis using (dT-dG)_n-specific probe. The approximate copy number of (dT-dG)_n is estimated to be 10^5 per human genome, assuming that the average size of n is 25.

3. Evolutionary conservation of Z1-element. The presence of the (dT-dG)n sequence in various eukaryotic cells from yeast to human was surveyed using Southern blot hybridization and ^{32}P -labeled poly (dT-dG). poly (dC-dA) as a probe. The approximate copy number of the Z1-element was estimated to be 5×10^4 in human, 3×10^4 in calf, 10^5 in mouse, 4×10^3 in chicken, 10^5 in *Xenopus*, 2×10^5 in salmon, 2×10^3 in *Drosophila*, and 10^2 in yeast, indicating extraordinary evolutionary conservation of the Z1-element.

4. The structure and location of the Z1-element. The primary sequences of the Z1-element and the sequences flanking (dT-dG)n were compared between different genes. No significant homology was observed in the genes other than the stretches of (dT-dG)n sequence, indicating that the Z1-element is only of (dT-dG)n (n is variable) without any other sequence. The location of the Z1-element with respect to the coding region was random. The Z1-element is located not only in introns but also in other regions.

5. The presence of (dG-dC) alternating sequence in eukaryotic genomes. The presence of another purine-pyrimidine alternating sequence, (designated the Z2-element) a possible left-handed helical DNA sequence, was surveyed by Southern blotting analysis using ^{32}P -labeled poly (dG-dC) which we prepared as the probe. The (dG-dC)n sequence was moderately repeated in human, mouse and salmon genomes, but not found in the calf genome.

Significance to Biomedical Research and the Program of the Institute: The reversible interconversion of potential Z-DNA-forming sequences between B- and Z-form has been shown by physicochemical studies. The transition from B to Z or Z to B depends on the environment, such as ionic concentration and on the modification of DNA itself, such as by methylation and by carcinogens. In fact, methylation of cytosine residues in poly (dG-dC) and modification by a certain carcinogen stabilize the Z-form. On the other hand, it is also widely accepted that the extent of cytosine-methylation is inversely correlated with the transcriptional activity of genes. Thus, the modification of a potential Z-DNA sequence and a corresponding transition from B to Z-form could cause altered gene expression, which is a requisite for the process of cell differentiation and carcinogenesis. 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: We can take advantage of the cloned actin genes containing the Z1-element. We will determine the transcriptional activities of the intact gene and of the mutant gene in which the Z1-element is deleted, replaced, or modified by methylation or mutagens. DNA fragment with the Z1-element flanked by the usual B-form sequence can be used for physicochemical studies. It is also possible to ask how the Z1-element is packaged in the nucleosome structure.

Publications:

Hamada, H. and Kakunaga, T.: A potential Z-DNA forming sequence is highly dispersed in the human genome. Nature. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05242-01 LMC

PERIOD COVERED

September 1, 1981 to October 30, 1982

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibody Mapping of Cytochromes P-450 in Different Species and Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Fujino	Expert	LMC	NCI
OTHER:	H.V. Gelboin	Chief	LMC	NCI
	M. Protic-Sabljić	Guest Researcher	LMC	NCI
	S.S. Park	Visiting Associate	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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Metabolic Control Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.7

PROFESSIONAL:

1.5

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The multiple forms of cytochrome P-450 regulate the metabolism of a wide variety of xenobiotics and endogenous compounds in vivo. With the aid of monoclonal antibodies, we have demonstrated that human placental microsomes and lymphocytes contain more than two different forms of cytochrome P-450. We have also used monoclonal antibodies to show that murine 7-ethoxycoumarin deethylase behaves the same as that of human placenta. The monoclonal antibodies that inhibit enzyme activity can be used to specifically phenotype cytochromes P-450 containing specific antigenic sites in different tissues and species. These are examples of how monoclonal antibodies to cytochrome P-450 can be used as a tool for classifying P-450s. Making a detailed atlas of the cytochromes P-450 present in different species and tissues will help us in understanding the diversity of cytochrome P-450 and its role in tissue and species susceptibility to carcinogens.

Project Description

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 exact number and quantity of P-450s in various species and tissues is necessary. The sexual and developmental related characteristics of the cytochromes P-450 will also be examined.

Methods Employed: Monoclonal antibodies were prepared by the general methods of Kohler and Milstein. The 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 fluorospectrophotometric assay after incubation with monoclonal antibodies. The hydroxylation of benzphetamine was also assayed.

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, there is an apparent difference in inhibition by monoclonal antibodies between MC-treated 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 by monoclonal antibodies. 3) The hepatic 7-ethoxycoumarin deethylase of C57BL/6 mice was inhibited 43% which was not parallel to the degree of inhibition in AHH, while that of DBA/2 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 liver in both C57BL/6 and DBA/2 mice.

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, the genetic control of cytochromes P-450 and their relationship to drug and carcinogen metabolism, and the 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 the AHH and 7-ethoxycoumarin deethylase activities. The radioimmunoassay system will also be used. Through these investigations with monoclonal antibodies, we will make an atlas of the cytochromes P-450 along with a library of monoclonal antibodies which will differentiate different forms of the cytochromes P-450.

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. (In press).

ANNUAL REPORT OF THE LABORATORY OF MOLECULAR ONCOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1981 to September 30, 1982

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 Biological Carcinogenesis 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 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 Laboratory of Molecular Oncology was organized during this reporting year. Three sections previously existed (Biological Carcinogenesis, Cellular Transformation and Tumor Biochemistry) and for the most part have not changed their research objectives. The research direction of the Microbiology Section is being reorganized and one new section (Molecular Genetics) is being organized. A considerable number of personnel changes (and therefore projects) have occurred in Tumor Biochemistry which is reflected in the new and discontinued project reports.

A 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 two 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 determined, isolated and characterized the normal cellular homologs of these viral onc genes and have demonstrated that one of these genes, the cellular murine mos gene, can be activated to transform cells by a viral transcription control element. A major question being addressed is the mechanisms by which these cellular onc genes are transduced into retroviral genomes. Also, onc genes represent a family of conserved animal genes, and their expression in human tumors has been implicated in the transformation process. Yet it remains to be determined whether human homologs of onc genes are capable of transforming normal cells in a fashion analogous to the transcriptionally activated viral and cellular onc genes from other avian and mammalian species. We have also begun to address this problem.

The second approach we have initiated concerns direct isolation of sequences from human DNA which will enhance cellular transformation of known viral onc genes as well as the development of procedures for identifying dominant tumorigenic genes in mammalian tumors, both naturally occurring and those induced by a variety of carcinogens. Our initial efforts in these endeavors are very promising and they are addressed briefly in the following summary.

In the Tumor Biochemistry and Microbiology Sections we have analyzed a cloned transforming virus Moloney sarcoma virus (MSV) containing a transforming sequence v-mos, and its cloned cellular normal homologue c-mos. We are interested in identifying the sequences required for v-mos expression and in understanding the mechanism by which c-mos, a normal cell gene, can be activated to express its transforming potential. We have attempted to develop techniques for the efficient detection and isolation of transforming sequences present in human tumors and tumor-derived cell lines. In both cases we have utilized sensitive DNA transfection analysis in conjunction with recombinant DNA technology.

The provirus of the MSV contains both the MSV-specific transforming sequence (v-mos) and 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 high efficiency expression of the transformation potential of v-mos and its cellular homologue c-mos. The LTR acts to enhance v-mos transformation when linked either 5' or 3' relative to v-mos, and analysis of polyadenylated RNA indicates RNA synthesis either initiates or terminates in the LTR, depending on its location relative to mos. LTR-containing DNA clones also enhance v-mos transformation when two separate fragments are co-precipitated and co-transfected. We are characterizing the nature of mos-containing DNA and RNA in these transformed cells and are also analyzing the mechanism by which these fragments interact in order to develop a method for LTR-induced activation of other potential oncogenic sequences.

A single LTR introduced either 5' or 3' relative to mos is sufficient to enhance or activate transformation by mos. Cells transformed by transfection of these recombinant DNAs express polyadenylated RNA transcripts containing mos and have integrated copies of mos containing DNA consistent with the structure of the transfected DNA. The enhancing properties of the proviral LTR have been shown to reside within the 72 base repeat sequence present within the unique 3' region

of the LTR. We have also identified a cis-acting sequence within the 5' normal mouse DNA sequence flanking c-mos that inhibits activation of transformation by a 3' LTR element. Removal of this 5' normal mouse sequence permits activation of the transforming potential of c-mos by a 3' LTR. This is the first characterization of a negative acting cis control element. We have studied a conditional temperature sensitive mutant of MSV. This mutant expresses two size classes of mos-containing RNA at both permissive and non-permissive temperatures, while a spontaneous wt revertant of this mutant expresses only the smaller of the two RNA size classes. We are currently cloning the DNA provirus of this mutant in order to analyze the structure of the two RNAs and to identify the specific site of the lesion in the viral genome.

We are determining the DNA sequence upstream from the mos gene in two molecularly cloned MSV isolates in order to determine the origin of subgenomic mRNA for the mos gene product. We will also determine the sequence alterations which are responsible for the production of an altered gag gene product by one MSV isolate and the block in gag production of the second MSV isolate. Collectively these studies will provide us with a thorough understanding of the regulation of the expression of this transforming gene. Previously, these types of analyses have provided a preview of the types of molecular arrangements and modifications that can be identified in vivo in the molecular elements responsible for cellular transformation.

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 influences LTR activation of transformation. We have found that methylation of v-mos results in a reduction in transformation efficiency. A similar reduction is observed when the LTR is methylated. When both v-mos and LTR sequences are methylated, the reduction in transforming frequency is equivalent to the reduction observed when the entire MSV provirus is methylated. These studies suggest that the methylated state of a cell sequence with transforming potential will influence its activation by an integrated provirus LTR element.

A human DNA fragment containing a region homologous to the v-mos transforming gene of MSV (termed humos) was sequenced and compared to that of the mouse cellular homolog of v-mos (termed mumos). The humos gene contained an open reading frame of 346 codons that was aligned with an open reading frame initiated at an ATG found internally in the mumos coding sequence and terminated at equivalent opal codons. The polypeptides predicted from the DNA sequence to be encoded by humos and mumos were also found to be extensively homologous and 253 of 337 amino acids of the humos gene product were in common with those of mumos. Near the middle of the polypeptide chains, four regions ranging from 19-26 consecutive amino acids were conserved. However, we have not been able to transform mouse cells with transfected humos DNA fragments or with hybrid DNA recombinants containing humos and retroviral LTR elements. We have therefore developed techniques for isolating unique DNA recombinants between human genomic DNA and the mos region of Moloney sarcoma virus. We have characterized these recombinants by specific methods demonstrating that such recombinants contain both human and MSV mos genetic sequences. These recombinants will be tested for biological transforming activity in order to identify the region of the mos gene that is essential for focus formation.

The Biological Carcinogenesis Section has been primarily interested in avian acute transforming retroviruses, their transforming onc genes and the cellular

homologs of these genes. This section has studied two such acute transforming retroviruses. The avian myelocytomatosis virus (MC29) and avian myeloblastosis virus (AMV). The avian myelocytomatosis virus or MC29 has a broad oncogenic spectrum in vivo and transforms fibroblasts and hematopoietic target cells in vitro.

Integrated MC29 proviral DNA was isolated from a library of recombinant phage containing DNA from the MC29-transformed nonproducer quail cell line Q5. The cloned DNA was analyzed by restriction endonuclease digests and by electron microscopic visualization of R-loops formed between the cloned DNA and MC29 or helper virus RNA. It was found that the cloned DNA insert contains approximately 4 kb of the MC29-specific sequences and helper related sequences. Transfection of the cloned DNA into NIH 3T3 cells revealed that the MC29-specific sequences are functional.

The complete nucleotide sequence of the integrated proviral genome of MC29 coding for gag-myc protein was determined and compared to the helper virus as well as the cloned cellular myc locus in order to localize junction points between helper viral and v-myc sequences. These studies demonstrated that the LTR sequence of MC29 is very similar to that of RSV. The recombination between viral and cellular sequences occurred in the coding region of the gag p27 resulting in an open reading frame of an additional 1278 bp. This gag-myc hybrid protein terminates within the v-myc region 300 bases upstream from the v-myc-env viral junction.

The nucleotide sequence of the chicken cellular myc gene (c-myc) has been determined and compared to the MC29 v-myc gene. The cellular c-myc shares 1.6 kb homology with a viral-specific sequence and is interrupted by a 972 bp nonhomologous sequence. The latter sequence has consensus donor and acceptor splice signals that have been processed in the virus. The total number of viral-specific nucleotides in c-myc is identical to the number of v-myc. The cellular and viral genes differ by 15 nucleotides, none generating a new termination codon.

The second virus studied by this section, avian myeloblastosis virus (AMV) is replication defective and requires a helper retrovirus. AMV has undergone sequence substitution in the region coding for the viral envelope protein with cellular sequences responsible for leukemogenesis. The nucleotide sequence of the transforming region of AMV contains an open reading frame of 795 nucleotides within the cellular substitution. This would code for a protein of an approximate molecular weight of 30,000 daltons. Comparison of the nucleotide sequences of AMV and the helper indicates that the recombination site occurred near the 3' end of the polymerase gene. This sequence matches the consensus sequence for the 3' acceptor RNA splice sites. The preservation of the 3' splice signal in both AMV and MAV suggests that it may have been involved in the original recombination event, as well as being required as an acceptor site for LTR-promoted message synthesis. The amino acids predicted from the nucleotide sequences show that the carboxyl-terminus of MAV reverse transcriptase is different from that of AMV. It is not clear yet whether AMV produces a functional polymerase.

In vitro transcription studies were carried out to analyze the expression of the AMV and MC29 cloned DNAs. Initiation of transcription of AMV and MC29 by RNA polymerase II (α -Amanitin sensitive) in a eucaryotic cell-free system has been obtained and is located within the U₃ region of the LTR. In addition, initiation of transcription from a corresponding canonical Goldberg-Hogness sequence in the onc gene of AMV has been obtained, suggesting that this conserved sequence element may serve as an independent promoter.

The interests of the Cellular Transformation Section have been to correlate some of the transformed phenotypes with biochemical events. Malignant transformation by the avian Rous sarcoma virus is induced by virus specific coded protein (pp60 src). Characteristic morphological and metabolic features of transformed cells have been used as a basis for examining functions of the transforming protein and distinguishing between direct and indirect effects of the protein. Increased synthesis of ATP and utilization of energy by transformed cells has been observed to be a direct result of the virus-coded protein. Increased synthesis of macromolecules is not responsible for the increased energy utilization. We are now trying to identify the protein associated with the increased utilization of ATP by the transformed cells.

The members of the Tumor Biochemistry and Microbiology Sections have developed a screening system to identify dominant transforming DNA sequences based on the ability of such sequences to induce tumors in athymic nude mice. Transfection of NIH 3T3 cells with cloned MSV DNA, and with DNA from cells transformed by MSV and cellular DNA from human cells transformed by simian virus 40 (SV40) indicate that under the appropriate conditions tumor formation in nude mice is as sensitive an assay as is the conventional focus assays in tissue culture. We have identified MSV or SV40 sequences in tumors which arise following the injection of transfected cells and virus can be rescued or viral antigens detected in cell lines derived from tumor explants. We have screened a number of DNAs derived from primary human tumors and tumor derived cell lines using this assay and have identified three human tumor DNAs capable of inducing tumors in our assay. There are many advantages to performing the assay in this manner as compared to the conventional focus formation assay. First of all this is a direct determination of the tumorigenicity of such dominant transfected and transforming genes. Second, the tumors for the most part are monoclonal and therefore eliminate the necessity for subcloning and independently deriving foci. The assay is also more sensitive in detecting transforming genes since it can be expanded more easily and of course the handling in nude mice is far more efficient than cells in culture.

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 NIH 3T3 cells and scored for foci of transformed cells or injected into athymic nude mice. The DNA prepared from the MNNG transformed cells and the teratocarcinoma cells were able to morphologically transform NIH 3T3 cells, while cells transfected with DNA from the MNNG transformed cells and the fibrosarcoma gave rise to tumors in nude mice. Both the morphologically transformed NIH 3T3 cells and the tumors possessed DNA highly repeated human Alu sequences.

To rapidly isolate these sequences from the genome of the transfected tumor we have developed a novel DNA cloning vector, MT-5. This vector is used to generate eukaryotic genomic libraries that can be screened with the amber suppressible mini-plasmid VX and provides a rapid positive selection for the desired recombinants. Also the genome of the HT-1 MSV provirus has been modified by recombinant DNA techniques to contain a bacterial origin of DNA replication and a selectable drug-resistance gene. The covalently closed circular proviral DNA intermediate of this chimeric retrovirus is capable of replicating in bacteria as a plasmid and in eukaryotic cells as a retrovirus. It will be used primarily to introduce foreign DNA (cellular onc genes) into mouse NIH 3T3 cells under retroviral control and to study the effects of mutations on the process of reverse-transcription and integration.

While many biological phenomena have been amenable to investigation utilizing new technologies, developmental gene activation, german to the neoplastic process is difficult to investigate in mammalian species. A very simple system, the cellular slime mold *Dictyostelium discoideum*, is being used to study mechanisms which control developmental gene activation during normal differentiation. These organisms transcribe an additional 26% of their genome during developmental aggregation. Factors which regulate the coordinate expression of this large portion of the genome indicate that cell-cell interaction is a necessary pre-requisite for both the synthesis and stability of these mRNAs. Disruption of cell contacts results in the rapid and specific degradation of these mRNA species. Addition of cyclic AMP to cells which have been disaggregated restores the level of most, but not all, of these mRNAs. Thus, an event which initiates at the cell surface brings about a change in the nucleus which allows the transcription of an additional 26% of the *Dictyostelium* single copy genome. The actual rate of transcription and the subsequent stability of many of these mRNAs are then further regulated by a cyclic AMP mediated process.

DNA recombination is another process which is only recently being addressed in mammalian systems and is relevant to many of the systems which have been elucidated, and held to be responsible for neoplastic transformation, e.g. specific translocations of large segments of the chromosome have been associated with specific neoplastic diseases. Most of our knowledge and investigative insight into these processes have come from an understanding of recombination systems in prokaryotes. The coliphage λ integration paradigm has provided fundamental knowledge and understanding of site specific recombination and integration. In this regard we have been studying the interaction of a phage encoded topoisomerase I with the specific sites for DNA recombination using these sites cloned into a plasmid vector. Also an ELISA assay is being developed to detect nanogram quantities of the bacteriophage lambda coded *int* (integrase) protein. This development will enable the genetic expression of this important regulatory protein to be monitored in a variety of phage and host mutants.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04846-10 LMO

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemical Analysis of Viral Infection and its Control at the Cellular Level

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: B. I. Gerwin Chemist LMO, NCI

COOPERATING UNITS (if any)

J. Levin Chemist LMG, NICHD
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LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.45

PROFESSIONAL:

1.0

OTHER:

.45

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are utilizing a non-conditional polymerase mutant of B-tropic MuLV to study the mechanism of the reverse transcriptase reaction. This mutant synthesizes partial copies of the natural template in vitro. These products are being characterized in order to determine the nature of the enzymatic blockage to completion of the transcription reaction. At the same time, we are attempting to molecularly clone this mutant in order to completely characterize the genetic change which has led to the structural alteration in the polymerase. We are determining the DNA sequence upstream from transforming gene in two molecularly cloned sarcoma viruses which either do or do not efficiently splice a subgenomic mRNA for the transforming gene. In addition, attention will be given to determining the sequence alterations which are responsible for the production of altered gag gene products by one type of MSV and block production of any gag gene products by the second type of MSV.

Project DescriptionObjectives:

The goal of this project is an understanding at the molecular level of the life cycle of RNA tumor viruses, i.e., the expression of these viruses in cells infected both nonproductively and productively, as well as an understanding of 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) Partial copies of the viral RNA are the major products of reverse transcription by the polymerase mutant. These copies would indicate that DNA synthesis is blocked in the env region of the genome and that the mutant enzyme can progress beyond the synthesis of strong stop DNA.

(B) Major alterations in base sequence of sarcoma virus which splices inefficiently begin in the p10 region of the genome and continue to the mos specific onc.

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 actual location of the block of replication will be analyzed and located precisely on the genome in an effort to determine what enzyme function is altered or what transcriptional signal has been deleted. Cloning of the integrated mutant genome will allow direct analysis of the alteration of the pol gene.

(B) The region of interest for subgenomic splicing will be sequenced in both M-1 MSV (inefficient splicing) and HT-1 MSV (efficient splicing). Comparison of the two should yield important information on requirements for mRNA production and processing. In addition, M-1 MSV synthesizes an altered gag protein while HT-1 synthesizes no detectable gag protein. Sequence data should reveal the signals which account for translation of the gag gene in M-1 and a block to translation in HT-1.

Publications:

Rein, A., Lowy, D.R., Gerwin, B.I., Ruscetti, S.K., and Bassin, R.H.: Molecular properties of a (gag-pol-env+) murine leukemia virus from cultured AKR lymphoma cells. J. Virol. 41: 626-634, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04876-10 LMO

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Oncogenic Virus Influence on the Biochemical Events of Host Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Paul S. Ebert Research Chemist LMO, NCI
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COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

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TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

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(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Factors affecting the photodynamic inactivation of L1210 cells by light of defined wavelength and hematoporphyrin (HP) were investigated. Tumor cells containing HP were exposed to narrow bandwidths of white light and effects of the photons on subsequent cell growth was followed. 503 nm was the most effective wavelength followed by 397 and 531 nm. 636 nm, a wavelength outside the HP absorption spectrum, was about 10% as effective in killing cells as was 621 nm, the least effective wavelength in the HP spectrum. Succinylacetone (SA), dibucaine, and chloroquine increased the uptake of HP into L1210 cells and also decreased the requirement for HP to kill a given number of tumor cells. B-carotene, a potent singlet O₂ quencher, and vitamins E and C were ineffective in preventing damage to L1210 cells by light and HP. Though SA causes a decrease in heme levels in murine erythroleukemia cells, in L1210 cells heme levels are increased. Cell growth in L1210 cells is inhibited at concentrations of SA above 2 mM despite the finding that intracellular heme levels are not reduced. A visual test for colonies of transformed cells on a cell sheet of normal cells was developed. Colonies of cells transformed by Moloney sarcoma virus against a background of 3T3 cells fluoresced upon exposure to UV light when the cultures were treated with HP or HPd.

Project Description

Objectives:

To determine the effectiveness of various wavelenghts of white light which correspond to peaks of the HP absorption spectrum curves to kill malignant L1210 cells treated with HP. To determine why various malignant and transformed cells show an affinity for porphyrins such as heme, HP, and hematoporphyrin derivative (HPd). To investigate the characteristics of uptake of porphyrins in malignant and normal cells. To determine how various biological compounds can modify the uptake of HP into malignant cells.

Methods Employed:

Malignant L1210 cells grown in culture were incubated with HP and the uptake of this porphyrin was determined by a fluorimetric method. L1210 cells treated with HP were exposed to white light of various wavelengths from a lantern slide projector or a dye laser generator and then incubated in fresh medium for 3-4 days to determine their resultant growth patterns. The effects of various anti-oxidants and inhibitors on the phototherapy process were examined by treating the malignant cells with these compounds prior to HP and light administration.

Major Findings:

1. Effect of various wavelengths of white light on the photochemical killing of L1210 cells. Characteristics of the photochemical inactivation of L1210 cells by light of defined wavelength and HP were studied. HP exhibited five peaks of maximum light absorption. L1210 cells were incubated with HP, which is selectively taken up by malignant cells, and then exposed to light of narrow bandwidths corresponding to the peaks of light absorption of HP. The resultant growth of the cells was followed and the exposure time yielding 90% inactivation of cells was calculated. The decreasing order of effectiveness of photons of various wavelengths killing 90% of cells containing HP was as follows: 503>397≥ 531>621 nm. A wavelength beyond the 621 nm peak of HP absorption, 636 nm, was 10% as effective in killing tumor cells as was 621 nm, but showed dependence on the presence of HP for killing. These studies show, surprisingly, that the largest absorption peak, the Soret band (400 nm) did not contain the most effective photons for killing tumor cells in the presence of HP. These studies also show that the wide bands of white light used by many oncologists contain wavelengths that are relatively ineffective for inactivating tumor cells and may contribute to undesirable side effects.

2. Effect of various chemotherapeutic agents and antioxidants on the photochemical killing of L1210 cells. The effects of various HP uptake enhancers and modifiers on the photochemical inactivation of L1210 cells treated with HP and light were investigated. Dibucaine and chloroquine, therapeutic agents which can affect membranes, increased the uptake of HP in L1210 cells in vitro, while vitamins A and C could not. Both dibucaine and chloroquine were able to decrease the amount of light needed to kill L1210 cells incubated with HP. B-carotene, a potent singlet O₂ quencher, and two antioxidants, vitamins E and C were ineffective in limiting or preventing any photochemical damage to tumor cells by the treatment

of HP and light. These findings suggest that the primary mechanism for photo-dynamic killing of tumor cells by light and HP is by some mechanism other than that of the generation of singlet O_2 .

3. Augmented killing of tumor cells with HP and light by SA. The effect of SA on the uptake of HP into L1210 cells in vitro and its effects on the photochemical killing of tumor cells with light and HP was studied. Though SA has been shown to decrease the heme levels of murine erythroleukemia cells, heme levels in SA-treated L1210 cells were increased somewhat. Both hematin and HP uptake were markedly increased in SA-treated, porphyrin-supplemented medium. Preincubation of L1210 cells with SA decreased the amount of illumination needed to kill tumor cells exposed to HP and light. The increased uptake of HP into tumor cells was probably responsible for the enhanced photodynamic killing by light and HP.

4. Effect of SA on respiration of L1210 cells. When L1210 cells at high cell density were incubated with 1-4 mM SA, no cell killing was observed after 1 day, but after 3 days of incubation increased cell death was observed in the presence of 2-4 mM SA. Decreased endogenous respiration with reference to untreated L1210 cells was observed in SA-treated cells after 3 days of incubation with concentrations of SA above 2 mM.

5. Development of a rapid screening test for transformed cells on tissue culture plates using HP and HPd. The selective uptake of HP or HPd by malignant or transformed cells is well known. The presence of colonies of MSV-transformed cells on a background of NIH-3T3 cells can be detected by fluorescence upon exposure of HP or HPd-treated cells to UV light. The test can be repeated several times without deleterious effects, if the plates are not exposed to white light. The fluorescence can be amplified by pretreatment of the cells with dibucaine.

Significance to the Biomedical Research and the Program of the Institute:

Investigations on the best wavelength of light to kill HP-treated malignant cells should increase the potential of this potent and specific technique to eradicate a large range of discrete malignant tumors occurring in humans. Co-administration of HP and various agents which increase the uptake of porphyrins should reduce the necessary dose of porphyrin or light to kill a given number of malignant cells. Enhanced uptake of porphyrin would decrease the dose of porphyrin required to kill tumor cells and allow more effective penetration of light for photochemical killing. The studies with antioxidants should lead to a better understanding of the mechanism of killing of tumor cells by phototherapy.

Proposed Course:

Utilizing the technology learned in tissue culture, attempts will be made to eradicate various ascites malignancies and carcinomas in mice by the administration of HP and light. We will determine if SA and dibucaine also enhance the photodynamic inactivation of tumors in animals. We will attempt to determine the mechanism of action of the enhanced uptake of HP in malignant cells by SA, and if SA can also amplify the uptake of certain therapeutic agents as methotrexate. Covalently bound heme-methotrexate complexes may also be taken up selectively to a greater extent than methotrexate itself. Further attempts will be made to optimize the visual test for transformed cell colonies. Different types of transformed cells will be examined and optimal conditions with minimal

cell toxicity will be determined. Experiments to improve the efficiency and reproducibility of the transfection assay will be begun. DNA extracts from various human tumors will be studied by transfection techniques.

Publications:

Ebert, P.S., Bonkowsky, H.L. and Wars, I.: Stimulation of hemoglobin synthesis in murine erythroleukemia cells by low molecular weight ketones, aldehydes, acids, alcohols, and ethers. Chemico-Biol. Interactions 36: 61-69, 1981.

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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemical Events in Tumor Virus Replication and Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George F. Vande Woude	Chief, LMO	LMO, NCI
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	M. L. McGeady	Staff Fellow	LMO, NCI
	M. A. Oskarsson	Chemist	LMO, NCI
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COOPERATING UNITS (if any)

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TOTAL MANYEARS:

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A human placental DNA fragment containing a sequence homologous to the transforming gene (v-mos) of Moloney murine sarcoma virus was molecularly cloned. The DNA sequence of the homologous region of human DNA (termed humos) was compared to that of the mouse cellular homolog of v-mos (termed mumos) as reported by Van Beveren, et al. (Cell 27: 97-108, 1981). The humos gene contained an open reading frame of 346 codons that was aligned with the equivalent mumos DNA sequence. The aligned coding sequences were 77% homologous and terminated at equivalent opal codons. The humos open reading frame initiated at an ATG found internally in the mumos coding sequence. The polypeptides predicted from the DNA sequence to be encoded by humos and mumos were also found to be extensively homologous and 253 of 337 amino acids of the humos gene product were in common with those of mumos. In addition, near the middle of the polypeptide chains, four regions ranging from 19-26 consecutive amino acids were conserved. However, we have not been able to transform mouse cells with transfected humos DNA fragments or with hybrid DNA recombinants containing humos and retroviral long terminal repeat (LTR) sequences.

Project Description

Objectives:

To investigate biochemical events associated with tumor virus replication and to determine their relationship to neoplastic transformation of host cells.

Methods Employed:

Oncogenic viruses are propagated in tissue culture. Focus assays and plaque assays are used for the detection of some propagated and purified viruses. Other assays are: agarose gel diffusion for the detection of viral antigens, polyacrylamide gel electrophoresis and guanidine-agarose chromatography for the purification and identification of virus-specific proteins, in vivo and in vitro labeling of purified viruses using radio-labeled amino acids and nucleic acids. Sensitive peptide mapping techniques are developed for use in comparing virion polypeptides. Endonuclease restriction enzyme cleavage of viral and cellular DNA. Cloning in bacterial and mammalian vectors of specific virus-related sequences of viral and host cell origin.

Major Findings:

The nucleotide sequence and predicted protein structure of the human genomic homolog of the Moloney sarcoma virus mos onc gene are strikingly similar indicating that this gene has been strongly conserved in the estimated 70 M yr. since evolutionary divergence of man and mouse. Nucleotide sequence homology is greater in the open reading frame sequence of human mos than in the region 5' to its first possible initiation codon. The greatest homology between these sequences occurs in the middle portion of the human mos sequence. Other features of similarity are that both mos genes appear to lack intervening sequences, their open reading frames terminate in the same two amino acids and opal codons and the human mos initiation codon begins in a conserved block of five amino acids found internally in the murine mos sequence. The murine mos RNA has not been detected in any mouse cells tested which raised the question whether this gene is normally expressed. The strong selection for maintaining conserved sequences in the cellular open reading frames suggests that both proteins are essential.

Four ATGs are found before the beginning of the human mos open reading frame. The DNA sequences of both human and murine mos lack intervening sequences in the open reading frames. It is possible, however, that these genes represent exons of cellular transcripts.

Homology between the predicted amino acid sequences of the viral mos gene product and that of the avian sarcoma virus (ASV) src gene product has previously been described. These amino acid sequences were estimated to be 23% homologous in the c-terminal region of the ASV src gene product. The blocks of amino acids conserved between ASV src and murine mos gene products are also common to the human mos gene product.

It would seem plausible that the human mos gene could be activated to transform mouse NIH 3T3 cells in DNA transfection analyses in a manner analogous to the

activation of the transforming potential of the murine mos gene. The human mos gene was found to be inactive in DNA transfection/transformation assay and unlike murine mos could not be activated by insertion of LTR elements 5' to the coding sequence. Moreover, a hybrid gene consisting of the entire 5' region MSV fused at a common BglI site in the viral mos genes to 3' sequences from human mos, was also inactive in the transformation assay.

It is not possible to conclude on the basis of these results whether the human mos gene has, or has not, oncogenic potential. The human mos gene product may fail to interact with the mouse cell components in the same way as the murine viral mos or cellular mos gene products. It may well be appropriate to assay for mos transforming potential in a human cell line rather than the heterogenous NIH3T3 cells. Alternatively, evolutionary pressures may have contrived to select for mos-related human genes with a lower oncogenic potential while retaining the normal, presumably regulatory, role of the polypeptide product.

Significance to Biomedical Research and the Program of the Institute:

The formation of hybrid molecules between transcription control elements and cellular genes with transforming potential can serve as a model for identifying the molecular elements essential for transformation. Results suggest a model whereby retroviral integration at multiple sites in the host chromosome could serve to activate expression of normally quiescent cellular sequences with transforming potential mediated through an LTR-like transcription control element. This may be a useful model for other, non-viral, transformation events. Thus, normal cell sequences with functional properties of an LTR (e.g. transposons) could be transposed adjacent to transforming sequences or activated to express adjacent transforming sequences as a result of a genomic insult. Viral onc gene homologs detected in human genomic DNA have to be systematically tested for their transforming potential. Even though these genes are highly conserved in animal species, does not, a priori, mean that the transforming potential is conserved.

Proposed Course:

Specific cellular onc genes from human genomic DNA will be isolated, characterized and tested for their ability to transform cells.

Publications:

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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Transforming Genes of Avian RNA Tumor Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have determined the nucleotide sequence of the transforming gene of AMV region. Within the cellular substitution there is an open reading frame of 795 nucleotides starting with the initiation codon ATG and terminating with the triplet TAG. This would code for a protein of 265 amino acids with a molecular weight of approximately 30,000. The complete 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 junction points between helper viral and v-myc sequences. These studies demonstrate that the LTR sequence of MC29 is very similar to that of RSV, the viral genome has suffered extensive deletions in the gag, pol and env genes, the gag region can code for p19 and part of p27, 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 1278 bp resulting in a gag-myc hybrid protein, the open reading frame terminated within the v-myc region, 300 bases upstream of v-myc-helper viral junction, and the v-myc-helper viral junction at the 3' end occurred in the middle of env gene, rendering it defective.

Project Description

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) Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging. Isolation of phage 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. Isolation of cloned integrated proviral DNA sequences. Sucrose gradient-purified restriction fragments from a complete *EcoRI* digestion of Q5 DNA, ranging in size from 5 to 15 kb, were ligated to λ gt10- λ B arms. After banding in CsCl, phage particles produced from the ligation reaction in vitro packaging were adsorbed in *Escherichia coli* LE392 as described by Blattner et al. and spread on 150-cm agar plates at density of 5000 plaques per plate. Nitrocellulose filters were lifted from the plates and plaque plaques were identified by hybridization to an AMV(MAV) [³²P]cDNA probe.

(B) Construction of recombinant plasmids. Double stranded cDNA of AMV genome was prepared. It was converted to perfect duplex molecules with blunt ends by digestion with single-strand nuclease S₁. It was then incubated with *E. coli* DNA polymerase I. The product of the S₁-DNA polymerase reaction was ligated to BamHI linkers with T4 DNA ligase. pBR322 was digested with BamHI, treated with alkaline phosphatase. It was then ligated to double stranded cDNA linked to BamHI linkers.

(C) Transformation and identification of recombinant clones. Construction of chimeric plasmids and the transformation of *E. coli* X1776 by these plasmids was performed in a P2 physical containment laboratory. X1776 was transformed by a transformation procedure. Transformed colonies containing AMV sequences were identified by colony hybridization. The colonies were screened with ³²P-labeled AMV cDNA. Strongly hybridized colonies were selected and replated, single colonies were picked and grown.

(D) Sequences of integrated viral transforming genes and Large Terminal Redundancies (LTR). The "leuk" gene and the LTR from AMV clones were sequenced by using the chemical cleavage method of Maxam and Gilbert. DNA fragments were

labeled on the 5'-end with $\gamma^{32}\text{P}$ -ATP and polynucleotide kinase under standard conditions. The DNA sequences of the LTR from the MC29 clone was done by using the cloning and sequencing procedure with M13mp7.

(E) Computer analysis. Computer analysis of nucleotide sequences was performed by utilizing the computer program originally developed by Korn et al.

Major Findings:

(A) Avian myeloblastosis virus (AMV) is defective in reproductive capacity, requiring a helper virus to provide viral proteins essential for synthesis of new infectious virus. The genome of AMV has undergone a sequence substitution in the region normally coding for the viral envelope protein which has been replaced by cellular sequences responsible for leukemogenesis. We have determined the nucleotide sequence of this region. Within the cellular substitution there is an open reading frame of 795 nucleotides starting with the initiation codon ATG and terminating with the triplet TAG. This would code for a protein of 265 amino acids with a molecular weight of approximately 30,000.

(B) The complete 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 junction points between helper viral and v-myc sequences. These studies demonstrate that (1) the LTR sequence of MC29 is very similar to that of RSV, (2) the viral genome has suffered extensive deletions in the gag, pol and env genes, (3) the gag region can code for p19 and part of p27, (4) 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 1278 bp resulting in a gag-myc hybrid protein, (5) the open reading frame terminated within the v-myc region, 300 bases upstream of v-myc-helper viral junction, and (6) the v-myc-helper viral junction at the 3' end occurred in the middle of env gene, rendering it defective.

(C) In an effort to better understand how retroviruses acquire cellular transforming genes, the nucleotide sequence of the chicken cellular myc gene has been determined and is compared to the MC29 v-myc gene. The cellular locus shares 1.6 kilobase homology with viral specific sequences, interrupted by an internal 1.0 kilobase sequence. The junctions between v-myc and the internal cellular sequence have been determined. The 5' sequence, ...TCGG/GTGAG..., at which the v-myc gene diverges from c-myc, demonstrates homology with that of the consensus donor sequence, AG/GTRAG, and is identical to the donor sequence of β -globins. The 3' sequence of the interrupting cellular DNA is ...TTTTTTCCTGTGCAG/AAGA, homologous to the consensus acceptor sequence, Y-YYY-CAG/. From this sequence data, the 1.0 kilobase sequence in c-myc that is not homologous to v-myc can correctly be defined as an intervening sequence. This is the first molecular evidence that the region of non-homology that interrupts some cellular gene homologs of viral onc genes is an intervening sequence.

(D) The nucleotide sequence of the integrated avian myeloblastosis virus (AMV) long terminal repeat (LTR) has been determined. The sequence is 385 bp long and is present at both ends of the viral DNA. The cell-virus junctions at each end consist of a six-base pair direct repeat of cell DNA next to the inverted

repeat of viral DNA. The LTR also contains promoter-like sequences, a mRNA capping site, and polyadenylation signals. Several features of this LTR suggest a structural and functional similarity with sequences of transposable and other genetic elements. Comparison of these sequences with LTRs of other avian retroviruses indicates that there is a great variation in the 3' unique sequence (U₃) while the 5' specific sequences are highly conserved.

(E) DNA-mediated gene transfer of integrated MC29 proviral DNA into NIH/3T3 cells results in the induction of two morphologically distinct classes of transformed foci. Focal cells are neoplastically transformed as assayed by the ability to grow in soft agar, and by increased uptake of 2-deoxyglucose. The frequencies of neoplastic transformation for different defined subgenomic fragments are reported. The frequency is highest when the onc gene of MC29 (*myc*) is covalently linked to the proviral long terminal repeat (LTR) sequences. Separation of the *v-myc* gene from the LTR sequences results in a 300- to 500-fold reduction in the number of transformed foci. Transformation frequency is markedly enhanced when the plasmid containing *v-myc* is co-transfected with another plasmid containing a proviral LTR. The results of the co-transfection experiments suggest that proviral LTR sequences may have become covalently linked to *v-myc* during the transfection process.

(F) Polyadenylated RNAs of certain human tumor cell lines are shown to contain transcripts related to the cell-derived transforming onc genes of molecularly cloned primate, murine or avian transforming retrovirus genomes. Thus, analogues of retroviral transforming genes are both present and frequently expressed in human neoplastic cells.

(G) Total cellular poly(A) enriched RNA from a variety of fresh human leukemic blood cells and hematopoietic cell lines was analyzed for homology with molecularly cloned DNA probes containing the onc sequences of Abelson-MuLV (A-MuLV), Harvey-MSV (HaMSV), simian sarcoma virus (SSV) and avian myelocytomatosis virus (MC29). Results with the fresh blood cells paralleled those obtained with the cell lines. With A-MuLV and HaMSV, multiple RNA bands were visualized in all cell types examined without significant variation in the relative intensities of the bands. When SSV was used as probe, expression related onc sequences were absent in all hematopoietic cell types examined except for one neoplastic T-cell line (HUT 102) which produces the retrovirus HTLV. In this cell line a single band (4.2 kb) was observed. With MC29 as probe, a single band of 2.7 kb was visualized in all cell types examined with only a one- to two-fold variation in intensity of hybridization. An exception was the promyelocytic cell line, HL60, which expressed approximately 10-fold more MC29-related onc sequences. With induction of differentiation of HL60 with either DMSO or retinoic acid a marked diminution in amount of MC29-related, but the A-MuLV-related, onc message was observed.

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:

Current research is conducted toward defining the mechanism of action of malignant transformation in cells. Efforts are concentrated towards:

1. Definition of specific sequences required for transformation by MC29. We already have shown that a fragment of 9.1 kb transformed 3T3 mouse cells. This assay will be used to clearly establish the minimum number of nucleotides required for transformation. The importance of "gag" LTR in the transformation process will clearly be established by deleting such regions and in turn testing its biological activity.
2. We have already initiated studies to synthesize the transforming protein MC29 utilizing bacterial promoters in well-defined plasmids obtained from M. Ptashe. This will enable us to see if MC29 specific protein is formed where the MC29 sequences are expressed.
3. We are also involved in synthesizing the AMV transforming protein. The task here is relatively easy since we already know the nucleotide sequence.
4. We are presently characterizing endogenous chicken MC29 clones and six human clones all isolated from human libraries.

Publications:

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Eva, A., Robbins, K. C., Andersen, P. R., Srinivasan, A., Tronick, S. R., Reddy, E. P., Ellmore, N. M., Galen, A. T., Lautenberger, J. A., Papas, T. S., Westin, E. W., Wong-Stall, F., Gallo, R. C., and Aaronson, S. A.: Transcription of retrovirus onc gene analogues in human solid tumor cells. In Yohn, D. S., and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research. Amsterdam, Elsevier/North Holland, 1981, pp. 381-384.

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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. (in press).

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Papas, T. S., Shultz, R. A., and Chirikjian, J. G.: Enzymatic synthesis of duplex DNA by avian myeloblastosis viral reverse transcriptase: In Papas, T. S. and Chirikjian, J. G. (Eds.): Analysis of Nucleic Acid Structure by Enzymatic Methods. New York, Elsevier/North Holland, 1981, pp. 1-16.

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Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Samuel, K. P., Baluda, M. A., and Reddy, E. P.: Nucleotide sequence of integrated avian myeloblastosis virus (AMV) long terminal repeat (LTR) and their host and viral junctions: Structural similarities to transposable elements. In Chandra, P. (Ed.): Biochemical and Biological Markers in Neoplastic Transformation. New York, Plenum Press, 1982.

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Rushlow, K. E., Lautenberger, J. A., Baluda, M. A., Perbal B., Reddy, E. P., Chirikjian, J. G., and Papas, T. S.: Nucleotide sequence of the transforming gene of avian myeloblastosis virus (AMV). Science, 1982 (in press).

Westin, E., Wong-Staal, F., Gelmann, E., Baluda, M., Papas, T. S., Eva, A., Reddy, E. P., Tronick, S., Aaronson, S., Gallo, R. S.: 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. Amsterdam, Elsevier/North Holland, 1981, pp. 405-407.

Westin, E. H., Wong-Staal, F., Gelmann, E. P., Dalla Favera, R., Papas, T. S., Lautenberger, J. A., Eva, A., Reddy, E. P., Tronick, S. R., Aaronson, S. A., and Gallo, R. C.: Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79: 2490-2494, 1982.

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PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04902-09 LMO

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Purification Properties of 5-methyl-deoxycytosine DNA Methylase Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
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TOTAL MANYEARS:

2.5

PROFESSIONAL:

1.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have purified specific DNA methylating enzymes from a variety of Haemophilus species capable of methylating eukaryotic DNA at specific cytosine containing loci in cell-free reactions. Additionally, we have partly characterized the enzymatic assay as to its reaction kinetics and substrates: S-adenosylmethionine (S-AM) and a variety of DNAs both prokaryotic, viral and eukaryotic species. The methylated DNA products of the cell-free reaction are essentially completely protected from the corresponding restriction-endonuclease catalyzed cleavage and become useful probes as to the effects of site specific DNA modification vis-a-vis the efficacy of the modified DNA to perform a genetic function at a cellular level. Towards this end we have shown that certain specific loci of recombinant DNA vectors containing eukaryotic retroviral sequences are sensitive to specific methylase modifications. Specifically, certain methylated modification reduces the efficiency of cellular neoplastic transformation normally catalyzed by the retroviral sequences. Using a variety of methylated recombinant DNA constructs, we have been able to determine that hypermethylated retroviral DNA is less effective at promoting neoplastic cellular changes in vitro than its hypomethylated counterpart.

Project DescriptionObjectives:

To evaluate the region(s) of specific retroviral nucleic acid sequences that may be essential to its expression of oncogenic genetic information.

Methods Employed:

Large-scale cultivation of fastidious microorganisms, whole cell extraction using ultrasonic disruption combined with ultracentrifugational separation of particulate components. Fractionation of nucleoprotein complexed enzyme(s) by ion-exchange chromatography, coupled with enzymatic purification methods using affinity chromatographic techniques. Cell-free DNA dependent methylation reactions utilizing radioisotopic incorporation with tritiated S-AM, characterization of H³-methylated recombinant DNA molecules on agarose gels by preparative and analytic electrophoretic methods. Determination and evaluation of methylated locii using specific restriction endonuclease enzymes in conjunction with molecular sieve gel electrophoresis techniques. Evaluating the effect of methyl-modification reaction using cloned retroviral DNA into plasmid vectors and assaying the efficiency of its transforming potential by in vitro cell transfection techniques.

Major Findings:

- A. A variety of Haemophilus species of methylating enzymes are capable of stable purification, free of degradative nucleases and capable of catalyzing cell-free methyl group additions, at specific sites, to a variety of prokaryotic and eukaryotic DNA substrates.
- B. Methylations of specific locii of recombinant DNA substrates can effectively render them resistant to their correlating specific restriction endonuclease enzymes at $\pm 90\%$ levels.
- C. Retroviral DNA substrates are variously affected by methylation cell-free, namely, that certain sites appear to be sensitive to modification: such methyl group additions lowers their oncogenic transformation efficiency in vitro transfection assays.

Significance to Biomedical Research and the Program of the Institute:

These studies help corroborate the specific genomic location of the malignant cellular transformational information located in cloned retroviral sequences. Correlation of such modified sequences may delineate the minimal elements essential for the malignant potential encoded within retroviral genomes.

Proposed Course:

1. Methylation specific enzymes will be purified further and characterized as to substrate optima, macromolecular size and specific site of DNA locii modified.

2. Efficacy of transformation potential by methylated DNA using a variety of cloned retroviral oncogenic ("onc") sequences will be evaluated and compared to cloned "normal" cellular sequences homologous to "onc" sequences that have been methylated as well.
3. Analogous methylation modifications (ethylation) will be compared using purified DNA methylases and evaluated as to their oncogenic transforming potential.
4. Attempt at reversing the oncogenic transformation events with modified retroviral DNA that have been methylated by various specific methylase enzymes.

Publications:

None

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Herpesviruses: Immunologic and Biologic Studies in Relation to Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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R. Glaser The Ohio State University, Columbus, OH

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TOTAL MANYEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have prepared monoclonal antibodies against polypeptides associated with human herpesviruses and have employed these reagents for carrying out structural analyses of the viral polypeptides. Emphasis has been placed on determining structural and antigenic differences between polypeptides coded by herpes simplex type 1 and 2, and in developing tests which would differentiate these viruses. This year we have prepared over 40 monoclonal antibodies against 8 different HSV-2 proteins, including the major capsid component, an early phosphoprotein, an early DNA binding protein, and the major glycoproteins of the virus. These antibodies are being used in conjunction with a similar group of HSV-1 monoclonals, which we had previously prepared, for antigenic and structural comparisons of the HSV proteins. Monoclonal reagents have been developed against a number of cell membrane proteins which appear to be associated with transformation, either viral induced or by DNA transfection. These reagents will serve as diagnostic tools for the early identification of the transformed state.

Project Description

Objectives:

To develop monoclonal antibody reagents against viral-coded polypeptides and cell products. Studies include preparing monoclonal antibodies against polypeptides associated with human herpesviruses and employing these reagents for carrying out structural analysis of the polypeptides. Emphasis is placed on determining structural similarities and differences between polypeptides coded by herpes simplex virus type 1 and 2, and developing tests for differentiating these viruses. Monoclonal reagents are also prepared against "normal" cell proteins whose expression may be modified following malignant transformation. Similar reagents are prepared against "transforming proteins" associated with retrovirus-induced cell transformation. Additional studies are carried out using synthetic peptides for preparing monoclonal antibody reagents against epitopes of proteins which cannot be feasibly isolated in sufficient quantities.

Separate studies include microinjection techniques for introducing DNA sequences into cells to identify translational products and to assess transforming potential.

Methods Employed:

Standard tissue culture, virologic, and biochemical methods were employed with modifications as required.

Major Findings:

1. Monoclonal Antibodies to HSV-2 Proteins. Last year we reported the preparation and characteristics of 52 monoclonal antibodies against 10 HSV type 1 proteins. This year we have prepared about 40 monoclonal antibodies against 8 different proteins of HSV type 2. These proteins include the major capsid component (ICP-5), an early major phosphoprotein (ICP-6), an early DNA binding protein (ICP-8), glycoproteins gA/B, gD and gE, a 68,000 molecular weight protein, and a 75,000 molecular weight glycoprotein. The monoclonal antibodies against HSV type 2 ICP-8 are of special interest, since it has been reported that this antigen is present in cervical cancer cells.

Our antibody collection includes a non-neutralizing IgG₁ antibody (designated 104-S) which immunoprecipitates an HSV-2 glycoprotein(s) which migrates in SDS-polyacrylamide gels as a sharp band at about 65,000 molecular weight and as a broad band at about 75,000 molecular weight. Pulse-chase experiments indicate that the 65,000 molecular weight component is a precursor to the 75,000 molecular weight species. The precursor was sensitive to endoglycosidase H digestion, indicating the presence of O-linked glycosidic bands, whereas the 75,000 molecular weight product is sensitive to neuraminidase digestion pointing to the existence of sialic acid residues. This HSV-2 glycoprotein is very heat-labile in extracts of infected mouse cells, whereas it is heat resistant in extracts of infected human cells.

The most interesting property of the 104-S antibody is that it reacts with HSV-1 glycoprotein gC. This finding appears to be at variance with other studies which suggest that HSV-1 gC is type-specific. However, monoclonal antibodies were not used in these earlier studies. The high specificity and sensitivity of monoclonal antibodies have often revealed immunological relationships that were not previously detected. The cross-reactivity between HSV-1 gC and the 75,000 molecular weight HSV-2 glycoprotein was confirmed in experiments using guinea pig antiserum prepared against HSV-1 gC that was purified in a immunoadsorbent column containing monoclonal antibody. These findings indicate that HSV-1 gC contains a mixture of type-specific and cross-reactive antigenic determinants.

3. Microinjection. A recently developed technique, manual microinjection, has been introduced into the laboratory within the past year. Microinjection offers the advantage of introducing DNA into the nucleus of viable cells obviating the requirement for heterologous carrier DNA. Therefore, gene expression can be studied with specific DNA sequences in only a few cells and with precise control over the amount of DNA injected and the intracellular site of injection, either the cytoplasm or nucleus.

To determine if the microinjected cells were viable and expressing the injected DNA, B-2-1 and LTK⁻ cells were injected with HSV TK DNA cloned in the plasmid pBR322, as intact circular plasmid or after restriction endonuclease treatment to convert it to a linear form. Cells were selected in HAT medium and assayed for HSV specific TK activity and tumorigenicity in nude mice. The efficiency of biochemical transformation with the LTK⁻ cell line was about 10-fold higher than the B-2-1 cells by microinjection, which is in agreement with earlier studies using transfection. B-2-1 cell lines were obtained with specific viral TK activity after microinjection: 5 with uncut DNA, 11 cut with Hind III, 2 with Bam, and 1 with Pst. 1. None of the cell lines were tumorigenic in nude mice.

In collaboration with Dr. Donald Blair, a study was initiated to compare the transformation efficiency of the Moloney sarcoma virus oncogene, v-mos, when introduced into cells by microinjection. Foci were not detected following microinjection of the cloned ml-MSV provirus DNA into NIH 3T3 cells unless these cells were superinfected with replicating MuLV 24-48 hrs. later. Foci were observed in NIH 3T3 cells preinfected with MuLV and microinjected with v-mos in the same culture and in cells infected with the sarcoma virus rescued from the injected cells. These preliminary results suggest that virus replication and subsequent virus spread may be necessary to produce stably transformed cells following MSV microinjection. One possibility is that overproduction of the mos product in cells containing high levels of microinjected DNA could be lethal to the NIH 3T3 recipients. High multiplicity of MSV virus infections have been observed to be toxic in some cell lines. Currently studies are designed to quantitate the effective dose of v-mos for mammalian cells by microinjection.

In collaboration with Dr. Ronald Glaser, microinjection is being used to determine the sequences within the EBV genome responsible for the EBV-specific antigens: EA, VCA, and EBNA. CNE, a EBV-negative human nasopharyngeal cell line, is being used as recipient cells for microinjection of intact EBV DNA

(strains HR-1 and B95-8) and cloned fragments of B95-8. The cells are stained by indirect immunofluorescence for detection of viral antigen expression 2-4 days after microinjection of intact or subgenomic DNA. A hybrid cell line, HRD-3, is positive for EBV DNA but negative for expression of EBV proteins. In order to learn more about the role of this repressed EBV DNA and possible effect on expression of injected EBV DNA or subgenomic DNA fragments, a comparative study of the CNE and HRD-3 cells is being conducted. EBV-specific antigens have been observed in CNE and HRD-3 cells after microinjection of genomic and subgenomic EBV DNA. To determine the transforming region of the EBV genome, the current approach is to microinject human B-lymphocytes obtained from cord blood after attaching cells to the petri dish with ligand.

Significance to Biomedical Research and the Program of the Institute:

The development of monoclonal antibody reagents can be employed for studying protein structure and for identifying gene products expressed in transformed cells. These reagents will facilitate identification of expressed foreign DNA sequences transiently or permanently retained in transformed cells and will facilitate studies relating human viruses to various disease states.

Proposed Course:

This program will be phased down to reflect a change in direction.

Publications:

Eisenberg, R. J., Long, D., Pereira, L., Hampar, B., Zweig, M., and Cohen, G. H.: Effect of monoclonal antibodies on limited proteolysis of native glycoprotein gD of herpes simplex virus type 1. J. Virol. 41: 478-488, 1982.

Hampar, B.: Transformation induced by herpes simplex virus: A potentially novel type of virus-cell interaction. In Klein, G. and Weinhouse, S. (Eds.): Advances in Cancer Research. New York, Academic Press, 1981, Vol. 35, pp.27-47.

Heilman, Jr., C. J., Zweig, M., and Hampar, B.: Herpes simplex virus type 1 and 2 intracellular p40: Type-specific and cross-reactive antigenic determinants on peptides generated by partial proteolysis. J. Virol. 40: 508-515, 1981.

Showalter, S. D., Zweig, M., and Hampar, B.: Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. Infect. Immun. 34: 684-692, 1981.

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PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 04970-06 LMO

PERIOD COVERED

October 1, 1982 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemistry of Cellular Transformation by Avian Tumor Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John P. Bader Chief, Cellular Transformation Section LMO, NCI
OTHER: Robert Balaban Expert LKEM, NHLBI

COOPERATING UNITS (if any)

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TOTAL MANYEARS:

3.7

PROFESSIONAL:

1.2

OTHER:

2.5

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(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Malignant transformation by the avian Rous sarcoma virus is induced by a virus-coded protein (pp60src) which plays no role in virus reproduction. Attempts have been made to identify the metabolic function of this protein in transformation. Characteristic morphological and metabolic features of transformed cells have been used as a basis for examining possible functions of the transforming protein and a virus mutant which induces temperature dependent transformation has been useful in distinguishing between direct and indirect effects of the protein. Using these methods we have been able to separate late changes in phenotypic transformation from earlier changes induced by the functioning virus-coded protein and have eliminated from consideration several metabolic areas formerly implicated as primary virus-induced changes. However, an increased synthesis of ATP and utilization of energy by transformed cells has been observed, which may be a direct result of the virus-coded protein. An increased synthesis of macromolecules apparently is not responsible for the increased energy utilization, and the basis for this change is being pursued.

Project DescriptionObjectives:

To determine the biochemical function of the virus-coded protein responsible for the malignant transformation of cells by Rous sarcoma virus; to determine the primary physiological effects resulting from the functioning of the altered metabolic profile characteristic of malignant cells; to distinguish metabolic changes necessary to the maintenance of the malignant state from those which are irrelevant.

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, immunoprecipitation, immuno-affinity chromatography.
- (E) Dextran and sucrose density gradients, polyacrylamide gel electrophoresis, starch gel electrophoresis, autoradiography, fluorography.
- (F) Uptake of radioactive molecules into cells, incorporation of radioactive precursors into macromolecules, binding of radioactive molecules to specific cell-surface sites.

Major Findings:

Attempts have been made to determine the function of the virus-coded protein responsible for malignant transformation induced by Rous sarcoma virus (RSV). The Bryan "high titer" strain of RSV (RSV-BH) induces a unique and characteristic morphological change in infected cells. A mutant of this virus (RSV-BH-Ta), isolated in this laboratory, induces temperature-dependent transformation. At 41° infected cells appear nontransformed, but after shifting to 37°, a variety of morphological and biochemical changes occur, resulting in a transformed phenotype indistinguishable from cells transformed by wild type RSV-BH. We have identified several metabolic changes induced during transformation which require new RNA synthesis and protein synthesis including increased glucose consumption (caused by an increased availability of glucose carrier sites), increased lactic acid production (glycolysis), increased hyaluronic acid synthesis, increased protease (plasminogen activator), and increased sodium-ion uptake. Identified decreases in fibronectin (LETS protein), alkaline phosphatase, and adenosine deaminase in transformed cells were shown to be unnecessary for the development of other phenotypic properties of RSV-BH transformation.

Transformed cells have a higher glucose-uptake capacity than nontransformed cells as indicated above. Induction of increased uptake capacity in the temperature-sensitive system was partially inhibited by Actinomycin D, indicating a requirement for new RNA synthesis and synthesis of new carrier sites. Nonetheless, some increase occurs independently of RNA synthesis, and apparently is due to an increased utilization of ATP resulting from the activation of viral transforming protein.

In nontransformed cells, glucose deprivation, or treatment with substances which activate cytoplasmic or mitochondrial ATPases, induced an increased capacity for glucose uptake. The increase occurred independently of new RNA or protein synthesis, suggesting activation of existing cryptic molecules by the lowering of ATP levels. RSV-BH cells responded neither to glucose deprivation nor ATPase activators by increasing glucose uptake capacity, suggesting that ATP levels were already low. Measurement of ATP concentrations in glucose-deprived and ATPase activated nontransformed cells confirmed the lower values anticipated from these treatments, and RSV-BH cells typically contained lower amounts of ATP than nontransformed cells.

The lower amounts of ATP in RSV-BH cells, were in conflict with an apparent increased synthesis of ATP indicated by increased glucose consumption and glycolysis, with no alteration in respiratory capacity. The results suggested an increased utilization of ATP by transformed cells compared to nontransformed cells. Short-term inhibition of protein or RNA synthesis did not significantly affect ATP generation from glycolysis or respiration, demonstrating that macromolecular synthesis used only a small portion of the available ATP. These results suggest that the virus-coded transformation protein induces directly an increased utilization of ATP, either through its recognized protein kinase activity or in some other way.

Activations of Na-K-ATPase had been shown (see above) to activate glucose-uptake carriers, and the possibility that this enzyme was affected during transformation, and was responsible for the increased ATP utilization, was examined. Rates of RB-ion (a substitute for K-ion) uptake were indistinguishable in transformed and non-transformed cells, no differences in temperature sensitivity of Na-K-ATPase activity were observed, and ouabain binding capacity of transformed cells was similar to nontransformed cells.

The possibility that the Na-K-ATPase of transformed cells had a decreased efficiency, i.e., required more ATP to pump Na and K ions, was examined. By measuring changes in oxygen consumption due to K-ion uptake, the stoichiometric amounts of ATP necessary to transport K ion were determined. Three strains of tumorigenic cells (chick embryo RSV-BH, mouse Ehrlich ascites, hamster RSV-BH) were shown to be as efficient as normal cells with respect to Na-K-ATPase activity.

In another study, the relevance of glycosylation to transformation was examined using tunicamycin and 2-deoxyglucose, two inhibitors of glycosylation. Transformation by two strains of RSV developed irrespective of protein glycosylation, although infectious virus production was inhibited by the antimetabolites. By using similar treatments on cells chronically producing avian leukemia virus, infectious virus production was inhibited, and the interference with infection by related viruses was abrogated. Similar results were found when experiments were extended to murine leukemia viruses.

We have initiated experiments to identify the proteins coded by avian myeloblastosis virus using immunoselective techniques. Preliminary results have revealed an immunoreactive polypeptide in virus-induced myeloblasts, which is absent in embryonic cells, and which has a molecular size consistent with the available coding capacity of the transforming region of the viral genome.

Significance to Biomedical Research and the Program of the Institute:

The resolution of the function of a protein responsible for malignant transformation would be a major advance in our understanding of the nature of cancer cells. Recognition of a primary physiological imposition on the cell which converts that cell to a malignant form, and the identification of characteristic metabolic changes induced during the development of transformation, would allow a description of the entire sequence of metabolic events which culminate in malignancy. Such studies could lead to a determination of metabolic events necessary to the maintenance of the malignant state, but not to normal metabolism, and the possibility of therapeutic intervention in such metabolism.

Proposed Course:

To determine the primary function of the RSV-BH protein responsible for malignancy, and to describe the sequence of metabolic changes culminating in the phenotype characteristic of RSV-BH transformed cells. The immediate determination of the source of increased energy utilization by RBV-BH cells. Identification of the protein coded by avian myeloblastosis virus, and elucidation of its role in myeloblastosis.

Publications:

Bader, J.P., Brown, N.R., and Ray, D.A.: Increased glucose uptake capacity of Rous-transformed cells and the relevance of deprivation derepression. Cancer Res. 41: 1702-1709, 1981.

Bader, J.P., Okazaki, T., and Brown, N.R.: Sodium and rubidium uptake in cells transformed by Rous sarcoma virus. J. Cell. Phys. 106: 235-243, 1981.

Balaban, R., and Bader, J.P.: The efficiency of Na-K-ATPase in tumorigenic cells. Nature (in press).

Rein, A., Schultz, A.M., Bader, J.P., and Bassin, R.H.: Inhibitors of glycosylation reverse retroviral interference. Virology 119: 185-192, 1982.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Temperature-Sensitive Mutants of
Moloney Murine Sarcoma VirusNAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Thomas G. Wood	Senior Staff Fellow	LMO	NCI
	Marianne K. Oskarsson	Chemist	LMO	NCI

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TOTAL MANYEARS:

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied the genetics and biochemistry of ts-110, a Moloney murine sarcoma virus (MSV) temperature-sensitive (ts) mutant, which transforms cells at 34° but not at 39°. This mutant represents a deletion of at least 0.3 x 10⁶ daltons of genetic information when compared to its wild-type parent (MSV 349).

Project Description

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses and the function of specific gene products of MSV in this process.

Methods Employed:

We utilized focus and agar colony formation by MSV, as well as DNA transfection using cloned MSV sequences and whole cell DNA from transformed cells. We also used techniques of recombinant DNA cloning of integrated MSV sequences from transformed cells, restriction endonuclease mapping, and heteroduplex analysis of cloned sequences.

Major Findings:

Cells transformed by the wild-type parent of ts-110 (MSV 349) contained two size classes of mos-specific RNA sequence (6.9 and 5.8 Kb). Previous studies have shown that ts-110 transformed cells contain two size classes of mos hybridizing polyadenylated RNA (5.0 and 4.5 Kb). The two species were shown to be present at both permissive and non-permissive temperatures. However, a spontaneous revertant to ts-110 was found to contain only a 5.0 Kb mos-specific RNA.

Significance to Biomedical Research and the Program of the Institute:

Conditional mutants have been extremely useful in eliciting biochemical pathways in a number of cells and viral systems. MSV ts-110 represents one of the few stable, conditionally defective variants of a mammalian sarcoma virus. Analysis of this mutant at a molecular level should help to elucidate the mechanism of transformation by Moloney MSV.

Proposed Course:

This project has represented a rather low priority over the past year. It is anticipated that a major effort will be made to clone the integrated provirus from ts-110 MSV-transformed cells. This clone will be used to characterize the nature of two mos-specific RNA species found in ts-110 transformed cells. In addition, DNA transfections will be performed in order to generate temperature-sensitive transformed mouse cells. Antisera in preparation in this laboratory against synthetic mos-specific peptides will be used when they become available to determine the nature of mos gene product expression under permissive and non-permissive conditions.

Publications:

Horn, J. P., Wood, T. G., Murphy, Jr., E. C., Blair, D. G., and Arlinghaus, R. B. A selective temperature-sensitive defect in viral RNA production in cells infected with a ts-mutant of murine sarcoma virus. Cell 25: 37-46 (1981).

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Analysis of the Genome Structure and Organization of Avian Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHERS:	Takis Papas	Head, Carcinogenesis Regulation Section	LMO, NCI
	Nancy Kan	Visiting Fellow	LMO, NCI
	Kenneth Samuel	Visiting Fellow	LMO, NCI
	Dennis Watson	Staff Fellow	LMO, NCI

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1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Avian myelocytomatosis virus (MC29), a defective acute leukemia virus, has a broad oncogenic spectrum in vivo and transforms fibroblasts and hematopoietic target cells in vitro. We have used recombinant DNA technology to isolate and characterize the sequences which are essential in the transformation process. Integrated MC29 proviral DNA was isolated from a library of recombinant phage containing DNA from the MC29-transformed nonproducer quail cell line Q5. The cloned DNA was analyzed by Southern blotting of restriction endonuclease digests and by electron microscopic visualization of R-loops formed between the cloned DNA and MC29 or helper virus RNA. It was found that the 9.2 kb cloned DNA insert contains approximately 4 kb of viral sequences and 5.2 kb of quail cellular sequences. The viral sequences contain all of the MC29-specific sequences and 5' helper related sequences as well as part of the envelope region. The size of the cloned EcoRI fragment is the same as that of the major band in EcoRI-cleaved Q5 DNA that hybridizes to viral sequences. Transfection of the cloned DNA into NIH 3T3 cells, revealed that the MC29-specific sequences are functional, in that they induce foci of transformed cells.

Project DescriptionObjectives:

The scope of this investigation is to delineate the relationship between virus gene expression and the conversion of cells from a normal to a malignant state. A goal of these studies is to determine the molecular anatomy of known tumor viruses as well as to investigate: 1) the process by which viral oncogenes, as well as their cellular homologs, induce the activation of the metabolic processes and participate in malignant transformation, (2) at the molecular level the mechanism by which oncogenes act in concert with cellular factors to induce oncogenesis, and (3) the effect of functionally modified oncogenes transferred to specific target cells in an effort to analyze the function of their normal counterparts. The techniques of molecular cloning, DNA sequence analysis, and site specific mutagenesis will be used to implicate specific nucleotides in the transformation process.

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. Preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of virus-related sequences on these filters to probes prepared by nick translation of fragments of cloned viral DNA.
3. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging.
4. Isolation of phage from the libraries containing virus-related sequences by hybridization of labelled probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure.
5. Transfection of NIH 3T3 cells by exposing them to fragments of cloned viral DNA that had been co-precipitated with calcium phosphate.
6. R-loop and heteroduplex analysis of cloned DNA with viral RNA and cloned proviral DNA from other viruses.
7. Computer analysis of DNA sequences. We recently have installed a DEC MINC computer equipped with a high speed communications interface. This allows us to use existing DNA sequence analysis programs such as that of Korn and Queen and the SEQ program of the Stanford MOLGEN group and also to develop complementary programs for our machine. In addition, a program has been written for a Tektronix computer to produce dot matrix homology plots of the form described by Maizel and Lenk. We thus have the capacity to collect DNA sequences as computer disk files and to analyze them for restriction sites, control elements, self complementary regions, and for homology with other sequences.

8. DNA sequence 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. A program has been written so that a computer with a graphics tablet can automatically read gels. The sequence from each gel is sent to the NIH DEC-10 where it can be concatenated with other sequences by the NASSD program of R. Feldmann (DCRT, NIH).

Major Findings:

1. Construction of plasmids for expression of the *myc* protein in Escherichia coli. Several plasmids containing the 2.9 kb fragment of the *myc* gene have been constructed. These include insertion of *myc* sequences into the λ N gene of pKC30 and next to the λ cro initiation codon of pCQ2. Experiments are in progress for the identification of the *myc* protein in these bacteria. An intermediate in one of these constructions contained a 10 base pair synthetic oligonucleotide containing the sequence recognized by BamHI. This small insertion into the λ N gene has been used by the laboratory of Dr. Max Gottesman to study the role of the N gene in the regulation of transcription in E. coli.
2. The sequence determination of the AMV and MC29 LTRs demonstrates that these LTRs share much in common with the LTRs of RSV, RAV-2 and the endogenous ev-1 locus. Each of the avian acute virus LTRs contain R and U5 regions that are very similar. However, the U3 regions are more highly diverged. The extent of homology between each pair of these viruses has been determined by the computer program of Queen and Korn. This information has been used to compute divergence distances and an evolutionary tree. While the MC29 U3 region is very closely related to that of RAV-2 and RSV, the AMV U3 sequence shares very little homology with any other U3 region. However, the AMV U3 region is somewhat more closely related to that of ev-1 than the U3 sequences of the RSV-RAV-MC29 group.

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. Expression of viral proteins in Escherichia coli. The *myc* sequences from the cloned MC29 provirus will be placed in E. coli expression plasmids in order to produce the *myc* transforming protein in the bacterium. The transforming protein will be isolated and used to produce *myc*-specific antibodies. These antibodies will be used to identify and isolate the *myc* transforming protein from normal and malignant mammalian cells. Plasmids to be used are pKC30 (λ pL promoter + λ N gene), pCQ2 (λ pR promoter + λ cro initiation region), pvt75 (pBR322 β -lactamase promoter + synthetic ribosome binding site) as well as an inducible plasmid containing the λ pL promoter and part of the cII gene. This plasmid is presently under construction.

2. Molecular cloning of MHII DNA. MHII is an avian acute leukemia virus containing sequences related to the MC29 transforming gene. However, its neoplastic disease spectrum is different. We plan to clone MHII DNA from a λ library derived from nonproducer cells and to sequence this DNA. Comparison of the MHII with that of MC29 should provide insight into the relationship between DNA sequence and malignant disease at the nucleotide level.

3. Cloning of the MC29 *myc* sequences into transformation defective Schmidt-Ruppin strain of Rous sarcoma virus. The MC29 *myc* sequences will be inserted into a SRtdRSV clone that has been provided by Dr. A. Skalka. If the *myc* sequences are expressed, a non-defective virus with MC29's transformation ability could be produced. Thus the transformation of chicken or quail cells by an acute leukemia virus could be studied in the absence of helper virus. The cloned non-defective virus should also make it easier to localize specific regions on the genome responsible for transformation since it could be manipulated by site specific mutagenesis techniques.

4. Analysis of human tissue for variations and rearrangements of the *myc* gene. Southern blot hybridization will be used to screen various human DNAs to probe the structure of their endogenous *myc* sequences. Nick-translated MC29 *myc* sequences will be used as a probe. For each DNA at least three restriction enzymes will be used. The DNAs surveyed will come from a wide variety of sources including normal tissues and leukemic tissues.

5. The transforming regions of MC29 will be defined by further transfection experiments and by characterization of viral products within cells transformed by cloned viral DNA. NIH 3T3 cells will be transfected with specific restriction fragments. The overlaps between those fragments that form foci will define the necessary region. It will be of particular interest to know whether "gag" sequences are needed for transformation.

The nature of the viral DNA sequences within the transformed cells will be determined by the Southern procedure. The presence of viral sequences in bands not found in untransformed cells will confirm that transformation was mediated by the uptake of viral sequences. It will be of interest to determine if the transformed cells contain sequences related to the LTR and "gag" sequences in the cloned DNA.

Expression of viral sequences in the in vitro transformed cells will be studied by the Northern procedure. The cell RNA is electrophoresed in a denaturing gel and immobilized on a filter. Viral messages can be detected by hybridization to labeled cloned viral DNA. The proteins in these cells that contain viral sequences will be characterized by labeling the cells with ³⁵S-methionine and precipitating the viral proteins with antisera to AMV or RSV.

Publications:

Eva, A., Robbins, K. C., Anderson, P. R., Srinivasan, A., Tronick, S. R., Reddy, E. P., Ellmore, N. W., Galen, A. T., Lautenberger, J. A., Papas, T. S., Westin, E. H., Wong-Stall, F., Gallo, R. C., and Aaronson, S. A.: Cellular genes analogous to retroviral *onc* genes are transcribed in human tumor cells. Nature, 295:116-119, 1982.

Eva, A., Robbins, K. C., Andersen, P. R., Srinivasan, A., Tronick, S. R., Reddy, E. P., Ellmore, N. M., Galen, A. T., Lautenberger, J. A., Papas, T. S., Westin, E. W., Wong-Stall, F., Gallo, R. C., and Aaronson, S. A.: Transcription of retrovirus *onc* gene analogues in human solid tumor cells. In Yohn, D. S., and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research Amsterdam, Elsevier/North Holland, 1981, pp. 381-384.

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Tschlis, P. N., Spyropoulos, D. D., Pry, W. T., Rushlow, K. E., and Papas, T. S.: The transforming sequences of Avian myelocytomatosis (MC29). J. Supermolecular Structure 16: 193-207, 1981.

Lautenberger, J. A., Schulz, R. A., Garon, C. F., Tschlis, 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. (In Press).

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Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Samuel, K. P., Baluda, M. A., and Reddy, E. P.: Nucleotide sequence of integrated avian myeloblastosis virus (AMV) long terminal repeat (LTR) and their host and viral junctions: Structural similarities to transposable elements. In Chandra, P. (Ed.): Biochemical and Biological Markers in Neoplastic Transformation. New York, Plenum Press, 1982.

Papas, R. S., Schulz, R. A., Lautenberger, J. A., Pry, T. W., Samuel, K. P., and Chirikjian, J. G.: The nucleotide sequence of the large terminal redundancy (LTR) of avian myelocytomatosis virus (MC29). In Yohn, D. S., and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research. Amsterdam, Elsevier/North Holland, 1981, pp. 339-342.

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D. K., Baluda, M. A., Reddy, E. P.: Complete nucleotide sequence of the transforming genes of avian myeloblastosis virus (AMV). J. Cell. Biochem., 1982. (In Press).

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Watson, D. K., Beluda, 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. (In Press).

Rushlow, K. E., Lautenberger, J. A., Baluda, M. A., Perbal B., Reddy, E. P., Chirikjian, J. G., and Papas, T. S.: Nucleotide sequence of the transforming gene of avian myeloblastosis virus (AMV). Science, 1982 (In Press).

Rushlow, K. E., Lautenberger, J. A., Reddy, E. P., Baluda, M. A., Chirikjian, J. G., and Papas, T.S.: Nucleotide sequence analysis of the long terminal repeat (LTR) of Avian Myeloblastosis Virus (AM) and adjacent host sequences. J. Virol. 42: 840-846, 1982.

Westin, E. H., Wong-Staal, F., Gelmann, E. P., Dalla Favera, R., Papas, T. S., Lautenberger, J. A., Eva, A., Reddy, E. P., Tronick, S. R., Aaronson, S. A., and Gallo, R. C.: Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. USA 79: 2490-2494, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05156-03 LMO

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Expression of MSV Genetic Information

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomas Gordon Wood	Senior Staff Fellow	LMO, NCI
OTHER:	G. F. Vande Woude	Chief, LMO	LMO, NCI
	M. L. McGeady	Staff Fellow	LMO, NCI
	D. G. Blair	Expert	LMO, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION

Tumor Biochemistry Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have analyzed the polyadenylated RNA expressed in cells transformed by MSV. A single LTR introduced in either a 5' or 3' relative position to the mos sequence is sufficient for enhancement or activation of the transforming potential of mos. Southern analysis of DNA's containing the integrated mos sequences DNAs confirms that tandem integrations or rearrangement of the mos and LTR sequences is not required for expression of mos or cell transformation. The enhancement properties of the proviral LTR responsible for increasing the transforming efficiency of mos have been shown to reside in a region containing the 72 base repeat sequence present the unique 3' region of the LTR. Activation of the transforming potential of c-mos is influenced by a cis-acting sequence within the 5' normal mouse DNA sequence flanking c-mos. Removal of this region from the normal mouse sequences preceding c-mos permit the upstream activation of the transforming potential of c-mos by an LTR located downstream from the oncogenic sequence.

Project Description

Objectives:

The overall objective of the Laboratory of Molecular Oncology is to identify and characterize sequences responsible for neoplastic transformation. A specific goal of this laboratory is to assess the role of the retroviral long terminal repeat (LTR) in the expression of oncogenic sequences. Previously, we have shown that the transforming activity of the Moloney sarcoma Virus (MSV) proviral v-mos sequence is enhanced when LTR sequences are present in the transfected DNA. The transforming potential of c-mos, the normal mouse cellular homolog to v-mos, can be activated by introducing an LTR in either a 5' or 3' position relative to c-mos. Cells transformed by transfection of these DNAs express RNA transcripts containing mos sequences. One major objective has been to examine the arrangement of transfected DNA sequences present as integrated DNAs in transformed cells. Our specific interest has been to determine the structural relationship of mos and LTR sequences within the integrated DNAs. A second objective has been to assess the essential elements of the LTR required for the enhancement of the transforming activity of mos. Finally, we have initiated a series of experiments to examine the normal mouse cellular DNA sequences flanking c-mos. Our interest in this instance is to find a plausible explanation for the absence of transforming activity of the c-mos region as an autonomous DNA sequence.

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:

In each case where we have analyzed cells transformed by transfection of recombinant DNAs containing a mos sequence, we have demonstrated the presence of mos containing RNA transcripts in the polyadenylated RNA as well as mos containing DNA sequences in cellular DNA. Results from DNA transfection assays suggest a model where a single LTR can successfully enhance or activate the transforming potential of mos from either a 5' or 3' position relative to the mos sequence. Analysis of DNA from transformed cells by restriction endonuclease digestion and hybridization with various probes specific for sequences present in the transfected DNA demonstrate that a single LTR is sufficient for enhancement activation of mos transforming activity. Tandem integrations of mos-LTR DNA sequences, recreating a proviral-like structure, are not necessary for mos expression and cell transformation. The relative positions of mos and LTR

sequences within the transfected DNA are conserved in the integrated cellular DNA and have not undergone DNA rearrangement in the activation or expression of mos.

The transforming potential of mos can be enhanced or activated by introducing a proviral LTR 5' to the mos sequence, consistent with a downstream promotion model for the expression of oncogenic sequences. The LTR in this instance provides transcriptional control elements for the initiation of RNA transcription resulting in the expression of sequences downstream. Insertion of an LTR in a 3' position relative to mos is equally as efficient as a 5' LTR for enhancement of the transforming capability of mos. To evaluate the role of the 3' LTR in upstream enhancement or activation of mos, recombinant DNA clones containing v-mos and various sequences derived from the LTR, were tested for their transforming efficiency. These experiments show that the essential element within the LTR responsible for the enhancing properties of the LTR is a region containing the 72 base duplication. LTR sequences representing RNA initiation and polyadenylation consensus signals were not required for enhancement of v-mos transforming activity.

We have reported the absence of a detectable transforming activity for c-mos in DNA transfection assays. Transfection of cloned recombinant DNA containing an LTR 3' to c-mos exhibits a transforming efficiency significantly below the activity observed with similar cloned DNA constructs containing v-mos. In those instances when the transforming potential of c-mos was activated by a 3' LTR, the mos containing RNA transcribed in these cells showed limited hybridization to probes made from the normal mouse sequences preceding c-mos. Southern analysis of the DNA from these transformed cells revealed an integrated mos containing DNA devoid of detectible normal mouse sequences that were present in the transfected DNA suggesting that the low frequency transformation was associated with the loss of 5' normal mouse sequences. These results prompted a direct test of the effect that the 5' mouse sequences flanking c-mos have on activation of the transforming potential of c-mos. Recombinant DNAs were constructed containing an LTR 3' to c-mos with varying amounts of 5' normal mouse sequence and tested for their transforming efficiency in DNA transfection assays. The results from these experiments implicate a cis-acting sequence 1.5 to 2.0 Kb upstream from the 5' terminus of c-mos which functions through as unknown mechanism in suppressing the expression of c-mos. Recombinant DNAs which contained this sequence demonstrated little or no transforming activity. Cloned DNAs lacking this region of the normal mouse sequence preceding c-mos are capable of activation by a downstream LTR.

Significance to Biomedical Research and the Program of the Institute:

At least 16 different oncogenes have been identified and shown to be encoded in the genomes of various retroviruses. Most of these sequences have been shown to have a genetic counterpart within the genomes of normal cells. We have endeavored to examine one such oncogenic sequence, mos, which is responsible for the transforming activity of Moloney sarcoma virus. Using a combined biological and biochemical approach, we have shown that the transforming potential of the viral encoded sequence, v-mos, and its cellular homolog, c-mos, can be activated or enhanced by a single proviral LTR. Activation of c-mos is independent of the relative position of the LTR. This result implies that a cellular gene encoded

in the DNA of a normal cell is capable of inducing neoplastic transformation without changing the primary structure of the DNA sequence. Potentially, oncogenes may be 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 the oncogene results in neoplastic transformation. Alternatively, transposition of genetic control elements offers another pathway to oncogene activation.

Previously, we have shown that the transforming potential of mos can be activated by introducing an LTR in either a 5' or 3' relative position to the oncogenic sequence. We have now demonstrated that the essential element responsible for the enhancement properties of the LTR is a region containing the 72 base duplicated sequence. The mechanism through which this enhancement is effected is as yet unknown, however, the fact remains that a complete proviral LTR is not essential for oncogene expression and cell transformation.

The finding that activation of the transforming potential of c-mos is influenced by a cis-acting sequence present in the 5' mouse DNA flanking c-mos is an exciting observation. This result may explain the absence of detectable c-mos expression in normal cells and the inability of c-mos to induce cell transformation in DNA transfection assays. It may prove to be equally as important to gain an understanding of how a cell suppresses the expression of a gene as it is to explain the activation of one.

Proposed Course:

A continued analysis of the LTR's role in gene expression remains one of our primary research interests. Defining the precise limits of the LTR's enhancer sequence and determining the mechanism through which it influences gene expression represents immediate research goals. We plan to extend our studies of the LTR to an analysis of the effects of enhanced sequences on downstream promotion. Furthermore, by reconstructing proviral-like structures containing one complete LTR and a partial LTR sequence at the opposing position, we intend to assess the co-ordinate effects of LTRs on the expression of an intervening DNA sequence.

A detailed analysis of the cis-acting sequence present in the 5' normal mouse DNA flanking c-mos represents a major objective. Further characterization of the ability of this sequence to influence gene expression is required. In particular, experiments defining the precise element involved in this activity and the limitations of its field of influence are necessary. A collaborative effort to determine the primary DNA sequence of this region is in progress.

Publications:

None.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Transforming Genes of Avian Acute Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth Samuel	Visiting Fellow	LMO, NCI
OTHERS:	Takis Papas	Head, Carcinogenesis Regulation Section	LMO, NCI
	James Lautenberger	Expert	LMO, NCI
	Dennis Watson	Staff Fellow	LMO, NCI
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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Specific initiation, of transcription of AMV and MC29 cloned DNAs by RNA polymerase II (α -Amanitin sensitive) in a eucaryotic cell-free system has been obtained. The site of initiation of transcription is located within the U₃ region of the LTR sequences of both AMV and MC29. These results indicate that the information necessary for retroviral transcription is located within the viral genomes. In addition, initiation of transcription from a corresponding canonical Goldberg-Hogness sequence in the myb gene of AMV has been obtained, suggesting that this conserved sequence element may serve as an independent promoter. Current studies are designed to elucidate the role of 5'-extragenic regions in the expression of retroviruses. Deletion mutants harboring mutated upstream sequences are being constructed using the Bal-31 enzyme system and recombinant plasmid technology.

Project Description

Objectives:

The scope of this investigation is (a) to delineate the relationship between virus gene expression and conversion of cells from the normal to malignant state; (b) to study the molecular anatomy of known tumor viruses; (c) to investigate the process by which viral oncogenes, as well as their cellular homologs, induce the activation of the metabolic processes of tumor viruses and their participation in malignant transformation; (d) to delineate at the molecular level the mechanism by which oncogenes act in concert with cellular factors to induce oncogenesis; and (e) to introduce functionally modified oncogenes to specific target cells in an effort to analyze the function of their normal counterparts. The technique of molecular cloning, DNA sequence analysis, and site specific mutagenesis will be used to implicate specific nucleotides in the transformation process.

Methods Employed:

(A) Construction of recombinant phage libraries by ligation of restriction fragments of eucaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging. Isolation of phage of libraries containing virus-related sequences by hybridization of specific DNA probe to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the Benton-Davis procedure.

(B) Construction of recombinant plasmids. Double-stranded cDNA of AMV genome was prepared. It was converted to perfect duplex molecules with blunt ends by digestion with single-strand nuclease S_1 . It was then incubated with *E. coli* DNA polymerase I. The product of the S_1 -DNA polymerase reaction was ligated to BamHI linkers with T4 DNA ligase. pBR322 was digested with BamHI, treated with alkaline phosphatase. It was then ligated to double-stranded cDNA linked to BamHI linkers.

(C) Construction of MC29 subclone. A partial Xho-I restriction enzyme digestion of the MC29 recombinant plasmid was performed. A 4.8 kb (XhoI-EcoRI) fragment of MC29 DNA is repaired with DNA Polymerase I. EcoRI linkers are then added, the product digested with EcoRI, and then ligated to alkaline phosphatase treated pBR325 linearized with EcoRI. *E. coli* C600 is transformed with circularized plasmid and colonies picked and screened for MC29.

(D) Transformation and identification of recombinant clones. Construction of chimeric plasmids and the transformation of *E. coli* X1776 by these plasmids was performed in a P2 physical containment laboratory. X1776 was transformed by a transfection procedure. Transformed colonies containing AMV sequences were identified by colony hybridization. The colonies were screened with ^{32}P -labeled AMV cDNA. Strongly hybridized colonies were selected and replated; single colonies were picked and grown.

Major Findings:

The specific initiation of transcription of avian myeloblastosis virus (AMV) and myelocytomatosis virus 29 (MC29) by RNA polymerase II (sensitive to low levels

of α -aminitin) was obtained in a eucaryotic cell-free system using cloned AMV and MC29 DNAs as templates. The site of initiation of transcription is located within the U₃ region of the long terminal repeat (LTR) sequence. This result indicates that the information necessary for AMV and MC29 virus transcription lies within the viral genome. In addition, the cloned transforming gene of AMV also supports the specific initiation of transcription in this cell-free system. Since this gene has been shown (by DNA sequence analysis) to contain a canonical Goldberg-Hogness or ATA box, it could serve as a putative independent promoter for expression of the AMV myb gene.

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. To subclone the entire MC29 DNA in pBR322 without its 5'-flanking quail cellular sequence and use of this subcloned DNA in the construction of deletion mutants.
2. To characterize the deletion mutants of AMV and MC29 using in vitro transcription and expression of viral RNA in vivo using DNA transfection assay.
3. To devise a coupled in vitro transcription/translation system in an effort to synthesize the transformation protein of both AMV and MC29.
4. To delimit the specific region within the transforming gene sequence of AMV and MC29 that may be responsible for the specific spectrum of tumorigenicity directed by these viruses.

Publications:

Papas, R. S., Schulz, R. A., Lüttenberger, J. A., Pry, T. W., Samuel, K. P., and Chirikjian, J. G.: The nucleotide sequence of the large terminal redundancy (LTR) of avian myelocytomatosis virus (MC29). In Yohn, D. S., and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research. Amsterdam, Elsevier/North Holland, 1981, pp. 339-342.

Papas, T. S., Rushlow, K. E., Lautenberger, J. A., Samuel, K. P., Baluda, M. A., and Reddy, E. P.: Nucleotide sequence of integrated avian myeloblastosis virus (AMV) long terminal repeat (LTR) and their host and viral junctions: Structural similarities to transposable elements. In Chandra, P. (Ed.): Biochemical and Biological Markers in Neoplastic Transformation. New York, Plenum Press, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05221-02 LMO
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) DNA Recombination in Prokaryotic and Eukaryotic Systems		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Susan E. Bear Staff Fellow LMO, NCI Other: Donald Court Chief, Molecular Genetics Section LMO, NCI Anamaris M. Colberg-Poley Postdoctoral Fellow NIADDK		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Molecular Oncology		
SECTION Molecular Genetics Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.4	PROFESSIONAL: 1.4	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The interaction of a <u>phage encoded topoisomerase I</u> with the specific sites for DNA recombination is <u>studied using sites cloned into a plasmid vector</u> . An <u>ELISA assay</u> is being developed to detect <u>nanogram quantities of the bacteriophage lambda</u> <u>coded int protein</u> . This development will enable the <u>genetic expression of this regulatory protein</u> to be monitored in a variety of <u>phage</u> and <u>host mutants</u> .		

PROJECT DESCRIPTION

Objectives

The objectives of this project are in threefold: 1) to examine the interaction of a bacteriophage encoded DNA topoisomerase I with a specific DNA substrate. The product of the lambda int gene, this topoisomerase I is required for the site specific DNA recombination event that occurs when λ integrates into the host E. coli chromosome; 2) to examine the control of lambda int gene expression in different phage and host mutants, and 3) to identify control regions in the genome of Herpes simplex type 1 (HSV-1) present in DNA of defective HSV-1 particles. This DNA is isolated from virion capsids as a repeated sequence and most probably contains active replications and packaging sites. It has been classified as a member of the major class group I dDNA (Denniston, K.J. et al., Gene 15: 365-378, 1981). In addition, it is known to carry two promoters for immediate early messenger RNA synthesis (Watson, R.J. et al., J. Virol. 37: 431-444, 1981). Work in this laboratory has also suggested the presence in a 9.5 kb DNA fragment of the terminal "a" sequence which may be active in HSV recombination (Z01 CP 05102-03 LMV).

Methods Employed

The specific sites for recombination: attP for the phage and attB for the bacterial chromosome have been cloned in inverted orientation in the plasmid vector pBR322. In addition, the junction sequences (hybrid attachment sites attL and attR) between the integrated prophage and the E. coli chromosomal DNA have also been cloned in inverted orientation in pBR322. These cloned sites act as substrates for the recombination event in vivo. (integration = P x B; excision = L x R) The phage gene products required for integration (int protein) and excision (int and xis proteins) are provided by a phage infection of the cells carrying either plasmid. The recombination event results in DNA rearrangement and can be demonstrated by restriction endonuclease digestion and examination of the DNA fragments after gel electrophoresis.

The experimental design is based on an observation by Kaiser and Masuda (J. Mol. Biol. 47: 557-564, 1970), who showed an inhibition of the excision reaction (L x R) when certain phage carrying int mutations were used to cure a heteroimmune lysogen by superinfection. Wild type int protein was provided by the prophage. This inhibition was termed negative complementation. This inhibition, not observed when the infecting phage carried deletions or amber mutations in the int gene, may co-occur with the production of a protein that, although it cannot promote recombination alone, can act to inhibit the wild type int from binding a cleaving of the DNA.

A mixed phage infection of cells (carrying either the P-B or the L-R substrates) with λ wild type and each of several phage with mutations that have been genetically mapped within the λ int gene (Enquist, L. W. and Weisberg, R. A., J. Mol. Biol. 111: 97-120, 1977) will be used to study the effect on recombination of interaction of mutant protein with the wild-type protein.

Proposed models for the inhibition by mutant protein of the recombination event involves 1) competition of the two proteins for DNA binding sites or 2)

inhibition of the wild type *int* binding to and/or cleavage of the DNA. One prediction of either of these models is that in a certain percentage of the reactions, the recombination event does not go to completion. The evidence that *int* does not turn over in the reaction leads to the proposal that suspected intermediates of recombination, *chi*-forms or Holliday structures (Nash, H. W., Ann. Rev. Genet. Vol. 15) may be frozen in this configuration and be detectable. These can be observed as new bands of plasmid DNA after enzyme digestion and agarose gel electrophoresis.

II. To detect nanogram quantities of *int* protein in *E. coli* cell extracts, the enzyme-linked-immunospecific-assay (ELISA) was chosen. *Int* protein was purified and inoculated into a 6-week old female New Zealand rabbit. The ELISA assay for *int* protein was developed and rabbit antibodies were detected. The parameters of a competition ELISA assay are being determined.

III. A 9.5 kb EcoRI dDNA fragment (major class I), and the phage vector (λ gtWES-B) carrying the 9.5 kb EcoRI fragment (λ WES::12-7) have been described previously (Z01 CP 04882-06 LMV and Z01 CP 05102-03 LMV).

To test for an origin of replication and a possible packaging site, this hybrid phage is circularized and transfected in VERO cells. To provide the necessary helper functions, the cells are then infected with HSV-1 (Patton). When HSV plaques are evident (48 hrs), both total DNA and DNA from Herpes virus capsid are isolated. Supernatants are passaged, and after fifth passage the virion DNA is isolated again. These DNA are restricted, electrophoresed in agarose. The gels are dehydrated and hybridized to a 32 P-labeled λ DNA. A putative HSV origin of replication in the 9.5 kb EcoRI insert would allow for amplification of these and the adjoining phage sequences. If replication proceeds by a rolling circle mechanism, it is proposed that this would generate long concatemers of this molecule. This may be seen as an "amplification" of phage sequences, detectable over any background hybridization in control groups. Control groups include the λ vector alone and a hybrid vector containing a separate HSV insert. In addition, a packaging site in the insert may promote the incorporation of these λ hybrid concatemers (3 λ equivalents are the size of 1 HSV genome) into virus capsids.

Major Findings:

I. Using the attachment site substrates cloned into the plasmid vectors, intermediate structures that represent *chi* forms have been generated in vitro, and their mobility defined in agarose gel electrophoresis.

The mixed infection studies have generated two pieces of information regarding site specific recombination studied in this way: 1) the presence of *xis* gene may be inhibitory to P x P and P x B recombination, and 2) only two of the seven mutants selected from the Kaiser and Masuda study appear to inhibit a P x B recombination event. The remainder of the mutants lose their ability to negatively complement when an *xis* mutation is crossed into the phage.

II. A competition ELISA assay is being perfected to determining the level of *int* protein produced among different phage mutants.

III. Analysis of capsid DNA 48 hrs after HSV infection reveals λ sequences in all groups, excepting that group representing a simple HSV-1 infection. At the fifth

passage, DNA isolated from virions shows λ sequences only in the group transfected with the λ hybrid carrying the 9.5 kb EcoRI insert.

Significance to Biomedical Research and the Program of the Institute:

I. DNA recombination reactions most likely are similar throughout eukaryotic and prokaryotic life forms. Specifically, 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 viruses. In the RNA tumor viruses, this integration event is a key element in the disruption of the cellular physiology, that leads to the generation of a tumor. An understanding of the process of DNA recombination in prokaryotes provides a basis for work using eukaryotic viruses, many of which are relevant to diseases in humans.

II. This project investigates the control of the expression of a bacteriophage gene, the product of which is required for specific DNA recombination events. These DNA recombination events are most probably similar to those that occur in eukaryotes. Application of the results of this research may be to the study of the control of eukaryotic gene expression in general, as well as to the study of factors required for DNA rearrangements in eukaryotes.

III. The results indicate that an origin of replication in a packaging site may be present in the DNA that is represented as a concatemer in defective HSV particles. The detection of λ sequences in virion DNA in λ control groups 48 hr after infection raises the possibility that the packaging of DNA into Herpes capsids may occur in a head-full fashion, rather than by a site specific mechanism. After 5 passages of the virus, DNA with no biological role in the Herpes virus life cycle may be eliminated (i.e., λ DNA from control groups), while DNA with active sequences (i.e. containing an origin of replication) is replicated, amplified, and represented at the fifth passage. The localization of the origin of replication in Herpes virus, and studies of the control of viral replication and packaging, will aid in the understanding of the life cycle of this medically relevant virus. Studies of the control of the initiation of viral replication may be relevant to the problem of latency with this virus.

Proposed Course:

I. The results will be confirmed and the experiments extended to the L x R recombination event. A selected group of phage mutants will be used to study recombination intermediates.

II. Analyze genetically well-defined phage and host mutants for levels of int protein produced. The production of monoclonal antibodies, and the use of the ELISA assay to study the functional domains of the int protein are possible.

III. These results can lead to the development of a very useful eukaryotic viral vector system as well as reveal the mechanism of HSV replication and packaging mechanisms. We will determine the physical structure of the replicated HSV- λ recombinants.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05238-01 LMO

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structural Analysis of the Transforming Genes of Acute Leukemia Viruses
and Their Cellular Homologs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dennis Watson	Staff Fellow	LMO, NCI
OTHER:	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

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:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In an effort to understand the organizational structure of retrovirus genomes, the nucleotide sequence of the chicken cellular myc gene (c-myc) has been determined and compared to the MC29 (Avian myelocytomatosis virus) v-myc gene. The cellular gene shares 1.6 kilobase homology with viral-specific sequences, interrupted by 972 non-homologous sequence. The interrupting sequence can be identified as a true intervening sequence by the presence of consensus donor and acceptor splice signals. The total number of viral-specific nucleotides in c-myc is identical to the number of v-myc. The protein termination signal which is located within myc-specific sequences, is conserved in c-myc. Cellular and viral genes differ by 15 nucleotides, none generating a new termination codon. In addition, c-myc contains polyadenylation signal and polyA-acceptor signals located in the 3' flanking cellular sequences.

Project Description

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 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 on 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 the cDNA probe to virus-related sequences on these filters.
5. Construction of recombinant phage libraries by ligation 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 sequence 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. Preparation of aminophenylthioether filters containing immobilized polyA selected glyoxylated RNA and hybridization of specific nick-translates (P^{32}) DNA probes to detect expressed sequences.

Major Findings:

1. The myc-specific sequences are highly conserved between v-myc and c-myc. The total number of bases, 1568, is identical, although the c-myc clone contains 707 and 861 bp exons. The viral myc gene termination signal is preserved, suggesting identity of open reading frame.
2. The junctions between the myc-specific and cellular non-viral homologous regions have all been determined. A) Between the 5' and 3' myc exons, a 972 bp intervening sequence, identified by consensus donor and acceptor splice signals,

is found. B) The sequence of the c-myc clone 5' to myc specific sequences shows little homology to viral gag sequences. C) The sequence of the c-myc clone 3' to myc specific sequence shows little homology to viral env sequences.

3. Polyadenylation (AATAAA) and polyA acceptor (CACA) signals have been identified by c-myc 3' to myc-specific sequences.

4. Mouse DNA digested by EcoRI has been found to contain multiple myc-related sequences. DNA has been enriched for myc sequences by fractionation on RPC-5A columns.

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 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 terminal 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 will be used to make antibodies. These antibodies will be used to characterize both the viral and cellular myc proteins.
3. The extent of homology between 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.
4. The sequence of a human c-myc homolog will be determined.
5. Human leukocyte DNA will be restricted by multiple restriction enzymes followed by Southern transfer and hybridization to detect myc homologous sequence. Multiple individuals will be analyzed in an effort to study polymorphism of the myc locus. Blood from leukemic patients will also be analyzed.
6. The mouse c-myc gene, useful for a) polymorphism analysis using 5' and 3' c-myc probes and b) chromosomal location of the myc gene, will be cloned.
7. Expression of myc-related sequences will be examined using Northern analysis of polyA RNA. The first question is to examine processing of the cellular myc gene. Using defined probes detecting either myc-specific sequences or intervening sequences, it will be possible to determine whether the intervening sequence recognized by consensus signals is indeed spliced out (processed) in vivo.

Publications:

Papas, T.S., Rushlow, K.E., Lautenberger, J.A., Watson, D.K., Baluda, M.A., Reddy, E.P.: Complete nucleotide sequence of the transforming genes of avian myeloblastosis virus (AMV). J. Cell. Biochem. 1982. (In Press).

Papas, T.S., Rushlow, K.E., Lautenberger, J.A., Watson, D.K., Beluda, 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. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05239-01 LMO
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Structural Analysis of the Avian Myeloblastosis Virus Genome		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: OTHERS:	Nancy C. Kan Takis S. Papas James Lautenberger Dennis Watson	Visiting Fellow Head, Carcinogenesis Regulation Section Expert Staff Fellow
		LMO, NCI LMO, NCI LMO, NCI 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: 1.0	PROFESSIONAL: 1.0	OTHER: 0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Avian myeloblastosis virus (AMV) is defective in reproductive capacity and thus requires a helper such as myeloblastosis associated virus (MAV). The genome of AMV has undergone a <u>sequence substitution</u> in the region normally coding for the viral envelope protein which has been replaced by cellular sequences responsible for <u>leukemogenesis</u> . Comparison of the nucleotide sequences of AMV and MAV indicates that the <u>recombination site</u> occurs right after the sequence TTTTGCAGGC 80 nucleotides downstream from a KpnI site near the 3' end of the polymerase gene. This sequence matches well with the consensus sequence for the <u>3' RNA acceptor site</u> . The preservation of the 3' splice signal in both AMV and MAV suggests that it may have been involved in the original recombination event, as well as being required as an acceptor site for LTR-promoted message synthesis. Furthermore, the amino acids predicted from the nucleotide sequences show that the <u>carboxyl-terminus</u> of MAV <u>reverse transcriptase</u> is different from that of AMV. We assume the MAV reverse transcriptase is a functional protein, because 60 of the 64 amino acids encoded from the KpnI site to the terminator are identical to those of Rous sarcoma virus (RSV). It is not clear yet whether AMV produces a functional polymerase with its cellular-derived carboxyl-terminus.		

Project Description

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:

- (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 γ [³²P]ATP and polynucleotide kinase. Preparation of 3'-end labeled DNA fragments using α [³²P]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, located 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:

- (1) The predicted COOH-termini of AMV and MAV reverse transcriptase are different. After the point of divergence, there are 27 amino acids in AMV prior to a TAG terminator and 36 amino acids prior to a TAA terminator in MAV.
- (2) The MAV reverse transcriptase is a functional protein. The AMV reverse transcriptase with its cellular-derived carboxyl-terminus is defective because (a) 60 of the 64 amino acids encoded from the KpnI site to the end of the polymerase gene are identical between MAV and RSV, and (b) no reverse transcriptase activity has been detected in AMV-nonproducer cells.
- (3) The sequence TTTTGCAGGC found at the recombination site matches well with the consensus sequence of Y-YYY-CAG+ for the 3' RNA acceptor site. Since in vitro

transcription studies (K. Samuel, unpublished results) suggest that the AMV subgenomic RNA is spliced from the genomic RNA, we conclude that this sequence is involved in the generation of the AMV and MAV subgenomic RNA species transcribed from the 3' region of the viral genomes.

(4) The overlap of the putative RNA splicing site and the recombination site leads us to speculate that subgenomic RNA species may have been involved in the original recombinational event between the non-defective helper and the cellular sequence.

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 ³⁵S-methionine and analyzed on SDS-polyacrylamide gels. The origin of the viral proteins will be confirmed by immunoprecipitation with antisera against AMV transforming protein.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05286-01 LMO

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Identification of transforming genes in human tumors and in chemically transformed human cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Colin S. Cooper	Visting Fellow	LMO, NCI
Other: Donald G. Blair	Expert	LMO, NCI
George F. Vande Woude	Chief, LMO	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: 1

PROFESSIONAL: 1

OTHER: 0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 NIH 3T3 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 NIH 3T3 cells and that cells transfected with DNA from the MNNG transformed cells and the fibrosarcoma give rise to tumors in nude mice. Both the primary morphologically transformed NIH 3T3 cells and the tumors possessed DNA highly repeated human Alu sequences.

Project Description

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 NIH 3T3 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 NIH 3T3 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 efficiencies of 0.02 and 0.05 focus forming units per μg 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 teratocarcinoma 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 all secondary transfectants.

The presence of dominant transforming genes was also investigated in experiments in which NIH 3T3 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. More detailed analysis of these DNA samples is in progress.

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 a 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 transformation 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 secondary transfectants prepared in these studies will be further investigated and the human transforming genes present in these cells cloned by standard cloning procedures.

Publications:

Blair, D., Cooper, C. S., Oskarsson, M. K., Eder, L. A. and Vande Woude, G. F. "Tumor induction by freshly transfected NIH3T3 cells in nude mice; a sensitive method for the detection of transforming sequences present in cellular DNA" Submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05287-01 LMO
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
DNA Replication, Integration and Gene Expression of MSV

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Robins	Staff Fellow	LMO NCI
Other:	G. F. Vande Woude	Chief, LMO	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: 1.0	PROFESSIONAL: 1.0	OTHER: 0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genome of HT-1 MSV is modified by recombinant DNA techniques to contain a bacterial origin of DNA replication and a selectable drug-resistance gene. The covalently closed circular proviral DNA intermediate of this chimeric retrovirus is capable of replicating in bacteria as a plasmid. This novel retrovirus vector will be used 1) to introduce foreign DNA into mouse 3T3 cells under retroviral control and 2) to study effect of mutations on the process of reverse-transcription and integration.

Project Description

Objectives:

The goal of this project is to construct a retrovirus vector which will facilitate the direct molecular cloning of its own DNA genome in bacteria. This property will be useful in answering several fundamental questions about the origin and replication of these oncogenic viruses.

1) Using this vector system I will be able to introduce eukaryotic genes into mouse NIH 3T3 cells under retrovirus control. Specifically, I plan to introduce cellular proto-onc genes (i.e. c-myc) into this vector in order to study the arrangement of viral and cellular sequences necessary to activate the oncogenic potential of certain genes. Those mutational events which cause a normal cellular gene to become oncogenic can then be molecularly cloned and analyzed.

2) This vector will also be used to analyze the effects of various mutations on the synthesis and structure of proviral DNA. These studies should be able to delineate the viral sequences required for proper DNA replication and integration.

Methods Employed:

Recombinant DNA technology is used to modify in vitro the molecularly cloned DNA genome of MSV. The biological activity of these DNA molecules is measured by standard DNA-mediated-gene-transfer (transfection) experiments into mouse NIH 3T3 cells. Infectious virus is reconstituted from the cloned DNAs by co-transfection or rescue with a replication-competent helper virus.

Major Findings:

I have identified a region of the HT-1 MSV genome that is dispensable and can be replaced with foreign DNA segments without a significant loss of transforming activity in mouse NIH 3T3 cells. This region lies within the non-functional gag gene of this virus and constitutes approximately 10% of the viral genome. Foreign DNA segments inserted in this non-essential region are retained in viral RNA when infectious virus is reconstituted by co-transfection with helper virus.

Significance to Biomedical Research and the Program of the Institute:

Oncogenic retroviruses have proven themselves to be a useful model system to study the molecular basis of carcinogenesis. Analysis of the genome structure of these viruses has indicated that their ability to transform eukaryotic cells is derived from the aberrant expression of normal cellular genes which have been assimilated into the viral genome. I am proposing to utilize this inherent ability to act as a natural vector to further our understanding of the molecular details of cellular transformation caused by these viruses and their cellular proto-onc genes. These studies will also provide a useful system to study the replication and integration of proviral DNA derived from these viruses. A more detailed understanding of these processes may give us insight into the normal cellular DNA recombination and repair pathways.

Proposed Course:

We intend to 1) develop the shuttle vector for purposes of rapid isolation of selectable eukaryotic markers, and 2) study the mechanisms of virus replication.

Publications:

Duesberg, P., Robins, T.S., Lee, W.-H., Bister, K., Garon, C., and Papas, T.: On the relationship between the transforming onc genes of avian rous sarcoma and MC29 viruses and homologous loci of the chicken cell. In Revoltella, R.F. et al. (Eds.): Expression of Differentiated Functions in Cancer Cells. Raven Press, New York, 1982, pp. 471-484.

Robins, T., Bister, K., Garon, C., Papas, T., and Duesberg, P.: Structural relationship between a normal chicken DNA locus and the transforming gene of the avian acute leukemia virus MC29. J. Virol. 41: 635-642, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05288-01 LMO

PERIOD COVERED

October 1, 1981, through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cell Interaction, cAMP and Control of Developmental Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: D. Blumberg Senior Staff Fellow LMO NCI

COOPERATING UNITS (if any)

Dr. Stephen Chung, Dr. Scott M. Landfear and Dr. Harvey F. Lodish,
Department of Biology, MIT, Cambridge, MA

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 preaggregation stage cells. Studies designed to identify factors which regulate the coordinate expression of this large portion of the genome indicate that cell-cell interaction is a necessary prerequisite for both the synthesis and stability of the late mRNAs. Disruption of cell contacts results in the rapid and specific degradation of the late mRNA species. Messenger RNA species whose expression is conserved throughout development are unaffected by disaggregation. Addition of cyclic AMP to cells which have been disaggregated restores the level of most, but not all, of the late mRNAs. Thus, an event which initiates at the cell surface brings about a change in the nucleus which allows the transcription of an additional 26% of the Dictyostelium single copy genome. The actual rate of transcription and the subsequent stability of many of the mRNAs transcribed off of this portion of the genome are then further regulated by a cyclic AMP mediated process.

Project Description

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 amoebae into a multicellular organism. Differentiation of amoebae within the newly-formed aggregates generates the three distinct cell types found in the mature fruiting body: spore cells, stalk cells and basal disks. Dictyostelium exhibits many features of development seen in more complex eucaryotic organisms. Specific cell-cell contacts are found, a homogeneous population differentiates into discreet 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. It is our specific objective to study factors which regulate the coordinate expression of this large portion of the Dictyostelium genome.

Methods Employed:

Three independent methods have been used to identify and quantitate the late messenger RNA species. These include in vitro translation followed by two-dimensional gel analysis of protein products, hybridization of gel-separated RNAs to cloned genomic DNAs, and hybridization of messenger RNAs to a cDNA probe specifically enriched for the population of late messenger RNA species.

Major Findings:

1. Cell-cell interaction is a necessary prerequisite for expression of the late mRNA species.

a. Analysis of mutants that are blocked at different stages of differentiation shows that induction of expression of these genes is correlated with the formation of tight cell-cell contacts that resist EDTA. In particular, mutants that exhibit chemotaxis and aggregate to form loose mounds but do not form cell-cell contacts that resist EDTA fail to induce these late mRNA and protein species. By contrast, mutants that form normal contacts but progress no further through development do express the late mRNA species.

b. When cells are developed by starvation for 20 hours in a rapidly shaken suspension culture, conditions which prevent stable cell-cell contacts from forming, significant levels of the late aggregation specific mRNA are not formed. The complexity and overall composition of the mRNA population from suspension starved cells is the same as that from growing and early developing cells.

Thus, interactions at the cell surface are a necessary prerequisite for developmental induction of this large group of co-regulated mRNAs.

2. Cell contact and cAMP control both the synthesis and stability of most of the late mRNA species.

a. Disruption of cell-cell contacts results in specific degradation of the late mRNA species. When plated cells, which have been allowed to form the cell-cell contacts, are disaggregated to a single cell suspension and vigorously shaken for several hours, the late aggregation specific mRNA species are rapidly degraded. Their intracellular concentration drops over 60-fold in less than 5 hours after the cells are returned to a suspension culture. The average half-life of the late developmental mRNAs under these conditions is 24-45 minutes. By comparison, the half life of total mRNA during normal growth and development is 4 hours. The mRNA species whose expression is conserved throughout development remain at the same intracellular concentration upon disaggregation of late developing cells. Thus, the formation or maintenance of cell-cell interactions is a necessary prerequisite for stability and/or synthesis of the late developmental mRNAs. Disruption of cell-cell interaction results in rapid and specific degradation of late developmental mRNAs without any noticeable effect on expression of the developmentally conserved mRNAs.

b. cAMP can stimulate the synthesis of the late mRNAs in disaggregated cells. The levels of most, but not all, of the late mRNAs are maintained when cells are disaggregated in the presence of 10-100 μ M cAMP. Furthermore, addition of cAMP to cells 2.5 hr after disaggregation restores normal levels of most, but not all, of these mRNAs after an additional 3 hr. This indicates that cAMP must stimulate the synthesis of these late mRNAs and may also prevent their degradation.

c. cAMP cannot stimulate synthesis of late mRNAs in cells that have not previously been in contact. Cyclic AMP stimulates the expression of aggregation-stage mRNAs only in single cells which have once formed cell-cell contacts. Cells which have been starved for 15 hrs in a vigorously shaken suspension culture in the same solution as used for differentiation are termed aggregation competent. These cells possess the cAMP receptor and phosphodiesterase required for cAMP cell signaling and when plated on a solid surface they rapidly form mounds with prominent tips (first fingers) within 7 to 9 hours, several hours faster than plated growing cells induced to differentiation. These suspension-starved, aggregation competent cells fail to induce significant levels of any late mRNA or protein, either in the absence or presence of cAMP added at 4, 8, 13 or 15 hours after initiation of starvation provided they are maintained as single cells.

d. A small subset of the late mRNAs are not regulated by cAMP. Among the genomic and cDNA clones of late genes, a small subset has been identified which contains genes encoding mRNA species which require cell interaction for their synthesis and are degraded upon disruption of the contacts. However, unlike the majority of the late mRNA species, the synthesis and stability of these mRNAs are completely unaffected by cAMP.

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 eucaryotic 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 this 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:

1. Blumberg, D. D., Chung, S., Landfear, S. M., and Lodish, H. F.: Cell-cell contact, cyclic AMP 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.
2. Blumberg, D. D., and Lodish, H. F.: Changes in the complexity of nuclear RNA during development of Dictyostelium discoideum. Devel. Biol. 81: 74-80, 1981.
3. Blumberg, D. D., Margolskee, J. P., Barklis, E., Chung, S. N., Cohen, N. S., and Lodish, H. F.: Specific cell-cell contacts are essential for induction of gene expression during differentiation of Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA 79: 127-131, 1982.
4. Chung, S., Landfear, S. M., Blumberg, D. D., Cohen, N. S., and Lodish, H. F.: Synthesis and stability of developmentally regulated Dictyostelium mRNAs are affected by cell-cell contact and cAMP. Cell 24: 785-797, 1981.
5. Lodish, H. F., Blumberg, D. D., Chisholm, R., Chung, S., Coloma, A., Landfear, S., Barklis, E., Lefebvre, P., and Zuker, C.: Control of Gene Expression. In Loomis, Jr., W. F. (Ed.): Dictyostelium discoideum - A Developmental System, Ed. 2. New York, Academic Press. (In press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05294-01 LMO
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Activation and Isolation of Transforming Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Tainsky	Staff Fellow	LMO,NCI
OTHERS:	G. F. Vande Woude	Chief, LMO	LMO,NCI
	A. Woodworth	Biologist	LMO,NCI

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Laboratory of Molecular Oncology

SECTION
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1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A novel DNA cloning vector, λ MT-5 has been constructed by recombinant DNA techniques. This vector is used to generate eukaryotic genomic libraries that can be screened with the amber suppressible mini-plasmid Π VX. This enables a positive selection of recombinants without a background of the parent vector. In an analogous fashion we have developed techniques for isolating unique DNA recombinants in human mos onc gene between human genomic DNA and the mos region of Moloney sarcoma virus. We have characterized these recombinants by specific methods demonstrating that such recombinants contain both human and MSV mos genetic sequences. These recombinants will be tested for biological transforming activity on NIH 3T3 cells.

Project Description

Objectives:

One aim of this project is to introduce a double amber mutation into the phage vector, λ L47, which possesses a number of existing features which are useful for constructing genomic libraries from entire collections of eukaryotic genes. This vector can then be used in conjunction with a suppressor selectable system for rapid screening of homologous sequences.

It is especially useful to rapidly generate recombinants between biologically active and inactive onc genes to identify essential regions responsible for transforming activity. The second aim of this project therefore is to develop efficient recombination systems.

Methods Employed:

The novel phage cloning vector was constructed by standard DNA recombinant techniques and analyzed by restriction endonuclease cleavage patterns. The phage amber mutations were selected for and confirmed by subsequent plaque formation analyses on specific E. coli K-12 strains; Q358, Q359, W3110 and LE392.

The human DNA fragment containing the murine mos gene homolog was cloned into mini-plasmid containing an amber suppressor (π VX). In vivo recombinants were generated using the Moloney sarcoma provirus HT-1 cloned into the coliphage Charon 4A. Recombinants generated were selected by growth on non-amber mutation suppressing hosts. The biological activity was assayed by DNA transfection into NIH 3T3 cells and scoring for morphologically transformed foci.

Major Findings:

The construction of the phage vector, λ MT-5, was performed by enzymatically cleaving the L47 genome into three specific regions using the restriction endonuclease KpnI. The central (1.2 kb) fragment and right arm of the phage were used intact, while the left arm was further subdivided by the enzyme AvaI. The smaller of the two Ava fragments, the 4.8 kb fragment, was replaced by the homologous region from the coliphage Charon 16A containing the A and B genes of the phage with amber mutations. This 4.8 kb-Charon 16A fragment was ligated to the 13 kb AvaI/KpnI fragment from L47 and the two remaining KpnI fragments from L47 were ligated and then mixed with the left arm ligation. This DNA mix was introduced in E.coli, JC strain by transfection and the resultant progeny phage was tested for plaque formation only on amber suppressing strains. The novel phage vector DNA was analyzed and confirmed by restriction endonuclease cleavage in conjunction with agarose electrophoretic techniques.

Recombinant DNA clones containing the requisite sequences were recloned into appropriate vectors necessary to perform these experiments. The human mos sequences were cloned into the mini-plasmid (π VX) vector in both possible orientations. In a model system we found such clones recombined in vivo at very low frequencies using a phage λ WES B vector. It was necessary therefore to reclone the MSV proviral clone HT-1 into another phage λ vector. For our purpose, Charon 4A was found to be most efficient, presumably because of the specific expression requirements of the suppressible amber mutation system. Subsequently

we were able to generate *in vivo* recombinants upon infection of the human *mos* Π VX containing cells with MSV prophage clone, coliphage λ HTE. Analysis of these novel phage recombinants, using specific restriction endonucleases, suggested that genetic recombination had occurred within the *mos* region of the proviral genome.

Significance to Biomedical Research and the Program of the Institute:

Use of this novel phage vector will enable the production of large eukaryotic genomic libraries from partial *Sau*3A digestions and allow the rapid screening with specific sequences inserted in the mini-plasmid Π VX; taking advantage of its homologous recombinant propensity and its subsequent selection by amber mutant selective techniques.

This study has generated a number of novel recombinants using a new approach which could potentially enable the activation of transforming genes derived from endogenous cellular sequences. This approach would lend itself to the analysis of functional domain(s) encoded within the human DNA sequences analogous to the MSV malignant oncogenic sequences.

Proposed Course:

This vector will be used to construct genomic libraries from NIH 3T3 cells that have been transformed with DNA from human tumors. These (phage vector) resultant libraries will be used to infect bacterial strains containing Π VX recombinant plasmids containing the human highly repetitive DNA sequences. By this method any homologous human DNA sequences in the mouse NIH 3T3 cells can be selected for and rapidly cloned. Presumably, any tumor derived DNA sequence can be efficaciously selected for and detected using this genetically engineered phage vector.

In vivo generated recombinants of MSV proviral (HT-1) and human *mos* sequences will be subcloned and assayed for biological transformation activity with the DNA directed transfections into NIH 3T3 cells. In addition, recently, another method of activation and expression of *onc* gene sequences has been developed. We have succeeded in activating the amino acid coding sequences for the *v-mos* gene by direct DNA transfection into NIH 3T3 cells in the presence of human DNA in an approach analogous to the activation by the MSV LTR. Cloning of these human activating DNA sequences will allow isolation of LTR-like enhancer sequences from human DNA which may function to activate human oncogenes.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05295-01 LMO
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies of Transformation by Cloned Recombinant MSV DNA and Human Tumor DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHERS:	Thomas G. Wood	Senior Staff Fellow	LMO	NCI
	Marianne K. Oskarsson	Chemist	LMO	NCI
	Colin S. Cooper	Visiting Fellow	LMO	NCI
	Stephen D. Showalter	Biologist	LMO	NCI
	George F. Vande Woude	Chief	LMO	NCI
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COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

2.5

PROFESSIONAL:

1.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The provirus of the Moloney murine sarcoma virus (MSV) contains both an MSV-specific transforming sequence (v-mos) and sequences [the MSV long terminal repeat (LTR)] which contain presumptive promoters for activation of RNA synthesis. The LTR acts to enhance v-mos transformation when linked either 5' or 3' relative to v-mos, and analysis of polyadenylated RNA indicates RNA synthesis either initiates or terminates in the LTR, depending on its location relative to mos. LTR-containing DNA clones also enhance v-mos transformation when two separate fragments are co-precipitated and co-transfected. We have developed a screening and selection system to identify dominant transforming DNA sequences based on the ability of NIH 3T3 cells transfected by such sequences to induce tumors in athymic nude mice. Under appropriate conditions this assay is as sensitive as conventional focus assays in tissue culture. Transfected sequences are present in tumors and virus can be rescued or viral antigens detected in cell lines derived from tumor explants. We have presently identified three human tumor DNAs capable of inducing tumors in our assay. We have also studied the genetics and biochemistry of ts-110, an MSV temperature-sensitive (ts) deletion mutant, which transforms cells at 34° but not at 39°.

Project Description

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) Activation of mos transformation by co-transfection is sensitive to the structural organization of both the mos and LTR plasmids. Previous results have shown that co-transfection of v-mos and LTR-containing plasmid clones into NIH 3T3 cells resulted in a 100 to 300-fold enhancement of the transforming efficiency compared to the activity observed with v-mos alone. The studies also showed that co-transfection with v-mos and LTR clones which contained adjacent gag coding sequences also resulted in increased frequencies of mos transformation. Furthermore, a fraction of these transformed cells contained a rescuable transforming virus. Further analysis indicates that the maximum efficiency of activation of the transforming potential of v-mos in co-transfection assays requires that both DNA components be present as linearized plasmids. The enhancement of transformation efficiency is markedly reduced when plasmids are linearized at sites near the 5' end of the v-mos insert. No enhancement is observed when the v-mos containing insert is removed from the plasmid, or when the plasmid is present as a circular DNA, as opposed to a linear molecule. Circular or free LTR inserts are only able to activate v-mos transformation at a markedly reduced efficiency. Initial analysis of cells transformed in LTR-v-mos co-transfections indicate all transformed cells contain multiple copies of mos in cellular DNA.

2) Cells transfected by v-mos linked to a single proviral LTR contain polyadenylated mos RNA which either initiates or terminates within the LTR sequence. Previous results have shown that both c-mos and v-mos sequences actively

transform when linked to a single LTR either 5' or 3' relative to *mos*. Cells transformed with DNA fragments containing the structural arrangement 5'-LTR-*v-mos*-3' express multiple polyadenylated, *mos* containing RNAs containing unique 5' (U5) sequences derived from the LTR, but no unique 3' (U3) sequences. Conversely, cells transfected with DNA fragments of the type 5'-*v-mos*-LTR-3' contain RNA species which encode *mos* and U3 specific sequences, but no U5 sequences. In most cases the *mos* containing RNAs are larger than those predicted from the size of the *mos*-LTR insert, and contain pBR322 specific vector sequences.

3) Cells freshly transfected with cloned MSV DNA or with cellular DNA from either MSV or SV40-transformed cells will form tumors in nude mice. Under appropriate conditions, the limit of sensitivity of tumor formation is approximately that of focus formation in tissue culture. A population of cells which in tissue culture will give rise to 1-10 foci, will, if injected in nude mice, give rise to a tumor in at least one mouse in five. Cell DNA from tumors induced by cloned MSV contains new copies of MSV-specific DNA sequences. Infectious MSV can be rescued from cell lines derived by explanting portions of these tumors into tissue culture. Cells transfected with DNA derived from SV80, an SV40 transformed human cell line, also induced tumors at high efficiency. Cells explanted from these tumors express SV40 T antigen detectable by fluorescent antibody analysis. Conditions for maximal efficiency of tumor formation are being established. Care must be taken in maintaining the cells used as recipients, and the numbers of transfected cells injected into mice must be kept low to avoid the spontaneous transformation of the recipient cells and the subsequent appearance of "background" tumors. Histologically, all tumors examined to date are undifferentiated fibrosarcomas.

4) Cells transfected with DNAs from certain human tumor-derived cell lines will induce tumors in nude mice. High levels of sequences homologous to the human repetitive "Alu" sequence family have been detected in a tumor induced by DNA derived from PANC-1, an established human pancreatic tumor cell line. Analysis of DNA from tumors induced by two other human DNAs is in progress. When DNA from this primary mouse tumor is transfected into NIH 3T3 cells and these cells injected into mice, new tumors arise. Parallel cells kept in tissue culture form foci with an efficiency of 0.5-5 focus forming units per μ g of tumor cell DNA. Analysis of DNA from four of these foci reveals a reduced number of human repetitive Alu family hybridizing fragments following R1 endonuclease digestion. The structure of the Alu bands present in secondary tumors is currently being investigated. Cell lines derived from primary and secondary tumors consist of mixtures of refractile transformed cells and normal cells. Tumors are induced when these cells are injected into BALB/c nude mice but few or no tumors are induced when they are injected into normal BALB/c mice. The explanted cells are capable in most cases of growth in agar suspension.

5) Cells transformed by the wild-type parent of ts-110 (MSV 349) contained two size classes of *mos*-specific RNA sequence (6.9 and 5.8 Kb). Previous studies have shown that ts-110 transformed cells contain two size classes of *mos* hybridizing polyadenylated RNA (5.0 and 4.5 Kb). The two species were shown to be present at both permissive and non-permissive temperatures. However, a spontaneous revertant to ts-110 was found to contain only a 5.0 Kb *mos*-specific RNA. Portions of this project were previously reported under discontinued projects Z01 CP 05153-02 LVC, and Z01 CP 05070-03 LVC.

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.

Conditional mutants have also been extremely useful in eliciting biochemical pathways in a number of cells and viral systems. MSV ts-110 represents one of the few stable, conditionally defective variants of a mammalian sarcoma virus. Analysis of this mutant at a molecular level should help to elucidate the mechanism of transformation by Moloney MSV.

Proposed Course:

DNA and RNA from cells transformed by co-transfected mos and LTR plasmids will be analyzed to determine the nature of the association between them and to identify the mechanism by which co-transfection leads to activation of cell transformation. Attempts will be continued to develop non-fibroblast and human cell transfection/transformation systems in order to study cellular and species specific effects on the process of co-transfection and LTR activation. The biological activity of fragments containing portions of the LTR linked to mos in different spatial orientation will be measured to determine the structural limits of oncogene activation by LTRs, the LTR sequences necessary and sufficient for mos expression, and the specific identity of any genetic sequences associated with c-mos which block its expression.

The nature of the transforming DNA sequences present in the human pancreatic carcinoma cell line (PANC-1) will be further characterized. DNA from secondary and tertiary tumors and foci will be analyzed with known viral oncogene probes to determine if it represents one of those oncogenic sequences previously identified. Attempts will be made to clone the sequence and to characterize it and its normal nontransformed cell homologue. Attempts will be made to isolate and compare transforming sequences from other pancreatic neoplasms. Similar analysis will be performed with DNA sequences from other human tumor cell lines and a comparison between those present in tumors and those isolated from morphologically transformed foci in tissue culture will be compared to determine if differences exist between sequences selected by the two techniques. Screening of DNAs isolated from primary human tumors, human tumor xenografts (obtained from Dr. K. Stromberg, LVC) and human tumor derived cell lines will continue. Various other cell lines will be tested to determine if other potentially more useful and interesting recipient lines can be developed. Attempts will be made to raise antisera to, and to identify tumor specific antigens in, cells transfected with human sequences in order to identify and characterize the gene products of the transfected human sequences. If such a response can be detected, monoclonal antibodies to these antigens can be prepared.

A major effort will be made to clone the integrated provirus from ts-110 MSV-transformed cells. This clone will be used to characterize the nature of the two mos-specific RNA species found in ts-110 transformed cells. Antisera in preparation in this laboratory against synthetic mos-specific peptides will be used when they become available to determine the nature of mos gene product expression under permissive and non-permissive conditions.

Publications:

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, in press.

Blair, D. G., Oskarsson, M. K., McClements, W. L., and Vande Woude, G. F.: The long terminal repeat of Moloney sarcoma provirus enhances transformation. In Neth, R., Gallo, R. C., Graaf, T., Mannweiler, K., and Winkler, K. (Eds.): Haematology and Blood Transfusion, Modern Trends in Human Leukemia IV. Berlin, Heidelberg, Springer-Verlag, 1981, Vol. 26, pp. 400-466.

Blair, D. G., and Vande Woude, G. F.: Moloney sarcoma virus, a model for transformation by endogenous cellular genes. In Klein, G. (Ed.): Advances in Viral Oncology, Advances in Viral Research and Therapy. New York, Raven Press, 1982, Vol. 1, pp. 189-205.

Horn, J. P., Wood, T. G., Murphy, Jr., E. C., Blair, D. G., and Arlinghaus, R. B. A selective temperature-sensitive defect in viral RNA production in cells infected with a ts-mutant of murine sarcoma virus. Cell 25: 37-46, 1981.

McClements, W. L., Dhar, R., Blair, D. G., Enquist, L., Oskarsson, M. K., and Vande Woude, G. F.: The long terminal repeat of Moloney sarcoma provirus. Cold Spring Harbor Symp. Quant. Biol. 45: 699-705, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure and Function of MSV Genome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marylou McGeady	Staff Fellow	LMO, NCI
Others:	G. F. Vande Woude	Chief	LMO, NCI
	R. Ascione	Research Chemist	LMO, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

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Tumor Biochemistry Section

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NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies were done to test the effect of cytosine methylation on the expression of a transforming gene. It was found that methylation of the MSV genome at HpaII sites significantly reduced its ability to transform NIH 3T3 cells in DNA transfection experiments, and the reduction in transforming activity after transfection of methylated DNA was reversed by the use of a specific inhibitor of methylation. A low level of transforming activity after transfection of methylated DNA was detected apparently because the methylation pattern of transfected DNA was not maintained during initial rounds of DNA replication in cells. Methylation of the structural gene (mos) was more inhibitory to expression of transformation in these assays than methylation of the promotor region (LTR). Experiments to elucidate the mechanism by which methylation effects gene expression in this system are in progress.

Project Description

Objectives:

This project aims to determine the effect of cytosine methylation on the expression of the transforming phenotype of Moloney sarcoma virus (MSV) in 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 non-expressed 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.

Methods Employed:

Plasmid DNA was methylated in vitro and the biological activity was assayed by transfection into cells and determining the number of foci of morphologically transformed cells. DNA from normal or transformed cells was digested with restriction endonucleases and analyzed by the Southern blotting technique. RNA from transformed cells was analyzed by the Northern blotting technique.

Major Findings:

Cloned Moloney Sarcoma Virus proviral DNA was methylated in vitro using a HpaII methylase. The DNA was transfected into NIH 3T3 cells and its efficiency of transformation was reduced by 70% compared to the control. 5-Azacytidine, which can inhibit methylation of replicating DNA, was added to cells after transfection with methylated DNA. The treatment resulted in the restoration of transforming activity and demonstrated that the decrease in MSV transforming efficiency was due to HpaII methylation.

The effect of HpaII methylation on the transcriptional control element region in the proviral LTR and structural mos gene was determined separately by cotransfection 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 NIH 3T3 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 results indicate that the effect of methylation is greater on the mos structural gene and that the transcriptional control element in the LTR cannot activate the methylated onc gene.

DNA from cells transformed by transfected HpaII methylated DNA was analyzed by Southern blotting. In every cell line examined the MSV DNA was no longer methylated at HpaII sites, demonstrating that the methylated sites were lost during DNA replication in cells.

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 methylation pattern, two cell lines were isolated which contained HpaII resistant copies of MSV DNA in a non-rescuable, nontransforming state. The genomic copies of MSV DNA in these cell lines show no differences with regard to DNaseI sensitivity when compared to cell lines containing non-methylated copies of MSV DNA.

Significance:

The correlation between the presence of 5 methylcytosine in a DNA sequence and the lack of transcription of that sequence is well established for several well-studied cellular genes. The work reported here extends these studies to cellular transforming genes. 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 NIH 3T3 cells, revealed that HpaII methylation significantly reduced its biological activity. Further studies revealed that the presence of a viral transcription control element in the form of the LTR was not sufficient to activate transformation by the HpaII methylated mos gene. This result suggests that the activation of endogenous transforming genes can be markedly influenced by their state of methylation.

Proposed Course:

We will determine the mechanism by which methylation inhibits expression of mos. RNA from cells transfected with methylated DNA will be analyzed for mos containing transcripts, and DNA will be analyzed by digestion with nucleases to determine any effect methylation may have on chromatin structure.

Publications:

None.

ANNUAL REPORT OF THE LABORATORY OF MOLECULAR VIROLOGY

NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

The Laboratory of Molecular Virology (1) analyzes the mechanism of gene expression in normal and transformed eukaryotic cells; (2) plans and conducts research on viruses to define their potential role in the development of cancers in animals and man; (3) develops and applies biological, biochemical and immunological procedures to obtain evidence for the presence of virus genetic information in cells; (4) investigates the mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells; (5) and uses viruses as tools for probing cellular regulatory mechanisms.

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 transforming genes from viruses and their protein products; evaluates the effects of viral infection on cellular control mechanisms in transformation and in lytic responses; and (3) compares viral transforming genes to homologous genes isolated from normal cells and identifies molecular elements responsible for transforming activities.

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, and (3) uses recombinant DNA techniques and molecular genetics to study the effect of gene expression. Specific eukaryotic genes are recombined in prokaryotic host-vector-systems and their expression and mode of regulation is examined in eukaryotic and prokaryotic hosts.

A major objective of the Laboratory of Molecular Virology remains the elucidation of the regulatory signals which are responsible for gene expression. In this regard we have defined an important regulatory element in the expression of the early SV40 mRNAs, specifically the 72 base-pair repeated segments located to the late side of the replication origin called the activator or enhancer sequence. This particularly interesting segment of the genome is unique in that it encodes no known polypeptides and appears to be free from nucleosomes as judged by sensitivity to nuclease digestion in SV40 chromatin. A number of studies are in progress both to define the specific nucleotides within this segment which are critical to transcriptional initiation and to determine the role of similar sets of nucleotides in other viral and cellular operons. An important recent observation was that the activator sequences appear to exhibit host cell specificity. Thus, we have defined a sequence from the Moloney sarcoma virus (MSV) which is also a 72 base-pair tandem repeat. When examined in parallel with the SV40 repeats, this MSV enhancer is more active in mouse cells, whereas the SV40 enhancer sequence is more active in monkey cells. Future studies will be directed at the ability of enhancer sequences to dictate not only host range, but also to participate in the developmental and tissue specific regulation of gene expression. A future goal in this regard will be an attempt to elucidate the mechanism by which these activator or enhancer sequences perform their functions and what role they play relative to other transcriptional regulatory signals.

A considerable effort has been directed toward an understanding of the molecules present on the surface of cells which may play a role in normal cellular recognition or in cellular transformation. In particular, a number of cDNA and genomic clones of the mouse histocompatibility antigen, H-2, have been obtained. Characterization of these clones led to the surprising observation that one of them could potentially encode a soluble form of the H-2 antigen. Studies are in progress to define the genomic segment responsible for this putative protein, to determine its genomic location, to characterize its specificity of expression, and to isolate a putative protein product. Preliminary studies indicate that the expression of this unusual gene may be specific to liver, and studies are in progress to confirm and extend this observation. The potential role of putative secretory forms of H-2 in tolerance or tumor immunity will serve as the basis for a number of future studies in this area.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05101-04 LMV

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Molecular Mechanisms for Malignant Transformation of Cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Gilbert Jay Visiting Scientist LMV, NCI
OTHER: George Khoury Chief LMV, NCI

COOPERATING UNITS (if any)

L. J. Old, Memorial Sloan-Kettering Cancer Center, New York, New York
I. Pastan, 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:

0.3

PROFESSIONAL:

0.3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

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 DescriptionObjectives:

Studies on the function of the transformation-specific protein p53 in cancer cells.

Methods Employed:

Proteins were detected either by indirect immunofluorescence or by immunoprecipitation using conventional or monoclonal antibodies.

Major Findings:

We have identified a transformation-related antigen, designated p53, which has the following characteristics:

- (a) p53 appears to be a constant feature of tumor cells of the mouse, whether these were transformed by X-ray, chemicals, DNA or RNA viruses or arose spontaneously. In contrast, levels of p53 in normal mouse cells are either extremely low or undetectable.
- (b) Immunofluorescence studies have localized p53 to the nucleus of transformed mouse cells.
- (c) p53-related components are found in transformed cells of a variety of species, including rat, hamster, rabbit and human. These components vary somewhat in molecular weight, ranging from 53K in mouse cells to 56K in human cells.
- (d) Rapidly growing cultures of certain "normal" human cells, such as early passages of kidney epithelium, were found to express p53. However, in contrast to the persistence of p53 in malignant cells when they reach confluence, p53 expression in the normal cell disappears upon contact inhibition of cell proliferation.
- (e) In SV40-transformed cells, p53 forms a complex with the viral tumor (T) antigen.
- (f) p53 components are immunogenic in their species of origin. Tumors differ, however, in their capacity to induce p53 antibodies.
- (g) p53 is a phosphoprotein and has an associated protein kinase activity.
- (h) p53 can be induced in normal spleen cells by treatment with lectins.

Significance to Biomedical Research and the Program of the Institute:

The biochemical events responsible for the transformation of normal cells to malignancy have not been defined. A central question in this regard is whether different classes of etiological agents transform cells by uniquely distinct

pathways. Certain chemicals, radiations and oncogenic viruses are known to induce neoplasms in laboratory animals. While each of these agents may act distinctly to initiate the transformation of cells, it is possible that once a cell is committed, by whatever mechanism, it will follow a common pathway leading to the loss of control of proliferation. Our identification of a transformation-specific protein common to malignancies of different etiologies allows us to test this hypothesis and provides us with a biochemical handle to study the molecular basis for transformation.

Proposed Course:

Attempts will be made to define the molecular mode of action of this protein during the process of malignant transformation. The possible application of our findings to clinical diagnosis and therapy will be entertained.

Publications:

Dippold, W. G., Jay, G., DeLeo, A. B., Khoury, G., and Old, L. J.: p53 transformation-related protein: detection by monoclonal antibody in mouse and human cells. Proc. Natl. Acad. Sci. USA 78: 1695-1699, 1981.

Jay, G., Khoury, G., DeLeo, A. B., Dippold, W. G., and Old, L. J.: p53 transformation-related protein: detection of an associated phosphotransferase activity. Proc. Natl. Acad. Sci. USA 78: 2932-2936, 1981.

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., 1982. (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 sarcoma viruses. Proc. Soc. Exp. Biol. Med., 1982. (In press.)

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Genetic Elements Regulating the Initiation of Transcription.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter Gruss	Visiting Scientist	LMV, NCI
OTHER:	Michael Kessel	Guest Researcher	LMV, NCI
	George Khoury	Chief	LMV, NCI

COOPERATING UNITS (if any)

Hans Weyer, Dept. of Molecular Biology, U. of California, Berkeley, Calif.
Paul Steele, Arifa Khan, and Malcolm Martin, NIAID, NIH

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

1.8

PROFESSIONAL:

0.8

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
- (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We identified a novel eukaryotic transcriptional control element which markedly increases the transcriptional activity of eukaryotic genes. This element has been termed activator or enhancer. We first characterized the activator in the SV40 genome and in the Moloney sarcoma virus (MSV). We have now been able to localize the core activity to a very short stretch of nucleotides (approximately 10 to 15 bp) within the SV40 72 bp repeat. Similar core sequences have been detected in the enhancer region of polyoma, BK and MSV. We have demonstrated that sequences flanking the coding genes of endogenous retroviral genes of either mouse, monkey or human origin also harbor enhancer activity.

Project Description

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:

1. Construction of deletion mutants and point mutants
2. Mapping of recombinant genomes
3. RNA analysis
4. Protein analysis
5. Cloning in pBR322 and derivatives thereof.
6. Expression of recombinants using the chloramphenicol acetyltransferase gene.

Major Findings:

1. The 72 bp repeat of SV40 is a cis essential element for the expression of viral genes.
2. Using point-mutants generated in single-strand bacteriophages in conjunction with deletion mutants, we identified a critical core sequence within the 72 bp repeat.
3. Similar enhancer elements have been identified in the controlling regions of MSV and BKV.
4. Enhancer sequences have been detected in the mouse, monkey and human genomes. Those 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 a prerequisite for the elucidation of controlling elements for activation of transformed genes and differentiation.

Proposed Course:

These projects will continue with an emphasis on the identification of the mechanism by which enhancer elements control gene expression. It should also be of interest to apply our assay systems to the control regions of the human bladder carcinoma genes and normal human c-ras genes. Furthermore it is planned to search for cellular enhancers.

Publications:

Gruss, P., Dhar, R., and Khoury, G.: Simian virus 40 repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. USA. 78: 943-947, 1981.

Gruss, P., Dhar, R., Maizel, J., and Khoury, G.: The SV40 72 base-pair repeat: a remote control element for early transcription. In Winnacker, E. and Schoene, H.-H. (Ed.): Genes and Tumor Genes. New York, Raven Press, 1982, pp. 39-47.

Gruss, P. and Khoury, G.: Papova viruses as vectors for the expression of eukaryotic genes. In Goebel, W. and Hofschneider, P. H. (Ed.): Current Topics of Microbiology and Immunology. Springer-Verlag, 96: 1982, pp. 159-170.

Gruss, P., Rosenthal, N., Konig, 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.): Viral Vectors. New York, Cold Spring Harbor Press, 1982, (In press.)

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.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05216-02 LMV

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

DNA Sequence of Important Tumor Virus Genetic Elements

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ravi Dhar Visiting Scientist LMV, NCI

COOPERATING UNITS (if any)

Edward Scolnick LTVG, NCI
Thomas Shih LTVG, NCI

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Harvey murine sarcoma virus is a retrovirus which transforms cells by means of a single viral encoded protein called p21 ras. We have determined the nucleotide sequence of the transforming gene which codes for p21. The nucleotide sequence has identified a potential amino acid sequence of two additional overlapping polypeptides which share their reading frames and C-termini with p21 but which contain additional N-terminal amino acids.

Project DescriptionObjectives:

1. To determine the structure of the p21 ras gene, and to try to correlate structure with function.
2. To analyze the regulatory signals such as transcriptional controls, protein processing sites and phosphorylation sites.

Methods Employed:

DNA fragments were labeled at their 3' or 5' end, then cleaved with restriction endonucleases. Single end labeled fragments were purified on polyacrylamide gels. These were sequenced by the chemical method of Maxim and Gilbert or the enzymatic method of Seif, et al.

Major Findings:

The Harvey murine sarcoma virus (HaMuSV) is a replication-defective transforming retrovirus. The 5.5 kb genome is tripartite; 3.5 kb are derived from rat 30S sequences which are retrovirus-like in nature. The 30S sequences are flanked on both sides by the murine leukemia virus long terminal repeat (LTR) sequences.

We have analyzed the genome sequences coding for p21 and its flanking regions. Three sequences, related to Goldberg-Hogness box (TATAAA), were found around positions 45, 137 and 165. This would put the putative 5' ends of the transcripts 25 nucleotides downstream from the 3' end of the consensus sequences. It is also consistent with the approximate locations observed for the primary transcripts from the SV40 Ha-MuSV recombinant-genomes.

Another important transcriptional feature was the localization of a potential RNA polymerase III promoter. In other genes that have been studied, two transcriptional control regions for RNA polymerase III are intragenic. The sequence between positions 53 and 64 of Ha-MuSV (5'-GUGUUUUGGGG-3') has 10 out of 12 nucleotides homologous to the first consensus control region and the sequence between positions 133 to 144 (GGGUUCGGCCC) has 10 out of 12 nucleotides homologous to the second consensus control region. This would place the 5' end of a putative RNA polymerase III transcript around positions 45 to 47. This location would be consistent with published data demonstrating an in vitro transcriptional start site observed for RNA polymerase III which maps approximately to that location. The three potential AUG initiation codons are in the same reading frame. On this basis, amino acids have been assigned to the sequence between nucleotides 185 and 910. From the above transcriptional information, it appears that the V ras sequences have at least three potential translational initiation codons, located at amino acid positions -53, -39 and +1. This suggests that three distinct polypeptides may be

specified by this viral gene. These polypeptides would be of sizes 30K (p 30, initiating at amino acid position -53), 29K (p29, initiating at -39), and 21K daltons (p 21, initiating at +1). We have compared the amino acid sequences of Harvey V-ras with Kirsten V-ras. The N-terminal region of the polypeptides shows very extensive homology (114/120 amino acids). There is very little homology (3/22 amino acids) at the respective C-termini. Nevertheless, overall the p21s share approximately 81% of their amino acids. The fact that these two distinct ras genes have been conserved in a wide range of species suggests that their physiological functions, although perhaps similar, may be distinct.

Significance to Biomedical Research and the Program of the Institute:

Recently the human counterpart of the Ha and Ki-ras sequences (isolated from bladder and colon carcinomas) have been shown to transform NIH-3T3 cells. This suggests the possibility that such sequences are involved in human carcinogenesis.

Proposed Course:

We are obtaining the nucleotide sequences of the two endogenous rat Harvey ras sequences. The comparison between V-ras and C-ras will help in elucidating the biological functions of different segments of the protein.

Publications:

David, P., Dhar, R., Furano, A.: The conservation of DNA sequence over a very long period of evolutionary time. Evidence against intergenic chromosomal transfer as an explanation for the presence of *E. coli* Tuf gene sequence in taxonomically unrelated prokaryote. Eur. J. Biochem. 120: 69-77, 1981.

Dhar, R., Ellis, R. W., Shih, T., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D. and Scolnick, E.: The nucleotide sequence of the p21 transforming protein of Harvey murine sarcoma virus. Science. 1982. (In press.) Science. 1982. In press.

McClements, W. L., Dhar, R., Blair, D. G., Enquist, L. W., Oskarsson, M., and Vande Woude, G. F.: The long terminal repeats of Moloney sarcoma provirus. Cold Spring Harbor Symposium on Quant. Biol. 45: 699-705, 1981.

Shih, T., 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 protein coded for by Harvey and Kirsten strains of murine sarcoma virus. J. Biol. Chem. 1982. (In press.)

Shih, T. Weeks, M. O., Gruss, P., Dhar, R. Oroszlan, S. and Scolnick, E. M.: Identification of a precursor in the biosynthesis of the p21 transforming protein of Harvey murine sarcoma virus. J. Virology 42: 253-261, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05217-02 LMV

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Regulation of SV40 Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Shigeko Nomura	Microbiologist	LMV, NCI
OTHER:	Gilbert Jay	Visiting Scientist	LMV, NCI
	George Khoury	Chief	LMV, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

0.9

PROFESSIONAL:

0.9

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The SV40 agnogene product (agnoprotein) was detected in the SV40 lytic infection of African green monkey kidney cells. Antibody to the agnoprotein was raised in rabbits. The agnoprotein was located in the cytoplasm, in the perinuclear region, and on the inner surface of the plasma membrane, but was absent from nucleus by immuno-fluorescence and subcellular fractionation analysis. A possible regulatory role of this protein in early gene expression is proposed.

Significance to Biomedical Research and the Program of the Institute:

Studies of SV40 gene products will hopefully provide an approach to the understanding of regulation of viral and host cell gene expression.

Proposed Course:

To investigate putative roles of the agnoprotein in transcription, processing and attenuation of the late SV40 mRNA in both lytic and abortive infection, or in the assembly of virions, the following investigations are in progress:

1. Identification of RNA species which encode the agnoprotein.
2. Determination of the specific DNA binding site of the agnoprotein.
3. Further evaluation of possible regulatory functions of the agnoprotein on early and late gene expression by comparing infections with the wild-type SV40 and agnoprotein mutants.

Publications:

Jay, G., Nomura, S., Anderson, C. W. and Khoury, G.: Identification of the SV40 agnogene product: a DNA binding protein. Nature 291: 346-349, 1981.

Project Description

Objectives:

To study the expression of the SV40 agnogene and the function of its product.

Methods Employed:

Antibody to the agnoprotein was raised in rabbits using both agnoprotein purified by SDS-polyacrylamide gel electrophoresis or a chemically synthesized part of the agnoprotein (14 amino acid residues at the N-terminus) conjugated with the carrier molecule (bovine serum albumin) as antigens. Detection and characterization of agnoprotein were carried out on 14C-arginine of 3H-leucine labeled cell extracts from SV40-infected African green monkey kidney (AGMK) cells by means of DNA cellulose chromatography, SDS-polyacrylamide gel electrophoresis and immunoprecipitation. Intracellular localization of agnoprotein was determined by immunofluorescence and subcellular fractionation. Analysis of viral transcripts selected by oligo(dT)-cellulose chromatography was performed by the Northern blotting technique followed by hybridization with ³²P-labeled total SV40 segment isolated by restriction endonuclease digestion. To test the specific SV40 RNA binding site of agnoprotein, cell extracts were electrophoresed on polyacrylamide gels and proteins were transferred by Western blotting. The specific SV40 binding site of agnoprotein band was identified by hybridization with labeled, restriction endonuclease digested SV40 DNA.

Major Findings:

1. Antibody against the agnoprotein was prepared in rabbits by inoculation with either gel-purified agnoprotein or a chemically synthesized oligopeptide to the agnoprotein. Both antigens produced antibody specific to the agnoprotein. Antibody to the synthetic peptide recognized a subset of the agnoprotein.
2. The intracellular location of the agnoprotein in SV40-infected AGMK cells was determined by immunofluorescence. The agnoprotein was localized to the cytoplasm, the perinuclear region and the inner surface of the plasma membrane, but not to the nucleus, 48 hours after infection. This observation was confirmed by subcellular fractionation, followed by immunoprecipitation.
3. Agnoprotein was absent from the SV40-transformed mouse cell lines 3T3WT, SVT2 and SV3T3.
4. Agnoprotein was not found to be phosphorylated.
5. We have previously suggested that the agnoprotein may have a regulatory function rather than a structural role. Several findings suggest that this protein may regulate SV40 early gene expression. We compared early translational products in AGMK cells infected with wild-type SV40 and mutants in the agnoprotein. There was relatively little (1/5 - 1/10) increase in T-antigen production after 48 hours of infection with mutants compared to infection with wild-type SV40.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05218-02 LMV

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Regulation of RNA Tumor Virus Gene Expression in Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Shigeko Nomura Microbiologist LMV, NCI

COOPERATING UNITS (if any)
R. H. L. Pang, Genex Corporation, Rockville, Maryland

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER: 0
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CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The phosphorylated transformation-specific protein p53 was detected by immunoprecipitation with monoclonal antibody in "normal" parental 3T3 mouse cells and in flat revertants in concentrations similar to those found in Moloney MSV-transformed S+L- mouse cells from which revertants were derived.

Project DescriptionObjectives:

To investigate the function of the transformation-specific protein p53 and viral proteins in cell transformation and reversion.

Methods Employed:

Proteins were detected by immunoprecipitation using monoclonal and conventional antibodies.

Major Findings:

The presence of p53 and MuLV p30 was investigated in "normal" parental cells (3T3FL), Moloney MSV-transformed S+L (S+L-3197) and its flat and MSV non-rescuable spontaneous revertant (SR-214) and the 5-fluorodeoxyuridine-induced flat revertant (FR-326) from which MSV was rescuable. The p30 determinant was expressed strongly in FR-326, S+L-3197 and also in similar amounts in the "normal" 3T3FL cells. Considerably less p30 was detected in SR-214 cells. The four cell lines all revealed similar amounts of p53 protein determined by immunoprecipitation of 35S-methionine labeled cell extract with monoclonal antibody. The identifiable p53 proteins from the four cells were phosphorylated to similar extents as determined by specific immunoprecipitation of P-32 labeled cell extracts.

Significance to Biomedical Research and the Program of the Institute:

Studies of the cellular and viral functions involved in transformation and reversion will facilitate the understanding of molecular basis for viral oncogenesis and the nature of cellular regulatory control in mammalian cells.

Proposed Course:

Quantitative and qualitative analysis of p53 present in normal, MSV-transformed S+L- and revertant cells will be determined after which this study will be terminated.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05219-02 LMV

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Construction of General Vector for Efficient Expression of Mammalian Proteins in Bacteria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Gilbert Jay Visiting Scientist LMV, NCI
George Khoury Chief LMV, NCI

COOPERATING UNITS (if any)
Ernest Jay, Department of Chemistry, University of New Brunswick, Fredericton, N. B., Canada

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.3	OTHER: 0
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CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords).

The goal of this project is to investigate the regulatory elements required for recognition by prokaryotic ribosomes during the process of initiation of protein synthesis. We have generated a plasmid vector containing a synthetic ribosome binding site which will assure the efficient expression of mammalian proteins in Escherichia coli.

Project DescriptionObjectives:

Use of a synthetic prokaryotic ribosome binding site for the efficient expression of SV40 t-antigen in bacteria.

Methods Employed:

Recombinant DNA techniques carried out under conditions specified by the NIH Guidelines were used in these studies. Nucleic acids were detected by combinations of hybridization, gel electrophoresis and electron microscopy. Proteins were analyzed by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

We have constructed a general plasmid vector carrying a chemically synthesized prokaryotic ribosome binding site which will assure the efficient expression of eukaryotic proteins in E. coli. In addition to the regulatory signals necessary for ribosome recognition, the synthetic segment contains, at one end, a Pst I cleavage site which will direct its insertion to pBR322 DNA and, at the other end, a Hind III site to facilitate attachment of the passenger eukaryotic gene. Using SV40 tumor (t) antigen as a model system, we have ligated the SV40 DNA fragment containing the entire t-antigen gene in tandem with the synthetic ribosome binding site to pBR322 DNA at the Pst I site, which lies within the coding sequence of the β -lactamase gene. Initiation of transcription at the β -lactamase promoter would produce a chimeric messenger RNA with the synthetic ribosome binding signals and the SV40 sequence flanked by β -lactamase coding sequences. The utilization of the synthetic regulatory signals for the initiation of translation is demonstrated by the efficient synthesis in bacterial transformants of authentic SV40 t-antigen. Excision of the entire SV40 insert by Hind III from those clones that have retained intact Hind III sites at the junction between the ribosome binding site and the SV40 sequence would allow insertion of other heterologous DNAs using Hind III linkers.

Significance to Biomedical Research and the Program of the Institute:

One of the main goals of recombinant DNA research is to produce significant quantities of medically and agriculturally important proteins whose genes have been cloned on bacterial plasmids.

With the premise that messenger RNAs transcribed in E. coli from cloned eukaryotic DNA inserts do not possess the necessary regulatory signals for recognition by prokaryotic ribosomes, we have constructed a general plasmid vector carrying a chemically synthesized prokaryotic ribosome binding site which will assure the efficient expression of eukaryotic proteins in E. coli.

This approach can be used for the expression of any eukaryotic protein whose RNA transcripts do not require post-transcriptional modifications specific to eukaryotic cells, or whose complementary DNA has been obtained by reverse transcription of its messenger RNA.

Publications:

Jay, G., Khoury, G., Seth, A. K., and Jay, E.: Construction of a general vector for the efficient expression of mammalian proteins in bacteria: use of a synthetic ribosome binding site. Proc. Natl. Acad. Sci. USA 78: 5543-5548.

Jay, G., Jay, E., Seth, A., and Khoury, G.: A novel procedure for the expression of eukaryotic proteins in *Escherichia coli*. In Keenbergh, M. (Ed.): Proc. Battelle Conf. Genetic Engineering, Vol. 4. Seattle, Battelle Seminars and Studies Program, 1982, 114-126.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05220-02 LMV

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Structure and Function of Cell Surface Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gilbert Jay	Visiting Scientist	LMV, NCI
	George Khoury	Chief	LMV, NCI
OTHER:	David Cosman	Visiting Fellow	LMV, NCI
	Michel Kress	Visiting Fellow	LMV, NCI
	Shigeko Nomura	Microbiologist	LMV, NCI

COOPERATING UNITS (if any)

L. J. Old, Memorial Sloan-Kettering Cancer Center, New York, New York.

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sequence analysis of mouse H-2 cDNA clones has suggested the existence of a novel class of H-2 (class I) 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 (class I) 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.

Project Description

Objectives:

Studies on the organization and expression of the genes coding for the histocompatibility H-2 antigens by molecular cloning technologies.

Methods Employed:

Recombinant DNA techniques carried out under conditions specified by the NIH Guidelines were used in these studies. Nucleic acids were detected by combinations of hybridization, gel electrophoresis and electron microscopy. Proteins were analyzed by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

Analysis of cDNA clones has allowed us to identify a novel class of H-2 related genes which could encode a shortened polypeptide. This protein would contain the entire biologically active portion of the H-2 antigen, located N-terminal to the transmembrane region, but probably would not be able to insert into the plasma membrane or to interact with cytoplasmic components of the cell. Such a molecule seems likely to be secreted.

The availability of a specific DNA probe, derived from the 3' non-coding region of a representative cDNA clone, has allowed us to study the expression of this novel H-2 related gene. A single size class mRNA about 1700 nucleotides long can be detected among total liver poly(A)+ RNA. Our estimate shows that this novel class of RNA comprises about one-fourth of all H-2 (class I) transcripts present in the liver, a substantial proportion in view of the fact that the class I antigens are encoded by a family of genes.

Of particular interest is the apparent tissue-specific expression of this novel class of H-2 related RNA. The liver is the only tissue where it is detected at significant levels, an observation that has been confirmed in at least three different inbred strains of mice (SWR, NZB and AKR). In contrast, the H-2K and H-2D products are expressed on virtually all cells in the body.

Significance to Biomedical Research and the Program of the Institute:

The classical transplantation antigens of the mouse are membrane glycoproteins composed of a single polypeptide chain about 346 amino acids long, noncovalently associated with a molecule of β^2 -microglobulin.

These antigens (class I antigens) are encoded by a family of genes, including the H-2K, H-2D, and H-2L loci, which have been mapped within the major histocompatibility complex (MHC) on chromosome 17. The cell surface presentation of these antigens is required for their role in allograft rejection, as well as in the associative recognition of viral and tumor antigens by cytotoxic T cells.

It is tempting to speculate that the putative protein product of our 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 determine what controls the expression of this novel H-2 related gene and to identify agents that can specifically affect its expression.

Publications:

Cosman, D., Khoury, G., and Jay, G.: Three classes of mouse H-2 messenger RNAs distinguished by analysis of cDNA clones. Nature, 295: 73-76, 1982.

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, 1982. In press.

Kress, M., Cosman, D., Khoury, G. and Jay, G.: Molecular cloning and expression of the gene for a novel transplantation-related antigen. In Pearson, M. L. and Sternberg, N. L. (Eds.): Gene Transfer and Cancer. New York, Raven Press, 1982. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05251-01 LMV

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Host Specific Activation of Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Laimonis Laimins	Guest Researcher	LMV, NCI
OTHER:	Peter Gruss	Visiting Scientist	LMV, NCI
	George Khoury	Chief	LMV, NCI

COOPERATING UNITS (if any)

Bruce Howard	LMB, NCI
Cornelia Gorman	LMB, NCI

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study examines 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, have been shown to be required for transcription. These sequences activate other genes placed 3' to the activators. Similar sequences have been identified in the long terminal repeat (LTR) of Murine sarcoma virus (MSV) and have been found to be capable of replacing the 72bp 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.

Project DescriptionObjectives:

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 acetyl transferase; cloning and restriction endonuclease analysis of DNA.

Major Findings:

1. MSV-derived 72 bp tandem repeat sequences enhance gene expression in a host specific manner. These sequences activate gene expression in mouse cells to a level approximately 10 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.
2. The SV40-derived 72 bp tandem repeats are not as host specific as are the MSV-derived repeats. In monkey kidney cells, the SV40 repeats enhance expression only 1.5 times the level seen in mouse cell lines. Similar results were also found in human HeLa cell lines.

Significance to Biomedical Research and the Program of the Institute:

The finding that some enhancer elements are host specific provides a model for one element which may play a role in the host range of DNA tumor viruses. Such sequences could also regulate eukaryotic gene expression.

Proposed Course:

The in vitro CAT assay will be used to study the exact sequences of the MSV repeats required for activation of expression. In addition, we will determine the effect of location and orientation of SV40 and MSV activation of expression.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05252-01 LMV

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
SV40 Mediated Expression of Cellular Transforming Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nadia Rosenthal	Guest Researcher	LMV, NCI
OTHER:	Peter Gruss	Visiting Scientist	LMV, NCI
	George Khoury	Chief	LMV, NCI

COOPERATING UNITS (if any)
Edward Scolnick Chief, LTVG, NCI
Ronald Ellis LTVG, NCI

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Md. 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 (200 words or less - underline keywords)

Two structurally distinct cellular genes from a rat genomic library can be identified by their close homology to the transforming gene of Harvey murine sarcoma virus. Experiments were designed to investigate the relative ability of these genes to direct the synthesis of the transforming gene product. Under the control of SV40 late regulatory elements, one of these genes (which contains several introns) produces both message and the transforming protein, whereas the other gene, which lacks introns, is transcribed efficiently but is translated only in greatly reduced amounts. This result correlates with the inability of the "intron-less" gene to transform NIH-3T3 cells under the control of a retroviral long terminal repeat and suggests that a mutation which reduces translational efficiency resides either within or very near the coding region of this gene.

Project DescriptionObjectives:

To study 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 1) which is closely homologous to the transforming gene of Harvey murine sarcoma virus. The viral gene, which encodes a 21K protein, synthesizes an unspliced message, whereas by comparison c-ras 1 contains several introns. A second rat cellular gene (c-ras 2) 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 1 gene transforms NIH-3T3 cells with high efficiency, whereas the c-ras 2 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 anti-sense orientation, are not transcribed. In vivo protein analysis demonstrated that the sense orientation of c-ras 1 synthesizes abundant 21 K protein. Surprisingly, the sense orientation construction of c-ras 2 produced greatly reduced amounts of a similar 21 K protein. In vitro translation of cytoplasmic poly A+ mRNAs synthesized by sense and anti-sense constructions of each gene yielded the same results, suggesting that the reduced level of c-ras 2 expression is due to a translational defect rather than the absence of a splicing event, and that this defect may underlie the low transformation efficiency of the c-ras 2 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 produce the transformed state, or in the case of c-ras 2, 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:

This study will be continued in the laboratory. The difference between the two genes that accounts for their differential expression will be determined by DNA sequencing, and further studies on expression will be based on these data.

Publications:

Rosenthal, N., Khoury, G., Ellis, R., Shih, T., Scolnick, E., and Gruss, P.: SV40 mediated expression of two cellular transforming genes. J. Cellular Biochem. Supp. 6: p. 251, 1982.

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Early Control Region of BKV

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nadia Rosenthal	Guest Researcher	LMV, NCI
OTHER:	Laimonis Laimins	Guest Researcher	LMV, NCI
	Peter Gruss	Visiting Scientist	LMV, NCI
	George Khoury	Chief	LMV, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The region of the human papovavirus BK, which corresponds to the early gene control region of SV40, includes a triplication of 68bp which may function in a way similar to the "activator" 72 bp repeats in SV40. To test their activating potential, the BKV repeats have been subcloned and are currently being tested for their activity in several systems available in this laboratory. A possible role of the repeats in determining the host range of the virus will be tested in human, monkey or mouse cells. Also, using the BKV 68 bp repeat region as a probe, experiments are in progress to isolate structurally similar sequences from a human genomic library.

Project Description

Objectives:

To characterize the early gene control region in BKV with respect to 1) its role as an activator of gene expression in eukaryotic cells, 2) its potential involvement in determination of the viral host range, and 3) its relationship to sequences in the human genome.

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.

Major Findings:

The structure and genetic organization of the human papovavirus BKV has been defined by complete sequencing (Seif, Khoury and Dhar, Cell 18: 963, 1979) and is similar to the monkey virus SV40, except for an area in the early gene control region where the two viruses are remarkably dissimilar. This region in SV40 harbors a pair of 72 bp repeats and has been defined by several laboratories as an "enhancer" or "activator" of early gene expression. In BK virus these repeats appear as a triplication of 68 bp. The dissimilarity between SV40 and BKV suggests that the early gene control region may play a role in the host range of these two viruses. To test this hypothesis the 68 bp repeat has been isolated from the BK genome and subcloned in a vector containing the bacterial gene coding for chloramphenicol acetyltransferase (CAT), an easily assayed enzyme. In this recombinant, synthesis of CAT in eukaryotic cells will be dependent on activation by the BK insert. By comparing the ability of the BK repeats to direct CAT synthesis in either CV1 (monkey) or HeLa (human) cell cultures, the potential role of the BK control region in host range determination will be investigated. If the BK repeats do indeed exhibit a host range effect, it may be that they are of cellular origin and are recognized by species-specific regulatory mechanisms in the human host cell. This hypothesis predicts that similar sequences may still exist in human or monkey cells and that they may retain an enhancer role in the host genome. Using the 68 bp repeat region of BK as a probe, experiments are in progress to isolate structurally similar sequences from a human genomic library.

Significance to Biomedical Research and the Program of the Institute:

BK virus 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 human cells.

Proposed Course:

We will continue to identify the regulatory elements required for expression of BK virus and its mutants. We will pursue the study of endogenous sequences homologous to those of the BKV.

Publications:

None.

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Insulin Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ruth Muschel	Medical Staff Fellow	LMV, NCI
OTHER:	Peter Gruss	Visiting Scientist	LMV, NCI
	George Khoury	Chief	LMV, NCI

COOPERATING UNITS (if any)

Dr. Lola Reid, Albert Einstein University Medical School, Bronx, New York

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
- (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Glucose concentration regulates the synthesis of insulin in pancreatic islets. We are studying the molecular mechanisms underlying this regulation, primarily at the mRNA level using both insulinoma cell lines and isolated pancreatic islets. We are in the process of developing cell conditions which will support the response of insulin synthesis to various stimuli. Preliminary results on stimulation of insulin synthesis by glucose indicate that while total amounts of cytoplasmic insulin RNA remain constant, there is an alteration in the size of these transcripts in response to changes in glucose levels. We are currently attempting to characterize the structural differences between these messages. In collaboration with L. Reid at Albert Einstein College of Medicine, we have demonstrated an approximately ten-fold increase in insulin-specific mRNA in rat insulinoma cells grown on extracellular matrix and serum-free medium as compared to cells grown on the usual tissue culture substrate in serum containing medium .

Project DescriptionObjectives:

1. To study molecular mechanism of the insulin response to glucose.
2. To investigate the structure of insulin mRNA and its precursors.

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:

1. Insulin specific mRNA is of different size depending upon glucose concentrations.
2. Growth of insulinoma cells on extracellular matrix in serum-free media markedly enhances levels of insulin mRNA.
3. Precursor mRNA for insulin may be of a larger size than predicted by the genomic sequence.

Significance to Biomedical Research and the Program of the Institute:

Insulin represents a gene of significant importance in normal eukaryotic metabolism and disease states. It also represents an excellent system for characterization of the expression of a highly regulated gene. An understanding of insulin gene regulation should provide an insight into a complex regulatory system.

Proposed Course:

Studies will be pursued with emphasis on the regulation of insulin gene expression.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05255-01 LMV

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Hormonal Regulation of Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael Kessel	Guest Researcher	LMV, NCI
OTHER:	Peter Gruss	Visiting Scientist	LMV, NCI
	George Khoury	Chief	LMV, NCI

COOPERATING UNITS (if any)

Gordon Hager	LTVG, NCI
Michael Ostrowski	LTVG, NCI

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To understand the regulatory elements which are involved in the initiation of transcription, we have studied a hormonally inducible element which is located on the long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV). Plasmids were constructed that contained the MMTV-LTR upstream from the prokaryotic gene chloramphenicol-acetyltransferase (CAT). After transfection into mouse L-cells, which were then grown in the presence or absence of the steroid hormone dexamethasone, the expression of CAT could conveniently be measured in cell extracts. Cells grown in the presence of dexamethasone produced about 9 times more CAT than hormone-free controls. Sequences of 300 bp or 1050 bp from the 5' end of the MMTV-LTR were deleted by a ClaI or a SacI cleavage, respectively. While the 300 bp deletion still maintained full inducibility, no induction was observed after the deletion of 1050 bp. We are currently investigating a series of exonuclease-generated deletions in order to precisely delineate the nucleotides required for inducibility.

Project DescriptionObjectives:

To understand the mechanism by which a hormonally regulated gene remains unexpressed in the absence of hormone and is activated in its presence.

Methods Employed:

Construction of chimeric genes, selective deletion of their regulatory sequences, DNA transfection into cultured eukaryotic cells, RNA analysis (Northern blots), and protein assays (chloromphenicol acetyltransferase determination in an enzymatic assay with TLC separation of the products, and quantification by liquid scintillation counting).

Major Findings:

1. The MMTV-LTR is able to confer hormonal inducibility to the CAT gene.
2. A second TATA box of SV40 origin between the CAT-gene and the LTR abolishes the inducibility rendering CAT production constitutive.
3. Deletion of 300 bp from the 5' end of the LTR retains inducibility, while deletion of 1050 bp abolishes active gene expression.

Significance to Biomedical Research and the Program of the Institute:

Understanding the mechanism of gene regulation is essential for approaching cell differentiation and transformation.

Proposed Course:

- (a) Fine mapping of the inducible region(s) on the MMTV-LTR by deletions generated with the exonuclease Bal 31.
- (b) Site-directed mutagenesis in the region(s) important for hormone inducibility (hormone receptor binding site).
- (c) Generation of constitutive mutations.
- (d) Functional comparison with other hormonally reactive sites.

Publications:

None.

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Mouse Lyt-2 Antigen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gilbert Jay	Visiting Scientist	LMV, NCI
OTHER:	George Khoury	Chief	LMV, NCI

COOPERATING UNITS (if any)

L. J. Old, Memorial Sloan-Kettering Cancer Center, New York, New York

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mouse Lyt-2 antigen is a T-cell marker, expressed on cytotoxic and suppressor (but not helper) T-cells. The importance of this surface antigen in T-cell functions is suggested by the finding that antibodies to Lyt-2 can block T-cell cytotoxicity in the absence of complement. In the present study, we have made use of monoclonal antibodies to determine the subunit structure of this antigen in thymocytes and in T-cell clones, and have entertained the possibility that it may be involved in the construction of one class of T-cell receptors.

Project DescriptionObjectives:

Studies on the molecular structure of the mouse Lyt-2 antigen by the use of monoclonal antibodies.

Methods Employed:

Proteins were analyzed by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

Thymocytes from BALB/c mice were radiolabeled in culture and the resulting cell extract was subjected to immunoprecipitation with a monoclonal anti-Lyt-2.2 antibody. Analysis of the immunoprecipitates on SDS-polyacrylamide gels in the presence of a reducing agent revealed three components that were specifically recognized by the monoclonal antibody; they have subunit molecular weights of 35,000, 30,000, and 28,000. By combining with polyacrylamide gel analysis in the absence of reducing agents, it was shown that there are two forms of the Lyt-2.2 antigen, each consisting of one molecule of the 28,000 M_r subunit covalently associated through disulfide bonds with either one 35,000 M_r subunit or one 30,000 M_r subunit. These two molecular structures are present in about equimolar ratios.

Significance to Biomedical Research and the Program of the Institute:

Three systems of surface antigens, Lyt-1, Lyt-2 and Lyt-3, characterize cells of thymic derivation in the mouse. As no other cell type has been found to express these components, Lyt antigens have been particularly useful as T-cell markers. Considerable interest in Lyt-2,3 antigens has been generated by the recent finding that conventional and monoclonal antibodies to these determinants in the absence of added complement can block T-cell cytotoxicity. It has been suggested that molecules bearing Lyt-2,3 determinants were in close spacial proximity to antigen receptors on T-cells, either on unrelated adjacent molecular structures or as an integral component of the receptor. The finding that Lyt-2,3 genes are tightly linked to structural genes for κ light chains adds support to the speculation that Lyt-2,3 components may be involved in the construction of one class of T-cell receptors in which κ chains and Lyt-2,3 chains would comprise the subunits of a functional recognition unit. Biochemical studies may provide an insight into the close linkage of the genes coding for Lyt-2 and -3 and kappa light chains and the meaning of specific blocking of cytotoxic T-cells by Lyt-2,3 antibody.

Proposed Course:

We are attempting to further define the molecular complexities of these surface antigens at both the structural and functional levels.

Publications:

Jay G., Palladino, M. A., 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.

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cloning of the Genes for Acetylcholine Receptor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ravi Dhar	Visiting Scientist	LMV, NCI
OTHER:	Amelia Nieto	Visiting Fellow	LMV, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have made a cDNA library from the electric organ of Torpedo californica. An 11 nucleotide primer which is complementary to a sequence about 150 nucleotides downstream from the encoded N-terminal amino acid was used to prime cDNA and electrophoresis of the product was performed on polyacrylamide gels. Individual cDNA bands were blotted on nitrocellulose filters and hybridized to a labeled probe of 14 nucleotides, representing the complementary strand. Three cDNA bands were positive and are presently under investigation.

Project Description

Objectives:

1. To isolate and sequence the clones coding for the acetyl choline receptor.
2. To study the regulation of the receptor in cells and its interaction with different chemical and pharmaceutical agents.

Methods Employed:

The standard cDNA and genomic cloning techniques have been employed in the generation of the libraries.

Major Findings:

We have made a cDNA library from the electric organ of *Torpedo californica*. This cDNA will be used to screen for acetyl choline receptor clones. The receptor consists of four known subunits of 65 K, 60 K, 45 K and 32 K daltons. The four subunits show extensive amino acid sequence homology at their N-termini. We are generating a probe to identify the subunits. An 11 nucleotide primer about 150 nucleotides downstream from the N terminus of the 60 K subunit was used to generate single stranded DNA. The cDNA was electrophoresed in polyacrylamide gels, and the 33 bands obtained were eluted and spotted on nitrocellulose filters. A 14 nucleotide complementary strand sequence was labeled at its 5' end with ³²P and hybridized to the dots on the filter. Three bands were positive. The sizes of the bands are approximately 210, 230 and 310 nucleotides. These bands have been cloned into pBR 322 and are now under further analysis.

Significance to Biomedical Research and the Program of the Institute:

It is believed that myasthenia gravis results from a defect in neuromuscular transmission which in turn is related to an autoimmune response to the receptor of motor end plates. This is also an optimal system for the study of gene regulation.

Proposed Course:

We propose to determine the structure of the four genes and to isolate the genomic clones. These will be used to study gene regulation.

Publications:

None.

ANNUAL REPORT OF THE LABORATORY OF TUMOR VIRUS GENETICS

NATIONAL CANCER INSTITUTE

October 1, 1981 through September 30, 1982

The Laboratory of Tumor Virus Genetics has continued to focus its studies on the characterization of the origin, structure and function of viral transforming genes and their gene products. Molecular clones of the Harvey and Kirsten murine sarcoma viruses have been used to study the transforming genes of these viruses (v-ras) genes) and their cellular homologues (c-ras genes). Differences have been found in the transforming efficiencies of two Harvey related c-ras genes when assayed by transfection of NIH 3T3 cells. Although both Harvey and Kirsten v-ras genes readily transform these cells, only the c-ras-1 gene could be shown to induce foci on NIH 3T3 cells; the c-ras-2 gene failed to do so. Reconstruction experiments between the viral Harvey ras and the cellular Harvey c-ras-2 genes revealed a translational defect in the 3' half of the latter gene. A novel ras translational variant, p28 ras, was found to be initiated from the rat-c-Harvey ras-2 promoter sequences. Both Harvey and Kirsten c-ras genes were found to be expressed by means of vastly different sized mRNAs which apparently differ in the lengths of their 3' untranslated regions. Experiments with the two different c-Kirsten ras mRNA species revealed no differences in their in vitro translational products. Nucleotide sequencing experiments have been initiated to understand the differences between the c-ras-1, c-ras-2 and v-ras genes and to determine the nature of the transforming defect in the c-ras-2 gene.

As a step toward understanding the molecular mechanisms of transformation, the biochemical properties of the p21 onc gene protein coded for by various members of the ras gene family have been investigated. The p21 proteins of Harvey v-ras and c-ras-1 genes have been purified to near homogeneity. While both proteins were found to bind guanine nucleotides, only Harvey v-ras-p21 autophosphorylates using GTP. The p21 of another ras gene containing virus, Balb-MuSV, was found to be similar to the p21 of Harvey c-ras-1. Amino acid sequencing studies on Harvey and Kirsten v-ras p21's have demonstrated similarities in both their in vitro and in vivo catalytic mechanisms and sites of autophosphorylation. Monoclonal antibodies to the p21 encoded by Harvey murine sarcoma virus have been produced and characterized. These reagents have been used to purify and study the expression of viral and cellular forms of p21 in several species including man. The ras gene of Harvey sarcoma virus has been cloned into a protein expression plasmid vector which allows the synthesis of p21 of *Escherichia coli*. The bacterial produced p21 retains its characteristic biochemical activities including GTP binding and autophosphorylation. Finally, cellular mutants resistant to transformation by Kirsten and Harvey murine sarcoma viruses have been isolated. NIH 3T3 cells containing two copies of Kirsten sarcoma virus and mutagenized with MNNG gave rise to flat revertant cells which contained both copies of the Kirsten virus genome, rescuable sarcoma virus, and high levels of the viral protein p21. The revertant phenotype was dominant in cellular hybrids formed after PEG fusion of flat revertants with Kirsten and Harvey virus-transformed cells. Fusion of the revertants with any of several cell lines transformed by other viruses, both RNA and DNA containing, and by chemically transformed cells

resulted in the formation of hybrid cells with a transformed phenotype. The results indicate that the Kirsten sarcoma virus revertants isolated here are mutants in a cellular gene.

Another family of transforming retroviruses has been used by members of the LTVG to study onc gene mediated tumorigenesis. Molecularly cloned provirus of the Snyder-Theilen (ST), Gardner-Arnstein (GA) and McDonough (SM) strains of feline sarcoma virus (FeSV) were used to characterize two viral oncogenes (v-fes and v-fms) responsible for FeSV-induced transformation and tumorigenesis. The complete nucleotide sequences of the ST and GA-FeSV v-fes genes, each of which encodes a tyrosine-specific protein kinase, have been determined. The predicted amino acid sequences of these proteins show that they are homologous to proteins encoded by other oncogenes (v-fps, v-src, and v-yes) which also exhibit tyrosine kinase activity. DNA transfection experiments using plasmids containing molecularly subcloned portions of GA-FeSV formally showed that v-fes sequences were necessary for transformation. Chimeric transforming viruses and mutants generated by recombinant DNA techniques were used to map viral functions which regulate expression of the transforming gene. The v-fms oncogene was shown to differ from v-fes and was demonstrated to encode a transforming glycoprotein. Monoclonal antibodies were prepared to the v-fms product and permitted assays of its steady state levels in transformed cells. Portions of the human c-fms proto-oncogene were molecularly cloned and partially characterized. Finally, FeSV was shown to transform spleen cells from fetal and newborn kittens. The transformed cells consistently gave rise to producer and nonproducer cell lines which contained FeSV transforming viral genes.

In other studies, the mouse mammary tumor virus (MMTV) has been used to study mammalian transcriptional control mechanisms. This system was suggested by earlier observations which showed that the rate of MMTV RNA synthesis was regulated by glucocorticoid hormones and that the recognition signals for this stimulation were part of the genomic structure of MMTV. During the last year deletion analysis of molecular chimeras between the MMTV LTR and the v-ras transformation gene from Ha-MuSV has been performed to identify and localize the steroid target site in the MMTV LTR. The hormone regulatory sequences are located within 200 nucleotides of the MMTV cap site. NIH 3T3 cells transformed with the MMTV v-ras fusions have been established in serum-free media. The transformation phenotype of these cells is under the control of glucocorticoids; concentration dependent phenotype switching parallels the binding curve for dexamethasone binding to the glucocorticoid receptor. This system is being exploited to select cellular mutants in the response to p21 transformation and in the hormone pathway. Finally, a new gene (pLTR) has been discovered at the 3' terminus of mouse mammary tumor virus (MMTV) within the long terminal redundancy (LTR). The presence of this gene has now been confirmed in exogenous C3H-S MMTV, and in two endogenous proviruses of C3H, Mtv-1 (unit V) and unit II. A pLTR message encoding the information for this gene is uniquely expressed in preneoplastic mammary tissue induced in Balb/c animals by the chemical carcinogen DMBA. The role of this gene in mammary carcinogenesis and/or MMTV regulation is under investigation.

Studies on Friend virus induced leukemia have continued. Friend spleen focus-forming virus (SFFV) is a replication defective virus which induces erythroleukemia in susceptible mice. Previous studies in this laboratory have localized the leukemogenic activity of this virus to the 1,500 bases of the viral genome coincident with the env gene. Some SFFV isolates induce polycythemia (SFFV-P) while others induce anemia (SFFV-A). Phenotypic differences in the in vitro erythroid effects

of these viruses have also been recently described in the LTVG. To understand these differences at the molecular level, the env genes of SFFV-P and SFFV-A were cloned, genetically recombined with non-leukemogenic helper viruses and tested *in vivo*. The pathology of the erythroleukemia in susceptible mice was entirely consistent with the type of SFFV env gene contained in the virus to which the animals were exposed. These experiments demonstrate that the env gene of SFFV is sufficient to induce disease and the biological differences between the polycythemic and anemic strains of these viruses map within this gene. In other studies on the Friend virus complex, certain mouse strains were found to endogenously express a novel glycoprotein, related to viral MCF gp 70, which confers resistance to leukemogenesis by a mechanism analogous to viral interference. Studies are in progress to determine how SFFV and Friend-MCF virus, which encode highly related envelope glycoproteins, interfere with erythropoiesis and why certain strains of mice are resistant to disease induced by these viruses. Erythroleukemia cell lines derived from spleens of mice infected with F-MuLV or the F-MuLV/SFFV complex were found to express high levels of a transformation-related phosphoprotein, p53. Studies are in progress to determine if an endogenous onc gene is expressed at high levels in these cells. Finally, an assay has been developed for a heretofore unidentified type of transformed cell encountered in the early stages of Friend-MuLV induced erythroleukemia. These erythropoietin sensitive cells may represent pre-leukemic elements and are being examined to determine their role in the pathogenesis of this disease. Conditions have been established for the routine derivation of hemopoietic cell lines from murine and human hemopoietic neoplasms. These cell lines can be used to study unique viral integration sites, tumor heterogeneity, and identification of factors which control the proliferation and differentiation of these cultured cells.

The molecular mechanism(s) of oncogenesis induced by retroviruses that lack a transforming gene have also been investigated. Two complementary approaches have been followed; (1) analysis of the genetics of avian retroviruses as they relate to the virus oncogenic potentials and, (2) analysis of the molecular mechanisms of oncogenesis in rats infected with Moloney MuLV. In the first part of these studies, the U₃ region of the viral LTR was shown to determine the ability of the virus to induce disease. However, sequences outside the LTR determined the spectrum of disease induced. A detailed genetic analysis is now in progress to identify the sequences responsible for the oncogenic spectrum of a given virus, and the mechanism by which their function is mediated. In the second part of those studies, two preferred virus integration events have been identified in rat thymic lymphomas induced by Moloney MuLV. Both of these integration events appear simultaneously in some tumors indicating at least two steps in the mechanisms for oncogenesis. The cellular sequences in the vicinity of the virus integration in one of these preferred integration events do not have the characteristics of an oncogene. In this sense, the mechanism by which viral integration influences the induction of disease in this system is other than "promoter insertion". In order to explain the results of these studies a model of "insertional mutagenesis" for retrovirus induced oncogenesis has been proposed.

In summary, this last year of operation of the LTVG under its present chief finds many of its research goals realized. Several retroviruses have been used to isolate and characterize, both molecularly and biologically, individual genes which are directly responsible for cell transformation *in vitro* and tumorigenesis *in vivo*. The protein products of these onc genes have been identified and their

role in malignant transformation has been unambiguously established. The existence of cellular homologues of these viral transforming genes in normal cells has been demonstrated and these highly conserved genetic elements have been shown to possess transforming activity. Incorporation of these cellular transforming genes into retroviruses by genetic recombination has been clearly shown. Recent work in several laboratories has pointedly underscored an important role for at least one class of these genes (ras) in several types of human cancer which collectively account for a high percentage of malignancies seen in the United States. Work with these viral systems has provided, for the first time, a rational approach and the necessary reagents for an in depth program to study the mechanisms involved in the initiation, diagnosis, prognosis and treatment of human cancer at the molecular level.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 04848-10 LTVG
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PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
RNA Tumor Viruses: Replication, transformation and Inhibition in Cell Cultures

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R. H. Bassin	Head, Viral Biochemistry Section	LTVG	NCI
Other:	M. Noda	Visiting Fellow	LTVG	NCI
	G. Hager	Head, Viral Immunogenetics Section	LTVG	NCI
	E. Scolnick	Chief, LTVG	LTVG	NCI

COOPERATING UNITS (if any)
Z. Selinger, Hebrew University, Jerusalem, Israel

LAB/BRANCH
Laboratory of Tumor Virus Genetics

SECTION
Viral Biochemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4.0	PROFESSIONAL: 2.0	OTHER: 2.0
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CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
 1. We have isolated cellular mutants resistant to transformation by Kirsten and Harvey murine sarcoma viruses. NIH/3T3 cells containing 2 copies of Kirsten sarcoma virus and mutagenized with MNNG gave rise to flat revertant cells which contained both copies of the Kirsten virus genome, rescuable sarcoma virus, and high levels of the viral protein p21. The revertant phenotype was dominant in cellular hybrids formed after PEG fusion of flat revertants with Kirsten and Harvey virus-transformed cells. Fusion of the revertants with any of several cell lines transformed by other viruses, both RNA and DNA containing, and by chemically transformed cells resulted in the formation of hybrid cells with a transformed phenotype. The results indicate that the Kirsten sarcoma virus revertants isolated here are mutants in a cellular gene.
 2. Cells transformed by a molecular chimera formed by the ligation of v-ras, a gene which codes for the p21 protein responsible for transformation by Kirsten and Harvey sarcoma viruses, and the Long Terminal Repeat of MMTV, which confers susceptibility to glucocorticoid hormone regulation on nearby genes, were adapted to growth in serum-free culture conditions in preparation for quantitative studies on hormone interactions with regulatory MMTV sequences.

Project Description

Objectives:

1. The primary objective of this study is the identification of cellular products which interact directly with the p21 protein kinase of Kirsten and Harvey viruses in the cell transformation process. A knowledge of the function of these cellular "target" molecules should lead to a better understanding of the transformation process itself. A secondary objective is to classify cells transformed by various agents according to functional criteria.

2. The objective of these experiments is to define the response of cells transformed with the MMTV-KiMSV chimera to various hormones in reproducible and quantitative terms and to isolate viral mutants, particularly in the MMTV LTR's which will be of use in defining the interaction of hormones with cellular receptors and DNA sequences.

Methods Employed:

1. In addition to the standard techniques of cell culture as applied to RNA tumor virology used previously in this laboratory, this project involves several additional techniques and methods. The use of ouabain to select revertant cells as well as the employment of Rb⁸⁶ to measure influx and efflux of potassium ions in cells has been developed. DNA hybridization following transfer to nitrocellulose filter paper (Southern blot technique) is used to measure the number of copies of viral DNA in revertant cells. The amount of viral p21 present in various cell lines is measured by the Western blot technique. Transfection of both cellular and viral DNA has also been used in this study. A major effort has been given to the development of fusion techniques whereby the phenotype of fused normal and transformed cells both in agar suspension cultures and in monolayers can be assessed. The characteristics of mutagenesis of cells with MNNG have also been worked out for Kirsten virus-transformed NIH/3T3 cells.

2. Published procedures for the adaptation of NIH/3T3 cells to growth in serum free medium have been duplicated in our laboratory. Techniques of cell mutagenesis acquired during the study of cell revertants described in Section 1 have also been adapted for use in this project.

Major Findings:

1. Initial studies showed that ouabain, a specific inhibitor of Na⁺/K⁺ ATPase, was able to kill Kirsten-transformed cells at a faster rate than it did normal NIH/3T3 cells. Using a ouabain selection system coupled with cells which were doubly infected with Kirsten sarcoma virus to reduce the number of viral mutants, we were able to isolate flat revertants from mutagenized Kirsten virus-transformed cells. Several criteria indicated that these flat revertants were in fact cellular mutants resistant to the transforming activity of Kirsten sarcoma.

The cellular DNA of the revertants contained two copies of Kirsten sarcoma virus DNA, as did the original transformed cells from which they were derived. In addition, virus could be rescued from all or nearly all of the revertant cells following superinfection with Moloney or Amphotropic murine leukemia viruses. Preliminary studies indicate that the Kirsten-specific p21 transformation-related protein is present in both revertant and Kirsten virus-transformed cells at approximately the same concentration. These studies all indicate that the revertant cells contain a normal Kirsten sarcoma virus genome(s) which produces normal amounts of viral p21. It seemed likely that these revertant cells were in fact mutants in a cellular gene whose product was involved in transformation by Kirsten sarcoma virus, perhaps as the direct "target" for the activity of the p21 viral protein.

In an effort to define further the nature of the mutation leading to expression of the revertant phenotype in these cloned cell lines, we fused them to a series of transformed cells and determined whether the fused heterokaryons exhibited a transformed or revertant phenotype, as determined both by colony formation in soft agar and by appearance in monolayer cultures. Revertants fused to Kirsten and Harvey transformed cells gave rise to heterokaryons which were flat, while revertants fused to polyoma, SV40 and a chemically transformed tumor cell line were transformed. Each of the two revertants tested in these fusion studies gave similar results. A large number of additional fusion experiments are contemplated.

2. NIH/3T3 cells can be adapted to serum-free medium using published procedures. Hydrocortisone is not necessary for the propagation of cells containing the MMTV LTR coupled to Kirsten MSV chimeric DNA. In this system, dexamethasone is active in causing expression of MSV-related cell transformation at 2.5×10^{-8} M or less. Preliminary data indicate that these cells can be grown in semisolid agar cultures in the absence of serum.

Significance to Biomedical Research and the Program of the Institute:

1. This program is designed to examine the cellular constituents which are involved in the transformation process. This could lead to insights into the mechanism of transformation, providing a rational basis for reversing the transformation process. In this light, we are currently investigating the differential effect of ouabain on the normal and transformed cells used in the experiments described here. In addition, the revertants described here can be used to define agents which transform cells by the same functional pathway, even though such agents may differ with regard to the means by which they effect the basic cell processes.

2. The development of a serum-free system for investigating the effect of hormones and other agents on the MMTV LTR-dependent expression of p21 in NIH/3T3 cells is important for the quantitative study of hormone effects on transcription of viral RNA as well as the isolation of cellular mutants representing alterations in the response of DNA sequences to glucocorticoid hormones.

Proposed Course:

1. Biological properties of cell hybrids formed between the revertants of Kirsten-transformed cells and a variety of transformed cells will be described. These studies will also be extended to primary tumor cells including human tumor cells. Patterns of transformation in cell hybrids will be noted in order to establish functional similarities among different groups of transformed and tumor cells, perhaps transformed by different oncogenic agents. The differential effect of ouabain on NIH/3T3 cells and their Kirsten sarcoma virus-transformed derivative will be examined. The role of Na⁺/K⁺ ATPase mutations in the derivation of flat revertants will be studied by using the K⁺ analog ⁸⁶Rb to measure the activity of the enzyme. Also, the behavior of ouabain-resistant mutants of normal NIH/3T3 cells in heterokaryons formed by fusion with a variety of transformed cells will be studied in detail. This project will thus advance in two directions: A study of the mechanism of transformation of Kirsten sarcoma virus and related agents (which appear to include the oncogenic agents involved in some human tumors), with emphasis on the role of Na⁺/K⁺ ATPase; and the use of revertants of transformed cells to identify groups of oncogenic agents which use the same functional mechanisms to transform cells.

2. Interactions of various hormones with MMTV LTR's will be assessed using the induction of Kirsten sarcoma virus RNA, p21 protein, and morphological transformation as parameters. We will attempt to isolate and characterize mutants which represent cellular genes altered in their response to glucocorticoid hormones using this system.

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 (In press).

Duran-Troise, G., Bassin, R. H., Wallace, B. F., and Rein A.: Balb/3T3 Cells Chronically Infected with N-Tropic Murine Leukemia Virus Continue to Express Fv-1^b Restriction. J. Virol. 112: 795-799, 1981.

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 Viral Vectors, Cold Spring Harbor Laboratory (In press).

Rein, A., Bader, J. P., and Bassin, R. H.: Inhibitors of Glycosylation Reverse Retroviral Interference. Virology 119: 185-192, 1982.

Rein, A., Lowy, D. R., Gerwin, B. I., Ruscetti, S. K., and Bassin R. H.: Molecular Properties of a Gag⁻ Pol⁻ Env⁺ Murine Leukemia Virus from Cultured AKR Lymphoma Cells. J. Virol. 41: 626-634, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04858-10 LTVG

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Control of Growth of Cells by Ras Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward M. Scolnick	Chief	LTVG	NCI
OTHER:	Douglas Lowy	Medical Officer	DCB	NCI
	Ronald Ellis	Senior Staff Fellow	LTVG	NCI
	Thomas Shih	Research Chemist	LTVG	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Molecular Virology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

4.0

PROFESSIONAL:

2.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The in vivo and in vitro properties of the transforming protein, p21 coded for by various members of the ras gene family have been investigated. The p21 coded for by v-ras-H has been purified to near homogeneity. The protein is a guanine nucleotide binding protein and autophosphorylates itself using GTP. The p21 coded for by c-ras-H, a normal cellular protoncogene has also been purified and found to be a guanine nucleotide binding protein. However, p21 coded for by c-ras-H-1 does not autophosphorylate. Thus, a major qualitative difference exists between the p21 of v-ras-H and the p21 of c-ras. The p21 of another ras gene containing virus, Balb-MuSV, is similar to the p21 of c-ras-H-1.

Objectives:

The goal of this project is to define the biochemical pathways involved in different forms of different cancers and to define the normal physiological roles for the p21 protein in cellular functions, especially homopietic cellular functions. The long range goal is to refine assays for p21 so that they can be used in diagnosis of human diseases including cancer.

Methods Employed:

A. Pulse labelling of cells with ^{35}S -methionine and immunoprecipitation using *Staphylococcus aureus* protein A. Analysis of precipitates on polyacrylamide slab gels. Fluorographic enhancement of protein bands. Tryptic peptide analyses using two-dimensional thin layer plates.

B. Restriction enzyme analyses of cloned and amplified sarcoma virus DNA. Southern transfer blotting assays and ethidium bromide containing cesium chloride gradients.

C. Biochemical assays for phosphorylation and dephosphorylation of proteins. Gel electrophoresis, charcoal extractions, butanol two-phase extractions, thin layer chromatography.

D. Column chromatography using ion-exchange, hydroxiapatite, and hydrophobic column chromatography. Analysis of peptides by two-dimensional thin layer chromatography.

E. Gene transfer studies using calcium phosphate precipitation; gene cloning using a variety of bacterial vectors.

Major Findings:

A biochemical purification method has been devised for obtaining purified p21 from transformed cells. A major purification is achieved by first purifying the plasma membranes of ras transformed cells. The p21 is then solubilized with nonionic detergent and subjected to four successive purification steps: ammonium sulfate salting out; DEAE cellulose chromatography; phenylsepharose chromatography; HPLC molecular size analysis. The p21 can be visualized on acrylamide gels stained with silver chloride and is over 50 percent pure.

All biochemical activities, previously associated with p21, copurify with the v-ras^H p21 molecules. These include GTP specific binding and GTP specific autophosphorylating activity. In contrast, the p21 of c-ras^H-1 contains only GTP binding activity and does not autophosphorylate.

Other p21 molecules, coded for by other ras genes, have also been studied. The p21 of a human c-ras-1 gene, Balb-MuSV, and the Rasheed sarcoma virus all share the GTP binding activity but lack the autophosphorylating activity. The results

indicate that GTP binding activity is common to all known ras gene p21 molecules and suggest that the binding property is necessary for the biochemical and biological activities of p21 ras.

Significance to Biomedical Research and the Program of the Institute:

Recently, we and others have shown that ras genes play a major role in the etiology of certain human epithelial cancers including bladder, lung, and colon cancer. Understanding how p21 ras works to cause malignant transformation is a pivotal question in cancer research and may be a rate limiting step in evolving new methods for the diagnosis and treatment of human epithelial cancers.

Proposed Course:

This intramural project will terminate as the principal investigator is leaving the National Cancer Institute.

Publications:

Hankins, W. D. and Scolnick, E. M.: Harvey and Kirsten sarcoma viruses promote the growth and differentiation of erythroid precursor cells in vitro. Cell 26: 91-97, 1981.

Ruscetti, S. K., Feild, J. A., and Scolnick, E. M.: Polycythemia- and anemia-inducing strains of spleen focus-forming virus differ in post translational processing of envelope-related glycoproteins. Nature 294: 663-665, 1981.

Shih, T. Y., Weeks, M. O., Gruss, P., Ohar, R. Oroslyan, S. and Scolnick, E. M.: Identification of a precursor in the biosynthesis of the p21 transforming protein of Harvey murine sarcoma virus. J. Virol. (In press).

Z01 CP 04963-07 LTVG

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemical Studies on Ha-MuSV/Ki-MuSV and Mechanism of Cell Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. :	T. Y. Shih	Research Chemist	LTVG	NCI
Others :	E. M. Scolnick	Chief	LTVG	NCI
	R. Ellis	Staff Fellow	LTVG	NCI
	M. Furth	Staff Fellow	LTVG	NCI
	M. Ruta	Staff Fellow	LTVG	NCI

COOPERATING UNITS (if any)

S. Oroszlan, Biological Carcinogenesis Program, FCRF, Frederick, Maryland 21701
G. Khoury, P. Gruss and R. Dhar, LMV, NCI
D. Lowy and E. Chang, DBCD, NCI

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Molecular Virology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The p21 proteins, the onc gene products of Harvey and Kirsten murine sarcoma viruses, are directly responsible for the virus-induced malignant transformation of the infected cells. As a step toward understanding the molecular mechanism of transformation, the biochemical properties of the p21 proteins were studied. The complete amino acid sequence of the p21 has been determined by two means: (1) DNA sequencing of the p21 coding region; (2) confirmation of the predicted sequence by direct amino acid sequencing of peptides derived from radio-labeled p21 protein. The p21 of Ha-MuSV possesses GTP-binding and autophosphorylation activities. By comparing the in vivo and in vitro sites of phosphorylation, it is concluded that the in vitro enzyme activity is responsible for the in vivo p21 phosphorylation. The amino acid sequences surrounding the phosphorylation site has been determined by sequencing the phosphopeptides. Both the Harvey and the Kirsten p21s share the same phosphorylation site.

Project DescriptionObjectives:

The scope of this project is to study the molecular biology of the Harvey and Kirsten strains of murine sarcoma viruses and the molecular mechanism of malignant transformation induced by these viruses. The major objective of this project is to characterize the biochemical properties of the p21 transforming proteins encoded by these viruses and their cellular homologues, and to investigate the structure-function relationship of the p21 proteins. From this model system, we hope to learn the biochemical steps leading to conversion of normal cells into cancer cells and to develop the rational basis for chemo-prevention and chemotherapy of p21-related human neoplasia.

Methods Employed:

1. Cells labeling and immunoprecipitation: Cells were labeled with ^{35}S -methionine or other radioactive amino acids for special purposes. The p21 proteins were identified by immunoprecipitation with antibodies to the p21 protein. The immunoprecipitated proteins were analyzed by SDS-gel electrophoresis and visualized by autoradiography. For studying the phosphorylation sites, the proteins were labeled by ^{32}P -orthophosphate.

2. Radiochemical sequencing of peptides: The p21 was labeled by in vivo incubation of the virus-transformed cells with ^3H -amino acid mixture containing lysine, leucine, phenylalanine, tyrosine and proline at high specific activity. The labeled p21 was then purified by SDS-gel electrophoresis following immunoprecipitation with antibodies directed against p21. The p21 protein bands were then eluted from gels, dialyzed and lyophilized. Peptides generated from V8 protease or trypsin digestion of the purified p21 were then resolved by the high performance liquid chromatography (HPLC). The amino acid sequences of these peptides were determined by the automated N-terminal Edman degradation in the Beckman sequencer.

3. Determination of the phosphorylation site: To determine the amino acid sequence surrounding the phosphorylation site, the p21 was labeled by autophosphorylation with γ - ^{32}P -GTP. The phospho-peptide generated by trypsin cleavage was subjected to automated N-terminal Edman degradation. From the location of the ^{32}P -labeled threonine residue from the N-terminal tryptic cleavage site, and the theoretical possible phospho-peptides predicted by the p21 amino acid sequence, it is possible to identify the phosphorylation site within the p21 molecule.

4. Peptide mapping: The peptides generated by protease digestion were also mapped by a 2 dimensional procedure on the thin-layer chromatography (TLC) plates. The first dimension is electrophoresis and the second dimension is chromatography.

5. Phospho-amino acid analysis: The ^{32}P -labeled p21 was hydrolyzed in 6N HCl and the phospho amino acids were identified by a 2-dimensional procedure.

Major Findings:

1. The amino acid sequence of the p21 protein of Ha-MuSV. As a baseline information for the structure-function studies of the p21, we sought to determine the reliable amino acid sequence of this protein. In collaboration with R. Dhar and his colleagues, the p21 coding region of the Ha-MuSV ras gene DNA has been sequenced. To ensure that the predicted amino acid sequence is correct, many peptides derived from V8 protease or trypsin cleavage were purified and the radio-labeled amino acids were sequenced.

Peptides covering most of the p21 molecules were sequenced, and we were quite confident that a reliable amino acid sequence of the Ha-MuSV p21 has been obtained. The Ha-MuSV p21 contains 189 amino acid residues. Comparison of the Ha-MuSV p21 sequence with the predicted amino acid sequence of Ki-MuSV p21 from the DNA sequence determined by Tsuchida et al, it is found that the amino acid sequences of these two p21 proteins are highly conserved, and the only regions showing substantial differences are the last 24 amino acid residues at the C-terminal ends. Despite the high conservation of these two protein sequences, the nucleotide sequences show substantial difference so that a DNA probe prepared from Ha-MuSV ras gene does not hybridize to the Ki-MuSV ras gene DNA under stringent DNA/DNA hybridization condition. Most of these nucleotide sequence mismatches derive from the third base differences in the codon usage for the same amino acid. This observation suggests that the p21 ras gene family may originate from a common ancestral proto-ras gene. Differentiation of these genes during evolution may be selected by the different regulating mechanisms rather than by the functions of the protein molecules.

2. The Phosphorylation Site of the Ha-MuSV p21. The p21 proteins of Ha-MuSV and the closely related Ki-MuSV are heavily phosphorylated in vivo. In the partially purified Ha-MuSV p21, the protein shows a guanine nucleotide-binding activity and, in addition, a very unique autophosphorylating activity at a threonine residue using as phosphoryl donor GTP but not ATP. We have compared the tryptic peptide maps of the Ha-MuSV p21 phosphorylated in vitro and in vivo. The results show that the major phosphorylation site is identical. Since the GTP-specific phosphorylation is very unique and distinct from all other known protein kinases, the present observation suggests that the in vitro enzymatic activity is responsible for the p21 phosphorylation in vivo.

We have analyzed the amino acid sequences surrounding the major phosphorylation site of the Ha-MuSV p21 by automated Edman degradations of the tryptic phosphopeptides. Threonine residue 59 from the initiator methionine residue 1 of the p21 protein is the phosphorylated amino acid residue, and the surrounding amino acid sequence is: NH₂...Thr-Cys-Leu-Asp-Ile-Leu-Asp-Thr-Thr(p)-Gly-Gln-Glu-Glu-Tyr...COOH. The p21 proteins of both the Ha-MuSV and the closely related Ki-MuSV share the same phospho-peptide. The amino acid sequences of the phosphorylation site is distinct from all other known protein kinases.

Although the physiological significance of p21 phosphorylation is unknown at present, the identification of the autophosphorylation site sequence will provide a handle to probe for the enzymatic activity of the p21 proteins such as the potential novel protein kinase activity whose target substrate proteins are still a mystery.

3. The Biosynthesis of Ha-MuSV p21. The pathway of p21 biosynthesis involves a precursor, pro-p21. Shortly after its synthesis on the free polysomes in the cytosol, the pro-p21 is processed into a mature form, the p21 then becomes associated with plasma membrane. Subsequently, the p21 is autophosphorylated into the phosphorylated form, the pp21. The precise biochemical nature of the p21 processing is still unknown. From the typtic peptide mapping of the p21 labeled with ³⁵S-cysteine, we found an extra peptide which is present in the pro-p21 but is absent in the processed p21. This experiment suggests that the processing may involve peptide cleavage.

Significance to Biomedical Research and the Program of the Institute: Very recently, several laboratories including R. Weinberg at MIT, G. Cooper at Sidney Farber Cancer Institute, M. Wigler at the Cold Spring Harbor Laboratory, M. Barbacid and S. Aaronson at NCI have independently found that the transforming genes cloned from human cancers such as bladder, colon and lung carcinomas are related to the *ras* genes of the Harvey and Kirsten murine sarcoma viruses. Presumably these cancer genes are cellular homologs of the viral transforming genes. This exciting finding represents one of the most profound advances in fundamental cancer research. The model systems we are studying in the animal viruses and the biochemistry of the viral transforming proteins are no longer just purely academic pursuits. The knowledge obtained from these studies is directly relevant to the understanding of the most prevalent forms of human malignancy. Studies on these problems will no doubt give us much better understanding on mechanisms of carcinogenesis; how chemical, physical and other environmental factors contribute to carcinogenesis; and the molecular mechanisms by which these transforming proteins convert normal cells into cancer cells. Eventually the knowledge obtained from these studies will provide us a rational basis to devise means for prevention and treatment of human cancers.

Proposed Course:

The biochemical properties of the p21 will be characterized further and its interaction with the cellular machinery will be studied in order to elucidate the biochemical mechanisms of malignant transformation of normal cells. Attempts will be made to understand how other carcinogenesis factors are involved in the *onc* genes and gene products in the mechanisms of carcinogenesis. Possible means of chemo-intervention in the carcinogenesis processes will also be explored.

Publications:

Shih, T. Y. Expression of the cloned p21 transforming *ras* gene of Harvey murine sarcoma virus. In Genetic Engineering Techniques: Recent Developments (Huang, P. C., Kuo, T.T., and Wu, R., Eds.): New York Academic Press. (In press)

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 (In press)

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Shih, T. Y., Weeks, M. O., Gruss, P., Dhar, R., Oroszlan, S. and Scolnick, E.M.: Identification of a precursor in the biosynthesis of the p21 transforming protein of Harvey murine sarcoma virus. J. Virol. 42: 253-261, 1982.

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PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Proteins Associated with the Biological Effects of Murine Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others:	E. M. Scolnick	Chief	LTVG, NCI
	L. Wolff	Postdoctoral Fellow	LTVG, NCI
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COOPERATING UNITS (if any)

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1.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Friend MCF virus is a crucial intermediate in the induction of erythroleukemia after infection of newborn mice with Friend MuLV. Certain mouse strains endogenously express a novel glycoprotein, related to viral MCF gp70, which confers resistance by preventing the replication and spread of MCF viruses by a mechanism analogous to viral interference. Adult mice of susceptible strains are also resistant to F-MuLV-induced disease as the result of the failure to replicate MCF viruses due either to a lack of a suitable number of target cells or an effective immune response against the MCF viral envelope. Studies are presently being carried out to determine how the spleen focus-forming virus (SFFV) and Fr-MCF virus, which encode highly related envelope glycoproteins, interfere with erythropoiesis and why certain strains of mice are resistant to disease induced by these viruses. Erythroleukemia cell lines derived from the spleens of mice infected with either F-MuLV or the F-MuLV/SFFV complex were found to express high levels of a transformation-related phosphoprotein, p53. Studies are in progress to determine if an endogenous onc gene is being expressed at high levels in these cells.

Project Description

Objectives:

- (1) To determine the mechanisms by which the spleen focus-forming and Friend MCF viruses alter erythropoiesis.
- (2) To determine the etiology of the second stage diseases induced by the spleen focus-forming and Friend MCF viruses.
- (3) To further understand the mechanisms of resistance of mice to diseases induced by murine leukemia viruses.

Methods Employed:

- (1) Preparation of specific antisera to viral-encoded proteins, particularly the preparation of monoclonal antibodies.
- (2) Use of specific antisera to precipitate proteins from cytoplasmic extracts of metabolically labeled virus-infected cells and tissues and analysis of immune precipitates by SDS-polyacrylamide gel electrophoresis and autoradiography.
- (3) Analysis of viral protein expression in individual cells by immunofluorescence.
- (4) Development of in vitro assays to study erythropoiesis.
- (5) Injection of various strains and genetic crosses of mice with murine leukemia viruses and analysis for development of leukemia.

Major Findings:

- (1) Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection.

We previously demonstrated that MCF viruses play a crucial role in the generation of erythroleukemia after injection of certain strains of newborn mice with ecotropic Friend MuLV and that resistance to this disease is mediated through the endogenous expression of an MCF/xeno-gp70-related protein that interferes with the replication and spread of MCF viruses. In order to test the possibility that this novel envelope protein is responsible for resistance to F-MuLV-induced disease by a mechanism analogous to viral interference, studies were carried out in collaboration with Dr. R. Bassin of this laboratory. It was shown that DBA/2 embryo fibroblast lines are resistant to infection with MCF viruses in vitro. Treatment of these cells with inhibitors of glycosylation, such as 2-deoxy-D-glucose and tunicamycin, which have recently been shown to drastically

reduce viral interference in MuLV-producing fibroblasts, rendered these cells significantly more susceptible to infection with several MCF isolates.

These drugs were shown by pulse-labeling and immune precipitation studies to effect the addition of sugar moieties to the MCF/xeno-related envelope glycoprotein expressed in the DBA/2 fibroblasts, and cell surface labeling followed by immune precipitation indicated that the expression of this protein at the cell surface was altered. These data indicate that the endogenous expression of MCF/xeno-related envelope glycoproteins in DBA/2 cells can confer on these cell resistance to MCF infection by a mechanism analogous to viral interference. This mechanism may be the basis for the resistance not only to Friend MCF-induced disease but also to other leukemias that involve the generation and spread of MCF viruses.

(2) Adult NIH Swiss mice are resistant to F-MuLV-induced erythroleukemia due to the failure of MCF viruses to replicate.

In contrast to newborn NIH Swiss mice, untreated adult mice are resistant to F-MuLV-induced erythroleukemia. Data generated in this laboratory have indicated that this increasing resistance with age is due to the failure to replicate MCF viruses due either to a lack of a suitable number of target cells into which the virus can integrate or an effective immune response against the MCF viral envelope.

(3) F-MuLV-induced murine erythroleukemia cell lines can be induced to differentiate into mature red blood cells.

Until recently, all Friend murine erythroleukemia cell lines (MEL) contained both Friend MuLV and the spleen focus-forming virus (SFFV). In 1981, Oliff et. al. described several transplantable cell lines, designated 57-MEL, derived from leukemic mice infected only with F-MuLV and subsequently shown to be free of SFFV. In order to determine if these 57-MEL lines, like the classical MEL lines, could be induced with various chemicals to differentiate along the erythroid pathway, studies were carried out in collaboration with Drs. F. Ruscetti and A. Oliff. It was shown that while many of the chemicals capable of inducing the classical MEL lines were unable to induce 57-MEL cells to differentiate, the latter cells could be induced to differentiate along the erythroid pathway with hemin. The occurrence of erythroid differentiation was judged by the development of benzidine positive cells, a markedly red color visible when the treated cells were pelleted, and hemoglobin synthesis.

(4) Erythroleukemia cell lines derived from mice infected with F-MuLV or FVP express high levels of a transformation-related protein.

It has been suggested that the erythroleukemias induced either by SFFV or Friend MuLV can be divided into two stages. Stage 1 is characterized by the rapid hyperplasia of erythroid precursor cells induced by the virus infection. These cells have a limited proliferative capacity and are not transplantable. Stage 2 is characterized by the appearance of transplantable malignant erythroblasts that can be grown as permanent cell

lines in culture. In an effort to determine whether biochemical differences could be found in cells obtained from these different stages, they were examined for the expression of a transformation-related protein, p53. This protein has been detected in a range of neoplastic cell types that were spontaneously transformed or transformed by viruses, chemicals or x-rays. When stage 2 cell lines derived from newborn mice infected with F-MuLV or adult mice infected with FVP were pulse-labeled and immune precipitated with a monoclonal antibody to p53, high levels of this protein were detected. No p53 could be detected in stage 1 spleens from mice infected 2-4 weeks previously with F-MuLV or FVP or in erythroid cell lines derived by in vitro infection of bone marrow cells with FVP. The p53 in the erythroleukemia cell lines is phosphorylated and is expressed in cells in both stationary and logarithmic phases of the growth cycle. When these lines are induced to differentiate into mature red blood cells, p53 levels are unchanged. These results indicate that p53 may be a marker for transformed erythroblasts present in the second stage diseases induced by F-MuLV or FVP.

(5) The endogenously acquired portion of Friend MCF gp70 maps to the N-terminus.

The env gene products of Friend MCF virus and SFFV were characterized using partial V8 protease digestion and immune precipitation. A MCF/xeno-specific monoclonal antibody precipitated 23K fragments from both Friend MCF gp70 and SFFV gp52. By comparing V8 protease digests of the Friend MCF envelope precursor, Pr80^{env}, and gp70, we have been able to show that the gp70 fragment precipitated by the monoclonal is derived from the amino-terminus. The data indicates, therefore, that the substitution that took place in the creation of Friend MCF occurred at the 5' end of the env gene and supports previous genetic studies.

(6) Localization of the pathogenic functions of F-MuLV and Friend MCF viruses to the env gene.

Studies carried out in collaboration of Dr. A. Oliff, Memorial Sloan Kettering Cancer Center, New York, N.Y., have attempted to localize the pathogenic functions of F-MuLV and Friend MCF virus to the env gene. This was done by molecularly cloning these viruses and generating novel recombinant viruses by co-transfection with the DNA from molecularly cloned amphotropic virus 4070. Viral proteins encoded by these hybrid viruses were characterized by immune precipitation with specific antisera and could be classified as being encoded by either F-MuLV or Friend MCF virus versus amphotropic virus. Animal studies with the F-MuLV/amphotropic virus recombinants have indicated that it is the F-MuLV env gene which contains the sequences required for disease. Animal studies are in progress to assess the pathogenicity of the Friend MCF/amphotropic virus recombinants and thus identify which Friend MCF env sequences are required for disease.

Significance to Biomedical Research and the Program of the Institute:

Understanding the proteins that are responsible for the biological effects of RNA tumor viruses is of great importance. The erythroleukemias induced in mice by the spleen focus-forming virus and the Friend leukemia virus are associated with the expression of specific viral proteins which are products of the spleen focus-forming virus and a generated recombinant MCF virus, respectively. It is

hoped that through further characterization of the biological parameters and biological effects will be found. Using reagents and information gained from studying these model systems may ultimately help us to develop immunoassays which would detect cross-reacting leukemia-specific proteins in primate species such as man. In addition, the study of the mechanisms of resistance to virus-induced leukemia in the mouse will also have relevance for the treatment of human leukemias.

Proposed Course:

(A) Studies to determine the mechanisms by which the spleen focus-forming and Friend MCF viruses alter erythropoiesis:

Data to date have indicated that the spleen focus-forming virus alters the sensitivity of erythroid precursor cells to the hormone erythropoietin (EPO). Since our molecular studies have indicated that the envelope protein of this virus, gp52, is the crucial element for biological activity, studies are being planned to understand how this protein modifies erythropoiesis. We propose that the protein, which can be detected in the cytoplasm and the plasma membrane, mimics the receptor for EPO. Thus, cells infected with SFFV will express more "EPO-receptors" and will thus be more effective than uninfected hematopoietic cells in binding small amounts of EPO present in serum. It has been observed that the polycythemia-inducing and anemia-inducing strains of SFFV, which differ in their sensitivity to EPO, differ in the processing of their envelope glycoproteins and consequently the amount that can be detected at the plasma membrane. Since an equal amount of gp52 can be detected in the cytoplasm, this data suggests that, if our hypothesis is correct, the receptor for EPO is at the cell surface. Using monoclonal antibodies to gp52 and its cross-reacting protein, MCF gp70, as well as purified SFFV gp52, we plan to test the hypothesis that a product of a murine leukemia virus, SFFV gp52, can alter the response of a cell, an erythroid precursor, to its hormone, erythropoietin. Using a protocol including the rat myeloma line Y3-Ag1.2.3 and spleen cells from rats immunized with Fr-MCF-infected cells, we have been successful in preparing a number of monoclonal antibodies to MCF gp70 which will be useful for these purposes.

Studies carried out at the molecular level comparing the anemia and polycythemia-inducing strains of SFFV may also give clues into the mechanisms by which these viruses interfere with erythropoiesis. Both of these viruses have been molecularly cloned and experiments have been initiated to engineer viruses that contain portions of each envelope gene and to obtain mutant viruses which will help us to localize the areas within the envelope gene that are involved in altering hormone sensitivity. Sequencing of the envelope gene of the polycythemia strain of SFFV has begun and plans are being made to sequence the product of this gene.

No *in vitro* assays have been developed to date which indicate how Friend MCF virus, a virus very closely related to SFFV, interferes with erythropoiesis. The fact that the animals infected with this virus, or its ecotropic parent, F-MuLV, become profoundly anemic suggests that Friend MCF virus may in some way block erythropoiesis. As with SFFV, the envelope glycoprotein of this virus, gp70, has been proposed as the crucial element for the biological activity of

this virus. We propose that MCF gp70, which cross-reacts with SFFV gp52, also mimics the receptor for EPO. However, since MCF gp70, unlike SFFV gp52, can leave the cell as part of the virion, we propose that it binds to EPO before it can reach the EPO receptors on the cell surface. Thus, hematopoietic cells infected with Friend MCF virus would be "blocked" from differentiating into mature red blood cells due to neutralization of EPO by the viral envelope glycoprotein. This hypothesis will be tested first by developing a good in vitro assay for studying erythropoiesis (the technique described recently by Dexter et. al. has been working in our laboratory) and showing how Friend MCF virus alters erythropoiesis in this assay. We also plan to test this hypothesis using monoclonal antibodies to MCF gp70 (and cross-reacting SFFV gp52) and purified MCF gp70 to determine if EPO can be neutralized by the product of a murine leukemia virus.

Our studies can be extended to other murine leukemia viruses, particularly those that have MCF virus intermediates. For example, AKR MCF virus may lead to thymomas by altering the response of thymocytes to T-cell growth factor (TCGF); viruses which cause myeloid leukemias may modify the response of a cell to any of the many factors involved in proliferation and differentiation along the myeloid pathway.

Also along these lines, we are interested in the target cell specificity of MCF viruses. Preliminary data from our laboratory indicate that a variety of MCF viruses (Friend, Moloney, AKR) have the ability to infect erythroid precursor cells, yet only Friend MCF virus can interfere with normal erythropoiesis. We plan similar experiments to determine if the various MCF viruses can infect T-cells and B-cells. If so, the data would suggest that target cell specificity is not due to types of hematopoietic cells. Instead, the data suggest that target cell specificity resides in the cell itself, i.e. a substrate with which the viral protein must interact in order to modify the proliferation and growth of that particular cell.

(B) Studies on the etiology of the second stage diseases induced by the spleen focus-forming and Friend MCF viruses:

Our results indicate that p53 may be a marker for transformed erythroblasts present in the second stage of diseases induced by F-MuLV or FVP. Since high levels of p53 are associated with transformation by a variety of oncogenes, this result could indicate that a cellular oncogene has been activated in hematopoietic cells of mice infected with F-MuLV or FVP and is responsible for the transformed phenotype of the stage 2 spleen cells. It has been proposed that lymphomas induced in birds by the avian leukosis viruses are the result of the promotion of the transcription of a particular cellular oncogene, and such a model may explain the stage 2 transplantable cell lines from F-MuLV or FVP-infected mice. Preliminary data have indicated that none of at least six known onc genes is expressed in cell lines derived from stage 2 spleens nor are any new RNA species detected using a probe for the viral LTR. Additional studies along these lines are planned to further investigate this model. Also, transplantable cell lines derived from leukemias induced by other murine leukemia viruses as well as human leukemia cell lines will be examined for expression of p53.

(C) Studies on the mechanisms of resistance of mice to diseases induced by murine leukemia viruses:(1) Resistance of certain strains of mice to erythroleukemia induced by F-MuLV:

Data accumulated in this laboratory in the past year are consistent with the hypothesis that MCF viruses play an important role in the generation of erythroleukemia after injection of certain strains of newborn mice with ecotropic Friend MuLV and that resistance to this disease is mediated through the endogenous expression of an MCF/xeno-gp70-related protein that interferes with the replication and spread of MCF viruses. Additional studies are being planned in order to further understand the basis for this resistance. This will include (a) genetic studies, carried out in collaboration with investigators at NCI and NIAID, to further document the association between resistance and the expression of endogenous viral proteins as well as to determine the number and location of genes involved in this resistance; (b) studies to determine if this resistance gene can be activated in susceptible cells by agents such as azacytidine and IudR; (c) studies to determine how this resistance can be overcome; and (d) studies to molecularly clone this endogenous envelope gene and the adjacent cellular sequences and determine its biological effects.

(2) Studies on the resistance to leukemia of susceptible strains of mice with age:

Data generated in this laboratory in the past year have indicated that the increasing resistance with age of NIH Swiss mice to F-MuLV-induced disease is due to the failure to replicate MCF viruses due to either a lack of a suitable number of target cells into which the virus can integrate or an effective immune response against the MCF viral envelope. These studies will be extended to determine if differences in the number of target cells can be found in newborn versus adult mice and whether soluble factors, such as those recently described in Fv-2r mice, effecting the cycling of the target cells in the adult mouse can be found. Also, the role of natural killer cells in the resistance of adult mice to F-MuLV-induced disease will be investigated.

Publications:

Anderson, S. J., Furth, M., Wolff, L., Ruscetti, S. K. and Sherr, C. J.: Monoclonal antibodies to the transformation-specific glycoprotein encoded by the feline retroviral oncogene v-fms. J. Virol. (In press)

Evans, L. H., Duesberg, P. H., Linemeyer, D. L., Ruscetti, S. K. and Scolnick, E. M.: Structural and functional studies of the Friend spleen focus-forming virus: Structural relationship of SFFV to dualtropic viruses and molecular cloning of a biologically active subgenomic fragment of SFFV DNA. In: Neth, R., Gallo, R., Graf, T., Mannweiler, K. and Winkler, K., (Eds.): Springer-Verlag, Berlin, Modern Trends in Leukemia IV, pp. 472-479, 1981.

Hoffman, P. M., Ruscetti, S. K. and Morse, H. C.: Pathogenesis of paralysis and lymphoma associated with a wild mouse retrovirus infection. I. Age and dose related effects in susceptible laboratory mice. J. Neuroimmunology 1: 275-285, 1981.

Linemeyer, D. L., Menke, J. G., Ruscetti, S. K., Evans, L. H. and Scolnick, E. M.: Envelope gene sequences which encode the gp52 protein of the spleen focus-forming virus are required for the induction of erythroid cell proliferation. J. Virol. 43: 223-233, 1982.

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Ruscetti, S.K., Feild, J.A. and Scolnick, E.M.: Polycythemia- and anemia-inducing strains of the spleen focus-forming virus differ in post-translational processing of their envelope-related glycoproteins. Nature 294: 663-665, 1981.

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PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Retrovirus; MMTV-Induced Oncogenesis; Mechanism of Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	R. Wolford	Microbiologist	LTVG	NCI
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4.0

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3.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new gene (pLTR) has been discovered at the 3' terminus of mouse mammary tumor virus (MMTV) within the long terminal redundancy (LTR). The presence of this gene has now been confirmed in exogenous C3H-S MMTV, and in two endogenous proviruses of C3H, Mtv-1 (unit V) and unit I. A pLTR message encoding the information for this gene is uniquely expressed in preneoplastic mammary tissue (hyperplastic outgrowth, HOG) induced in Balb/c animals by the chemical carcinogen DMBA. The role of this gene in mammary carcinogenesis and/or MMTV regulation is under investigation. Deletion analysis of molecular chimeras between the MMTV LTR and the v-ras transformation gene from Ha-MuSV has been performed to identify and localize the steroid target site in the MMTV LTR. The hormone regulatory sequences are located within 200 nucleotides of the MMTV cap site. NIH 3T3 cells transformed with the MMTV v-ras fusions have been established in serum-free media. The transformation phenotype of these cells is under the control of glucocorticoids; concentration dependent phenotype switching parallels the binding curve for dexamethasone binding to the glucocorticoid receptor. This system is being exploited to select cellular mutants in the response to p21 transformation and in the hormone pathway.

Project Description

Objectives:

- (1) Analysis of hormone regulated transcription of MMTV. Localization of hormone regulatory sequences and determination of mechanism of hormone action.
- (2) Structural analysis of MMTV genome, utilizing full-length molecular clone of endogenous Unit V Mtv-1 proviral DNA and clones of other MMTV strains.
- (3) Mechanism of mammary carcinogenesis, and role of MMTV in induction of this neoplasia.
- (4) Investigation of mechanism of v-ras mediated cellular transformation.

Methods Employed:

- (1) Molecular chimeras between the MMTV LTR and the v-ras gene of HaMuSV will be used to probe the regulatory regions involved in hormone induction of MMTV expression. Deletion analysis of molecular chimeras will be performed to localize these regions.
- (2) 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.
- (3) Fine restriction endonuclease maps and sequencing of the regulatory promoter and regulatory regions of MMTV proviral DNA will be performed.
- (4) 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 include 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.
- (5) 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.
- (6) 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 the the transformation response and in the hormone response.

Major Findings:

Transcription regulation of MMTV expression by glucocorticoids has been examined in detail. Fusions between the v-ras gene of HaMuSV and the MMTV 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. Using this assay, deletion analysis by molecular techniques has localized the site conferring hormone sensitivity on the MMTV LTR to within 200 nucleotides of the MMTV cap site.

NIH 3T3 cell lines transformed with the MMTV v-ras fusions have been established in serum-free media. The only components required in this medium in addition to the basic salt mixture are transferin, insulin and a poly-lysine coating of the support. Since the medium is completely free of glucocorticoids, we are in a position to study the interaction of steroids and the MMTV promoter at the cellular level in a totally defined culture environment. The concentration curve for dexamethasone induced phenotype switch from normal to transformed parallels exactly the binding curve for dexamethasone affinity to its cellular receptor, confirming that induction of the cellular phenotype switch occurs via glucocorticoid receptor mediated regulation of p21 gene expression. Two classes of cellular variants in which this switch no longer occurs could potentially arise. In one, cellular targets of the transformation protein could be altered; in the other, cellular components of the hormone response pathway could be impaired. Such variants have been derived and are currently being characterized.

A new gene has been discovered at the 3' terminus of the MMTV genome. This gene (pLTR) is completely encoded within the long terminal repeat of proviral DNA. The reading frame for this gene is maintained in each of three variants of MMTV that have been cloned and sequenced in the lab; these include the exogenous C3H-S strain, the endogenous Mtv-1 locus, and endogenous unit II. A message that contains only the coding information for this gene has been identified in preneoplastic stages of murine mammary cancer. Hyperplastic alveolar nodules can be induced in mammary tissue by either viral or chemical carcinogens. These abnormal tissues can be propagated by serial passage in animals as hyperplastic outgrowths (HOG). High levels of expression for the putative pLTR message have been detected uniquely in HOG lines and tumors developed from these lines. The role of this new gene in mammary neoplasia and/or MMTV regulation is under investigation.

Significance to Biomedical Research and the Program of the Institute:

The regulation of gene expression is becoming a central issue in the elucidation of mechanisms of oncogenesis. It is now established that mammalian oncogenic transformation, including human oncogenesis, is associated with the inappropriate activation of "normal" endogenous genes. Understanding the mechanisms by which these activation events occur necessarily requires a detailed knowledge of the regulatory mechanisms involved. We have established one of the first

systems in which the genetic mechanisms involved in the regulation of eucaryotic gene transcription are amenable to detailed analysis.

Proposed Course:

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 developed to identify the respective cellular targets.

Molecular mutagenesis techniques will be applied to the MMTV LTR v-ras fusion system to precisely characterize the hormone regulatory sequences.

Molecular clones of the MMTV regulatory element will be utilized with cell free transcription systems in attempts to reconstruct hormone responsive control in vitro. Mutants in the regulatory element derived as discussed above will be utilized in this approach to further probe the mechanism of hormone regulation.

Expression of the pLTR protein has been successfully demonstrated in bacterial expression vectors. "pLTR" specific polypeptides will be purified from these systems and used to generate antibodies to the protein. Various stages of murine mammary neoplasia will be examined for expression of the pLTR protein in efforts to understand the potential role of this gene in mammary oncogenesis.

Publications:

Battula, N., Hager, G. L. and Todaro, G. J: Organization of Type C Viral DNA sequences endogenous to baboons: analysis with cloned viral DNA. J. Virol. 41: 583-592, 1982.

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. (In press)

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Feline Sarcoma and Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

 (a1) MINORS (a2) INTERVIEWS

Molecularly cloned proviruses of the Snyder-Theilen (ST), Gardner-Arnstein (GA) and McDonough (SM) strains of feline sarcoma virus (FeSV) were used to characterize two viral oncogenes (v-fes and v-fms) responsible for FeSV-induced transformation and tumorigenesis. The complete nucleotide sequences of the ST- and GA-FeSV v-fes genes, each of which encodes a tyrosine-specific protein kinase, have been determined. The predicted amino acid sequences of these proteins show that they are homologous to proteins encoded by other oncogenes (v-fps, v-src, and v-yes) which also exhibit tyrosine kinase activity. DNA transfection experiments using plasmids containing molecularly subcloned portions of GA-FeSV formally showed that v-fes sequences were necessary for transformation. Chimeric transforming viruses and mutants generated by recombinant DNA techniques were used to map viral functions which regulate expression of the transforming gene. The v-fms oncogene was shown to differ from v-fes and was demonstrated to encode a transforming glycoprotein. Monoclonal antibodies were prepared to the v-fms product and permitted assays of its steady state levels in transformed cells. Portions of the human c-fms proto-oncogene were molecularly cloned and partially characterized.

Project DescriptionObjectives:

- (i) To study the structure and genetic organization of feline sarcoma viruses.
- (ii) To identify transduced viral oncogenes required for FeSV-induced transformation and tumorigenesis.
- (iii) To elucidate the role of virus-derived sequences in regulating the expression of recombined cellular transforming genes.
- (iv) To characterize cellular proto-oncogene sequences homologous to viral oncogenes transduced by FeSV strains.
- (v) To identify and characterize FeSV encoded proteins which are required for morphological transformation.
- (vi) To define the functions of oncogene and proto-oncogene gene products in both normal and malignant cells.

Methods Employed:

Cells infected with different strains of FeSV were cloned and characterized by Southern blotting analyses for the presence of integrated, transforming DNA proviruses. The DNA of cells containing single GA-FeSV and SM-FeSV proviruses was purified, digested to completion with the restriction endonuclease EcoRI, and subjected to preparative electrophoresis in agarose. Fractions enriched for proviral DNA-containing fragments were ligated into the bacteriophage vector λ gtWes. λ B and packaged in vitro into infectious bacteriophage particles. The libraries were plated on suitable E. coli hosts, and phage plaques were screened by blotting for the presence of sequences which hybridized to radiolabeled probes prepared from previously cloned feline leukemia virus (FeLV) proviral DNA fragments. Positive phage plaques were picked, re-cloned, and amplified from single particles, and the recombinant phage DNA was purified. Recombinant phages were characterized by restriction endonuclease mapping, R-looping with purified viral RNA, and heteroduplexing analyses. A previously obtained ST-FeSV clone as well as a transforming GA-FeSV DNA containing the same viral transforming gene were subjected to nucleotide sequence analysis using the technique of Maxam and Gilbert. Both DNA strands of each clone were independently sequenced several times and from different restriction sites, and all restriction sites used as starting points were also analyzed within overlapping fragments.

Segments of the GA-FeSV and SM-FeSV proviral DNA were electrophoretically purified and subcloned into the plasmid vector pBR322. Plasmid subclones were recovered by DNA transformation of permeabilized E. Coli hosts plated in the presence of suitable antibiotics (tetracycline or ampicillin as required). Positive colonies, identified by hybridization and rapid plasmid purification procedures, were expanded in one liter cultures, and supercoiled plasmid DNAs were purified by extraction and banding in cesium chloride gradients. Recombinant plasmids were characterized by restriction enzyme analyses, and where appropriate, by heteroduplexing procedures. In certain cases, genetically engineered viral DNA mutants were prepared following restriction endonuclease linearization. The ends of linearized molecules were either filled in by polymerase or digested with exonucleases, and the blunted termini were recircularized with high concentrations of T4 ligase. Plasmids containing either wild-type or mutant viral DNA

fragments were used to reconstruct complete DNA proviruses containing defined genetic lesions. Hybrid constructions were also prepared between FeSV and FeLV, and between FeSV and portions of Moloney murine leukemia virus (MuSV).

DNA proviruses of GA-FeSV and SM-FeSV, cloned either in lambda or plasmid vehicles, were tested for biological activity by transfection onto NIH/3T3 cells. The DNAs were admixed with carrier NIH/3T3 DNA and precipitated at neutral pH using the calcium phosphate procedure of Graham and Van der Eb. Transfected cells were shocked with DMSO or glycerol at concentrations empirically determined to give the highest efficiency of focus-induction. The cells were trypsinized 24 hours after transfection, fed at three day intervals, and scored at 2-3 weeks for the presence of foci of morphologically transformed cells. Transformed foci were subcloned using microcylinders, and the transformants selected by virtue of their rapid growth or ability to form colonies in soft agar. Recloned single-cell clones were used in biochemical and virological analyses. Transfection analyses were also performed with subgenomic proviral DNA fragments, proviral mutants, and chimeric proviral constructions.

Transfected NIH/3T3 subclones were characterized for the presence of rescuable, transforming virus after infection with ecotropic or amphotropic murine leukemia viruses (MuLV). Focus-forming activity was titered on both NIH/3T3 cells and mink CCL64 cells after serial ten-fold dilution of culture supernatants from infected cells. Previously prepared antisera to viral transforming polyproteins were used to immunoprecipitate viral-coded gene products from lysates of transformed cells. In general, the cells were metabolically labeled with various [³H]precursors prior to immunoprecipitation, and the denatured products were analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE). Alternatively, immune precipitates were assayed *in vitro* for protein kinase activity by following their ability to catalyze the transfer of radiolabeled phosphate from [γ -³²P]ATP into immunoglobulin heavy chains.

Genetically engineered viral mutants recovered from transformed NIH/3T3 cells were tested for their ability to induce disease in NIH mice. Newborn animals were inoculated intraperitoneally with stocks containing 3 to 4 logs of focus-forming virus per ml of inoculum. Control litters of mice were also injected with wild-type FeSV strains pseudotyped by the same MuLV helper viruses.

Monoclonal antibodies to viral-coded transforming proteins were prepared using a rat cell system. NRK cells transformed with SM-FeSV were inoculated into syngeneic animals, and sera from tumor-bearing recipients were screened for the presence of antibodies able to precipitate metabolically labeled viral proteins. Spleen cells from a hyperresponsive donor were fused to rat myeloma cells, and hybrids producing antibodies to viral proteins were identified by similar procedures. Hybrid colonies were subcloned twice in agar and established as stable cell lines. Cell lines producing monoclonal IgG were propagated in chemically defined, serum-free medium, and antibodies were purified to homogeneity from the culture supernatants. IgGs were radiolabeled with [¹²⁵I] and used in protein blotting experiments. In brief, lysates of virus-transformed cells were subjected to electrophoresis on SDS-PAGE, and the separated proteins were then transferred to nitrocellulose papers. The papers were reacted with labeled monoclonal

IgG, and after removal of the unbound immunoglobulins, were subjected to autoradiography. The expression of viral proteins could be quantitated by this technique, rendering results consistent with those predicted from assays of viral RNA expression ("Northern blotting" and liquid hybridization $C_{\text{r}}\text{T}$ analyses) in the same cells.

Major Findings:

The v-fes oncogene: ST- and GA-FeSV DNAs, independently cloned in bacteriophage lambda vectors, were subjected to nucleotide sequence analysis. These studies defined sequences which encode the transforming polyproteins of the two FeSV strains. The order of genes in both viruses was found to be:

5' LTR - tRNA_{pro} binding site - leader - agag - fes - Δenv - LTR 3'

in which only portions of the FeLV-derived gag and env genes, and none of the viral pol gene, are represented. The products of the fused gag and v-fes sequences are polyproteins which exhibit associated tyrosine-specific protein kinase activities. Nucleotide sequencing analyses predict that the actual molecular weights of the ST- and GA-FeSV products are 87 kilodaltons and 108 kilodaltons, respectively. The FeLV gag precursor is processed into four polypeptides whose order is NH₂-p15-p12-p30-p10-COOH. Both FeSV strains retain the complete p15 and p12 coding sequences and portions of the p30 sequences, although GA-FeSV encodes more p30 residues than ST-FeSV. In addition, the GA-FeSV strain contains more v-fes sequences than those present in ST-FeSV, thus accounting for the larger size of the GA-FeSV transforming protein.

The nucleotide sequences of v-fes indicate that both FeSVs encode products which are homologous to transforming proteins encoded by several other retroviral transforming genes. The latter include Fujinami sarcoma virus P130gag-fps, Y73 P90gag-yes, Rous sarcoma virus pp60src, and to a much lesser extent, the Moloney MuSV p37mos. The predicted homology between the FeSV products and FSV P130gag-fps is approximately 75%. Regions of homology are distributed throughout the entire v-onc gene-coded region of each protein, showing that the avian v-fps and feline v-fes oncogenes were derived from cognate cellular proto-oncogenes of birds and cats. By contrast, the 45% homology between RSV pp60src and the v-fes-coded domains is clustered within the carboxyterminal regions of the proteins, suggesting that only portions of the c-src and c-fes cellular proto-oncogenes are related to one another. These results indicate that sequences encoding different retroviral transforming proteins are ancestrally related and must be derived from a divergent family of tyrosine-specific protein kinase genes. The presence of conserved gene sequences in the DNA of both mammals and birds shows also that these highly conserved genes have been maintained within the germ lines of different vertebrates since their divergence from a common primitive ancestor.

The predicted homology between the amino acid sequences encoded by v-fes, v-fps, v-src, and v-yes allows a site of tyrosine phosphorylation to be assigned within the FeSV polyproteins. Although differences between the shared v-fes sequences of ST- and GA-FeSV include only five base changes out of 1322 nucleotides transduced in common, three of these substitutions lead to changes just upstream and

downstream of the predicted tyrosine phosphorylation site. One possibility is that tyrosine phosphorylation increases the oncogenic efficiency of the transforming protein. If viruses retaining certain regulatory sequences were more lethal in animals, these might be eliminated during horizontal transmission, such that more attenuated viruses might be selected. Alternatively, the ability of the polyproteins to be phosphorylated might be a fortuitous consequence of their enzyme activity, and might not represent a highly conserved function.

Mutant ST-FeSV strains which are transformation-defective (td mutants) were isolated from morphologically revertant cells, and were shown to encode polyproteins indistinguishable in size and antigenicity from the wild-type ST-FeSV P87gag-fes product. Several such revertants resegmented transformed colonies at a mutational frequency as determined by fluctuation analysis. In contrast to revertants containing td-ST-FeSV, the latter transformants released wild-type transforming virus after rescue, showing that correction of lesions within the ST-FeSV provirus resulted in reacquisition of the transformed phenotype. In each case, expression of the transformed phenotype was associated with the expression of P87gag-fes polyproteins with associated tyrosine-specific kinase activity, while the td-ST-FeSV products showed no detectable enzyme function and were themselves not phosphorylated in tyrosine. Thus, kinase activity appears to be required for initiation and maintenance of the transformed phenotype.

Several plasmids containing GA-FeSV proviral DNA were subjected to site-specific mutagenesis in order to formally demonstrate that the polyprotein coding regions were critical in transformation. Mutant plasmids were used to reconstruct subgenomic or full-length proviral DNA molecules which were tested for transforming activity by DNA transfection of NIH/3T3 cells. Those DNAs containing the 5' LTR and the intact P108gag-fes coding region were found to induce foci of transformed cells, each of which expressed P108-associated kinase activity. In particular, frame shift or asymmetric deletion mutations at an XhoI site in env, located within 100 base pairs of the putative P108 termination codon, did not affect the transforming activity of proviral DNA [all $>10^3$ focus-forming units/ug]. By contrast, deletions extending into the 3' end of the v-fes coding sequences abolished all transforming activity [< 1 focus/ug]. These results were consistent with nucleotide sequencing analyses showing that the 3' end of the v-fes gene specifies the tyrosine phosphorylation site in P108 and contains the most extensive regions of nucleotide sequence homology with other onc genes. A frame shift mutation located just upstream of the P108 initiating ATG codon also did not affect transforming activity. However, this mutation was found to affect the pattern on glycosylation of a minority of P108 molecules which normally incorporate sugars.

Foci of transformed cells derived by transfection of full-length GA-FeSV proviral DNA molecules yielded transforming viruses after rescue with either Moloney ecotropic murine leukemia virus (MuLV) or nonleukemogenic amphotropic MuLV (strain 4070). No infectious focus-forming virus was rescued from clones derived by transfection with subgenomic DNAs lacking the 3' LTR. Chimeric DNA constructions containing the 5' LTR and intact P108 coding region of GA-FeSV and the 3' LTR of Moloney MuLV also induced foci of transformed NIH/3T3 cells upon transfection

which gave rise to transforming viruses after MuLV rescue. Passage of the hybrid transforming viruses was found to stabilize the Moloney MuLV U3 region within both LTRs, thus generating focus-forming genomes whose activator and promoter sequences were derived from MuLV. The formation of these isolates lends support to current models for retroviral DNA synthesis, and now facilitates an assay of different regulatory sequences in the transcription of v-fes and in disease induction by GA-FeSV.

Chimeric DNA constructions were also prepared using the 5' LTR and gag leader of GA-FeSV and the gag - pol - env - LTR 3' portion of FeLV. These DNAs gave rise to infectious FeLVs after transfection of canine MDCK cells. Thus, although only full-length mRNA molecules have been detected in GA-FeSV transformed cells, the putative splice donor site within the FeSV gag leader can function in the formation of FeLV subgenomic mRNA. Constructions between FeSV DNAs containing the frame-shifted gag leader and FeLV structural genes also gave rise to infectious FeLV genomes upon transfection. As predicted from experiments with the FeSV mutants, the glycosylation pattern of the FeLV gag precursor, Pr65, was altered. The mutant FeLVs would be expected to express different antigens on the surfaces of FeLV-infected cells and might potentially show altered leukemogenicity in cats.

Plasmid subclones containing the major portions of ST- and GA-FeSV v-fes were labeled *in vitro* and were used to map proto-oncogene (c-fes) sequences in the DNA of uninfected cat cells by the Southern blotting procedure. The complexity of homologous cellular sequences was estimated to be about 4.5 kb. Restriction enzyme analyses suggested that the c-fes gene contained at least three introns which were not transduced during recombination with FeLV. The segmented nature of cellular proto-oncogene sequences suggests that spliced RNA transcripts served as intermediates in the formation of the two sarcoma viral genomes. The v-fes subclones have been used by several laboratories as probes to screen human DNA libraries for related human proto-oncogene sequences. As expected, the latter studies have confirmed that (i) cat and human c-fes genes are evolutionarily conserved and related with respect to their exon-intron organization and sites of restriction endonuclease cleavage, and (ii) the human c-fes and c-fps loci are identical genes.

The v-fms oncogene: The SM-FeSV strain contains viral oncogene sequences which are unrelated to those of ST- and GA-FeSV. Using techniques analogous to those described for GA-FeSV, a 10 kilobase pair DNA fragment containing the intact SM-FeSV provirus was cloned from transformed mink nonproducer cell DNA. Like ST- and GA-FeSV, the SM-FeSV strain has the gene order 5' agag-fms-env 3', in which only a portion of the FeLV gag gene and all of the env gene are retained. By heteroduplexing, the transduced v-fms sequences were estimated to be about 2.8 kilobase pairs in length.

SM-FeSV encodes several proteins which are detected in transformed cells. The largest of these is the gag-fms polyprotein, gP180, of a size which reflects the complete coding capacity of the SM-FeSV gag and v-fms sequences. In addition, an envelope glycoprotein precursor, gP85, can also be detected at low levels. The large polyprotein, synthesized from full-length SM-FeSV RNA, appears

The realization that certain mammalian and avian tumor viruses have transduced identical genes suggests that the number of proto-oncogenes may, in fact, be few, and that the role of different environmental factors in eliciting disease may be to impinge on the same restricted subset of cellular genes. In most cases, such studies have facilitated the identification of viral oncogene products as well as their cellular proto-oncogene analogues. The understanding of the role of these proteins in controlling growth and differentiation will be crucial in determining how their inappropriate expression or biochemical alteration can induce changes in cellular proliferation.

Proposed Course:

Chimeric GA-FeSVs containing transcriptional regulatory regions (U3) derived from the Moloney MuLV LTR will be tested for oncogenicity in mice. FeSV(MuLV) pseudotype stocks containing either chimeric or wild-type FeSV will be generated by rescue using nonleukemogenic amphotropic MuLV, and will be titrated for infectious focus-forming virus on both NIH/3T3 and CCL64 mink cells. Experimental and control stocks, matched for focus-forming activity and infectious helper virus titer, will be inoculated intraperitoneally into newborn mice and intravenously into weanling animals. The animals will be palpated for tumors, and studied for pathology where appropriate. Attempts will be made to reisolate virus from tumored animals using standard extraction procedures. In conjunction with in vivo assays, the chimeric and wild-type FeSVs will be studied in tissue culture for (i) their efficiency of focus-formation in different cell types (carnivore versus rodent), (ii) the transcription of viral RNA in focus-derived clones containing single proviral DNA copies, and (iii) the levels of expression of P108gag-fes. Preliminary experiments in vitro have already established that chimeric FeSVs containing rodent viral regulatory sequences induce more rapid proliferation of mouse cell targets than do wild-type GA-FeSV genomes.

Mutant FeLVs containing frame shifted sequences within the gag leader region will be assayed for their ability to code for glycosylated gag precursors. Metabolic labeling experiments will be performed using both sugar and amino acid precursors, and the various gag products detected by immunoprecipitation with appropriate antisera will be chemically studied by tryptic fingerprinting procedures. The ability of glycosylated precursors, if any, to localize at the cell surface will be studied by lactoperoxidase iodination of viable, FeLV-infected cells. These experiments will test whether the topology of viral proteins in infected cells can be prospectively altered by site-specific mutagenesis.

Synthetic peptides representing amino acid sequences encoded by v-fes are now being synthesized in collaboration with extramural investigators. The first peptides to be prepared will derive from regions of v-fes which are highly conserved and contain the regions of maximal homology to pp60src and P130gag-fps. These peptides will be conjugated to immunogenic protein carriers, and antisera will be raised in rabbits. The antibodies will be repurified by affinity chromatography and tested for their ability to immunoreact with various members of the tyrosine kinase family. In principle, such sera may be useful in identifying related products encoded by homologous genes which were never transduced by retroviridae, and may, in addition, facilitate simultaneous assays of several tyrosine kinases within a single cell type.

to be cleaved to two smaller polypeptides which include p60gag and gp120fms, derived from the amino- and carboxyterminal ends of the larger polyprotein, respectively. Tryptic peptide analysis of [³⁵S]methionine-labeled products confirmed that gp120 contains a subset of gp180 peptides. Neither gp180gag-fms nor gp120fms have been found to express protein kinase activity. Although both v-fms products contain sites for tyrosine phosphorylation *in vitro*, the proteins were not phosphorylated in tyrosine *in vivo* and did not produce an increase in the levels of phosphotyrosine in transformed cells. Metabolic labeling experiments with [³H]mannose and [³H]galactose showed that gp180 and gp120 are glycoproteins. Using tunicamycin, an inhibitor of glycosylation, it was found that the apparent molecular weights of the nonglycosylated proteins are 155kd and 95kd, respectively.

Monoclonal antibodies to v-fms-coded epitopes were prepared using a rat hybridoma system. Several independent monoclonal IgGs were purified to homogeneity. Two such preparations were found to react in solid phase immunoassays ("Western blotting" of proteins), and were used to quantitate the levels and locations of v-fms products in transformed cells. The v-fms proteins were found exclusively within the cytoplasm, and were quantitatively associated with sedimentable organelles. Fluorescent staining of fixed, permeabilized mink and rat cell transformants suggested that only a minority of the molecules were associated with the plasma membrane.

Subclones of SM-FeSV DNA containing v-fms sequences were prepared in the plasmid pBR322 and in M13mp8 phage. These were used as probes to screen a human DNA phage library for c-fms sequences. Four different phages were obtained containing v-fms-related sequences. Sequences homologous to the 3' 1.8 kb of v-fms were dispersed over 10 kb of human DNA. This region was found to contain at least seven exons, as determined by heteroduplexing with SM-FeSV DNA. More than 15 kb at the 3' end of the c-fms locus were also obtained. An additional nonoverlapping clone was selected using a probe representing the 5' end of the v-fms gene.

Western blotting analyses using monoclonal antibodies to v-fms epitopes detected a protein of 85 kd in uninfected cells. To date, this is the only candidate for a product encoded by the c-fms locus. Cell fractionation experiments showed that the 85 kd protein copurifies with gp120fms in transformed cells, but is expressed only at very low levels as compared to the viral gene product.

Significance to Biomedical Research and the Program of the Institute:

Genetic engineering techniques now permit detailed structural analyses of transduced cellular genes involved in malignant transformation. Of fifteen known retroviral transforming genes, at least two have been transduced by FeLV from the DNA of cats in the formation of FeSVs. Each of these viral oncogenes is homologous to evolutionarily conserved sequences in the DNA of other vertebrate species, including man. For the first time, such techniques can be used to identify and purify putative human proto-oncogene sequences, and to determine their role in the etiology of human cancer.

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 Dr. R. Gallo, LTCB, DCT, NCI
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 Dr. S. O'Brien, LVC, DCCP, NCI
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Extramural:

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 Dr. G. Cooper, Sidney Farber Cancer Center, Boston, MA
 Dr. D. Dina, Albert Einstein College of Medicine, Bronx, NY
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 Dr. J. Gilbert, Michigan State University, East Lansing, MI
 Dr. S. Gisselbrecht, Hopital Cochin, Paris, France
 Dr. H. Hanafusa, Rockefeller University, New York, NY
 Dr. W. Haseltine, Sidney Farber Cancer Center, Boston, MA
 Dr. W. Hayward, Memorial Sloan-Kettering Cancer Center, New York, NY
 Dr. N. Hopkins, Massachusetts Institute of Technology, Boston, MA
 Dr. S. Hu, City of Hope National Medical Center, Duarte, CA
 Dr. G. Hunnsmann, Institut fur Immunobiologie, Freiberg, W. Germany
 Dr. R. Kettmann, Universite Libre de Bruxelles, Bruxelles, Belgium
 Dr. Y. Li, Memorial Sloan-Kettering Cancer Center, New York, NY
 Dr. K. McClain, University of Minnesota, Minneapolis, MI
 Dr. J. Mullins, Harvard School of Public Health, Boston, MA
 Dr. F. Noronha, Cornell University, Ithaca, NY
 Dr. J. Nunberg, Cetus Corporation, Berkeley, CA
 Dr. H. Robinson, Worcester Foundation for Experimental Biology, Shrewsbury, MA
 Dr. P. Roy-Burman, University of Southern California, Los Angeles, CA
 Dr. D. Stehelin, Institut Pasteur, Lille, France
 Dr. A. Tavitian, INSERM U248, Paris France
 Dr. N. Teich, Imperial Cancer Research Fund, London, England
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 Dr. I. Verma, Salk Institute, San Diego, CA

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Dr. R. Weinberg, Massachusetts Institute of Technology, Boston, MA
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Publications:

Anderson, S. J., Furth, M., Wolff, L., Ruscetti, S. K., and Sherr, C. J.: Monoclonal antibodies to the transformation-specific glycoprotein encoded by the feline retroviral oncogene v-fms. J. Virol. (In press)

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PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Mechanisms Involved in Retrovirus Induced Oncogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Nancy Zeller	Guest Worker	LTVG	NCI
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COOPERATING UNITS (if any) H. Robinson, Worcester Foundation Shrewsbury, Mass.; J. Coffin, Tufts University, Boston, Mass.; A. M. Skalka, Roche Institute of Molecular Biology, Nutley, New Jersey; E. Jaffe, Hematopathology Section, LP, DCBD, NCI

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Viral Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD. 20205

TOTAL MANYEARS:

3

PROFESSIONAL:

3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two complementary approaches have been used to examine the mechanisms of retrovirus induced oncogenesis. A) Study of the genetics of oncogenesis by avian retroviruses, and B) Study of the molecular mechanisms of oncogenesis by MoMuLV in mammals. Our results showed that the U3 region of the viral LTR, is the major determinant of the oncogenic potential of the virus. However, other viral sequences determine the virus oncogenic spectrum. In addition, while "promoter insertion" may be important, it does not seem to be the only mechanism by which mammalian retroviruses induce lymphoid malignancies. In this system more than one virus related event may be essential for tumor induction.

Project DescriptionObjectives:

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.

The specific goals are:

1. To define the role of viral sequences in oncogenesis by nontransforming avian retroviruses.
2. To define the molecular mechanisms of retrovirus induced oncogenesis in a mammalian system.
3. To study the role of transforming gene activation in human malignancies.

Methods Employed:

Molecular cloning Southern and "Northern blotting" and hybridization, DNA sequencing, DNA transfection.

Major Findings:

1. The non-transforming avian retroviruses (avian leukosis viruses) can be divided into two distinct groups: The endogenous viruses that are present in the normal cell DNA and are transmitted vertically in a mendelian fashion, and the exogenous viruses that were originally isolated from spontaneously arising neoplasms. Biological differences between the two groups include the host range, determined by the env gene, and pattern of growth and oncogenicity. The exogenous viruses are oncogenic in vivo while the endogenous viruses are not. By generating recombinants between the two groups, we have shown in collaboration with Dr. John Coffin and Dr. Harriet Robinson, that both the growth potential differences and oncogenicity differences between endogenous and exogenous avian retroviruses are due to differences in the RNA sequences at the 3' end of the viral genome. One of the recombinants generated in these experiments (NTRE-7) has been instrumental in these studies because it appears to be identical to the endogenous parent RAV-0 with the exception of sequences at the 3' end that were derived from the exogenous parent tdPrRSV-B. In order to accurately define the viral sequences involved in the biological phenomena described, we have molecularly cloned the NTRE-7 recombinant and its RAV-0 parent and sequenced the viral 3' end sequences in collaboration with Dr. G. Hager and Dr. A. Skalka. These results have defined accurately the recombination event that generated NTRE-7 and have provided the structural basis for further studies regarding the function of the regulatory elements that reside in the 3' end sequences of the viral genome.

In other recent studies (also in collaboration with H. Robinson and J. Coffin) we have found that while the 3' end sequences of the viral genome determine the ability of the virus to induce disease, other viral sequences not present in the viral env gene are important in determining the spectrum of the disease induced.

2. A second major effort was to develop a system for studying the mechanism of oncogenesis by Moloney leukemia virus (MoMuLV). The experimental animal we used was the rat because this animal has a very low background of endogenous virus sequences homologous to the MoMuLV genome. This model represents a unique experimental approach for identifying the mechanism(s) by which viruses that lack a transforming gene induce lymphoid malignancies in mammals.

Analysis of the tumors at the DNA and RNA level has revealed a complex picture. Four tumor-related viral RNA transcripts have been identified. Two of these transcripts appear to be derived from recombinants generated between MoMuLV and other virus related sequences (probably rat endogenous virus) and two appear to contain cellular sequences in addition to viral sequences. (Tsichlis P.N. and Strauss P.G. in preparation). Analysis of tumor cell DNA revealed a complex pattern of as many as ten separate viral integration events. Junction fragments between the virus and the cellular genome have been cloned in bacteriophage lambda. Analysis of these clones so far has revealed the following: a) Many of the clones represent structures containing MoMuLV and other virus related sequences (probably rat endogenous virus) and therefore, represent recombinants between MoMuLV and rat endogenous virus sequences, and b) Two of the clones contain cellular sequences that appear to be rearranged in the majority of the tumors. Most interestingly, there are tumors in which we have found DNA rearrangements in the cellular DNA represented by both clones. Rearrangements in a domain of the cellular DNA in this system are due to virus integration within this domain. This means that we have so far identified two preferred integration events (Tsichlis P.N. Strauss P.G. and Hu L.F. in preparation) and that in some tumors both of these events have occurred (Tsichlis P.N. Strauss P.G. and Hu L.F. in preparation). In view of the fact that retroviruses are known to integrate randomly within the cellular genome, this indicates that cells which carry these integration events are selected during oncogenesis. The selection could be due to the activation of a transforming gene, the inactivation of a gene that plays an important role in cellular differentiation, or perhaps the interference with the sequential activation and inactivation of genes involved in T cell differentiation. Future experimentation using the clones we have generated will help us differentiate between these possibilities.

3. Based on genetic evidence we originally proposed a model according to which retrovirus induced oncogenesis is due to integration of the virus next to a cellular transforming gene. This gene is then transcribed efficiently from the viral promoter that resides within the U3 region of the viral long terminal repeat (Tsichlis P.N. and Coffin J.M. Cold Spring Harbor Symp. on Quant. Biol. Vol XII "Viral Oncogenes", p123, 1979; Tsichlis P.N. and Coffin J.M. J. of Virol. 33: 238-249, 1980). This model now referred to as the "promoter

insertion" model of oncogenesis has been confirmed in the case of avian leukosis virus induced bursal lymphomas, by the elegant studies of W. Wayward and H. Varmus. The cellular transforming gene activated by the integrated provirus in this case is the c-myc gene. Evidence from this as well as other laboratories indicates that activation of c-myc is specific for the avian leukosis virus induced B cell lymphomas. Other virus induced malignancies do not show activation of c-myc sequences.

We reasoned that if activation of c-myc is specific for B cell lymphomas, B cell tumors induced by a variety of oncogenic agents may show a similar activation. Using a v-myc probe we examined the expression of c-myc sequences in a variety of human lymphomas and normal tissues. Our results indicate that a modest 2-5 fold increase of myc specific mRNA is observed in B cell lymphomas when they are compared to normal human lymphoid tissues. Before we understand the significance of these results and the role the c-myc gene product may play in human malignancy it will be necessary to determine the normal function and regulation at this gene.

Significance to Biomedical Research and the Program of the Institute:

Our studies regarding the genetics of avian retroviruses have pointed out one of the fundamental mechanisms involved in virus induced oncogenesis, now referred to as "promoter insertion." Our recent results indicating that viral sequences, not yet well defined, determine the oncogenic spectrum of a given virus will allow us to address two issues of major significance to the understanding of tumor induction a) Mechanism of virus integration and, b) Tissue dependent specificity of transcriptional signals.

Our studies on the molecular mechanism(s) of oncogenesis by MoMuLV in rats have pointed out that tumor induction is a complex process and that "promoter insertion" however important it is, is not the sole mechanism of oncogenesis. We believe that these studies will soon reveal novel mechanisms for retrovirus induced disease.

Understanding of these animal models provides the conceptual framework for the study of nonviral tumors in humans.

Proposed Course:

1. a) Sequencing and cloning techniques will be utilized to define the sequences in the promoter region of the viral genome that determine the differences observed between endogenous and exogenous viruses. In addition to providing further insight into the molecular mechanism(s) of virus induced disease, these studies will also be important for understanding the structure and function of eucaryotic promoters.
- b) Similar techniques will be used to accurately define the viral sequences that determine the spectrum of disease induced. These studies will address

the mechanism of virus integration, since disease specificity could obviously be related to the type of oncogene activated by a given virus. A second issue of major significance in this regard is the possibility of tissue dependent specificity in transcriptional signals. This system provides an experimental model for addressing the much larger issue of tissue specificity for oncogene activation by a variety of carcinogens, including chemicals, radiation and other environmental insults.

2. a) The genomic clones will be further analyzed for a correlation between the RNA transcripts unique to the tumor and the cloned cellular sequences. This will generate a coherent picture concerning virus mediated events that lead to tumor formation.
- b) Sequences of interest identified by this approach will be further analyzed for their transforming potential and their informational content. Cellular sequences that will be important in this analysis are the cellular sequences of the two preferred integration events described.
- c) Sequences with oncogenic potential identified in MoMuLV induced rat thymomas will be examined for their importance in related systems (AKR mouse HRS/J mouse and spontaneous human thymomas).
- d) Preferred virus integration events in the tumors will be analyzed by DNA sequencing to potentially identify subtle specificity in the virus integration process.
- e) The role of viral recombinants during oncogenesis will be examined by identifying possible linkage between viral and cellular sequences with oncogenic potential.

Publications:

Robinson, H. L., Blais, B. M., Tschlis, P. M. and Coffin, J. M.: Two regions of the viral genome determine the oncogenic potential of avian leukemia viruses. Proc. Natl. Acad. Sci. U.S.A. 79: 1225-1229, 1982.

Tschlis, P. N., Donehower, L., Hager, G., Zeller, N., Malavarca, R., Astrin, S. and Skalka, A. M.: Sequence comparison of the crossover region of an oncogenic avian retrovirus recombinant and non-oncogenic parent: Genetic regions that control growth rate and oncogenic potential. Mol. Cell Biol. (In press)

Zeller, N., Cossman, J., Jaffe, E. and Tschlis, P.: Expression of c-myc sequences in human lymphomas. Environmental factors in leukemia and lymphomas. McGrath, B. Ramot and G. O'Connor. Raven Press. (In press).

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Analysis of Transcriptional Regulation of Murine Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.C. Ostrowski	Staff Fellow	LTVG, NCI
OTHERS:	H. Richard-Foy	Guest Worker	LTVG, NCI
	D.S. Berard	Microbiologist	LTVG, NCI
	R.G. Wolford	Microbiologist	LTVG, NCI
	G.L. Hager	Head, Viral Immunogenetics Section,	LTVG, NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Viral Immunogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

3.3

PROFESSIONAL:

2.3

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have shown that the Mouse Mammary Tumor Virus (MMTV) long terminal repeat (LTR) contains the target site for steroid hormone action associated with this virus. Subsequently, deletion analysis of MMTV LTR has indicated that approximately 100 out of the 1300 bp of the LTR mediate hormone regulation of transcription. However, while these experiments define the region of MMTV that is necessary for steroid hormone regulation of gene expression, they do not directly address the molecular mechanisms by which hormone-receptor complex, MMTV LTR, RNA polymerase II, and other nuclear protein components interact to produce the observed biological phenomenon of elevated rates of transcription. In order to begin to understand this complex problem, we feel that reconstitution of hormone regulated transcription in vitro will be required. Reconstitution attempts using current state-of-the-art technology have failed, most probably because they do not assemble the correct epigenetic structure. To overcome this problem, we are trying to introduce MMTV LTR into eukaryotic cells as an episome, by using the Bovine Papilloma Virus (BPV) as a vector. Such an approach will allow us to isolate MMTV LTR as nucleoprotein minichromosomes with their in vivo chromatin structure intact.

Project Description

Objectives:

- 1) Chimeric molecules will be made between MMTV LTR, v-ras, and BPV via molecular cloning. These chimeras will be transfected into susceptible cells and the resulting transformed cells will be analyzed. Attention will be focused on the state of the newly acquired DNA and the RNA initiated from the MMTV LTR.
- 2) Episomal chimeric DNA will be isolated from these cells so that nucleoprotein particles are obtained. These particles will be used in run-off transcription studies and in nuclease digestion studies to demonstrate that MMTV LTR remains transcriptionally active when mobilized on the BPV vector.
- 3) The enriched minichromosomes will serve as templates for in vitro transcription experiments. Various nuclear extracts and purified glucocorticoid receptor will also be used in these transcription experiments. The cloned, naked DNA is used as a control.
- 4) 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:

- 1) Chimeras are constructed from purified restriction fragments of cloned viral sequences using pBR322 as a bacterial cloning vector. The final structure of these molecules is determined by restriction mapping. Recombinants are introduced into cultured cells via calcium phosphate precipitation.
- 2) DNA from BPV transformed cells is analyzed by Southern blot analysis. MMTV LTR initiated transcripts are analyzed by Northern blotting and S1 mapping.
- 3) Minichromosomes are prepared from nuclei of cells identified as containing LTR episomes by salt or EDTA extraction procedures which rely on the small size of these episomes as compared to chromosomal DNA. These particles are further enriched by differential ultracentrifugation or gel filtration. Transcription run-off and nuclease digestions are performed as described in published procedures.
- 4) Nuclear transcription extracts are prepared by modifications of published techniques. Glucocorticoid receptor is purified from high speed supernatants of tissue culture cells by affinity chromatography.
- 5) Mutant LTR's are made by site-directed in vitro mutagenesis.
- 6) The nuclear components necessary for directing hormone-regulated transcription in vitro will be analyzed and purified by classical biochemical techniques.

Major Findings:

Chimeric recombinant plasmids have been molecularly cloned in E.coli K12. These plasmids contain 3 eukaryotic components: MMTV LTR, v-ras (the transforming gene of Harvey murine sarcoma virus) and BPV. The arrangement of components is such that MMTV LTR should drive the expression of v-ras. When these chimeras are introduced into murine cells by calcium phosphate transfection, 8000 transformed foci per μ -mole are obtained. A number of these foci have been picked from plates, subcloned, and the structure of the BPV-LTR hybrids determined by Southern blotting. Three important pieces of information are obtained from these experiments. First, the BPV-LTR-vras chimeras are found as episomes, not integrated into chromosomal information in the transformed mouse cells. Second, there are up to 200 copies of these chimeric episomes present per cell. Finally, the physical organization of these episomes as determined by restriction mapping remains the same in murine and bacterial cells. We have also found that steady state levels of p21, the v-ras gene product, are increased in these cells when they are grown in the presence of glucocorticoid hormones. Further, the 5'-end of p21 mRNA has been mapped by S1 nuclease analysis. We find that this RNA is initiated at the predicted MMTV LTR capsite and that the amount of p21 RNA present is increased approximately 10-fold by glucocorticoid hormones. The amount of MMTV LTR initiated mRNA in transformed cells as estimated by the S1 procedure is consistent with the elevated copy number of the chimeras present. We have been able to isolate BPV-LTR-v-ras minichromosomes from nuclei prepared from transformed cells by either EDTA extraction or 0.25M ammonium sulfate extraction followed by removal of the nuclei by centrifugation. We estimate that such isolated minichromosomes have been enriched at least 100-fold as compared to whole nuclei. Transcription run-off experiments, in which RNA chains initiated in vivo are elongated in vitro, indicate that these minichromosomes are responsible for the mRNA production measured in the S1 experiments. Nuclease digestion experiments with micrococcal nuclease and pancreatic DNase I are currently in progress. These experiments should confirm the nucleoprotein structure of the minichromosomes as well as their role as transcriptional templates in vivo. Once these minichromosomes are characterized by these types of experiments, attempts to use them as templates for in vitro initiation of hormone-induced transcription will be made. Other components of this in vitro system will be crude nuclear protein extracts and purified glucocorticoid receptor. Efforts are now underway to obtain both of these additional components.

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:

- 1) The purification of the MMTV-BPV minichromosomes will be expanded to include either differential centrifugation or gel permeation in order to increase the relative amount of the minichromosomes in the final preparation.
- 2) The structure of these minichromosomes will be examined by nuclease digestion procedures.
- 3) Glucocorticoid receptors from rodent cells will be purified to at least 50% homogeneity (5000-fold purification) by affinity-chromatographic techniques.
- 4) Whole cell and nuclear extracts that contain RNA polymerase II and various nonhistone nuclear proteins will be prepared from rodent cells.
- 5) Attempts will be made to reconstitute faithful hormone-regulated transcription in vitro by recombining the nucleorotein minichromosomes, prepared from cells grown both in the presence and absence of glucocorticoids, with purified receptor and appropriate extracts. S1 mapping will be employed to assay for successful initiation events.
- 6) MMTV LTR mutants will be constructed and tested in the in vitro system.
- 7) 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:

Hager, G. L., A. L. Huang, R. H. Bassin and M. C. Ostrowski: Analysis of glucocorticoid regulation by linkage of the mouse mammary tumor virus promoter to a viral oncogene. In Viral Vectors, Cold Spring Harbor Laboratory. (In press)

Ostrowski, M. C., D. S. Berard and G. L. Hager: Specific transcriptional initiation in vitro on murine type C retrovirus promoters. Proc. Natl. Acad. Sci. USA 78: 4485, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP -05122-03 LTVG
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PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Transforming Sequences of Rat-derived Sarcoma Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R. W. Ellis	Staff Fellow	LTVG	NCI
Other:	D. (DeFeo) Jones	Microbiologist	LTVG	NCI
	E. M. Scolnick	Chief	LTVG	NCI

COOPERATING UNITS (if any)
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D. R. Lowy Dermatology Branch, NCI

LAB/BRANCH
Laboratory of Tumor Virus Genetics
SECTION
Molecular Virology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The molecular biology of the Harvey and Kirsten murine sarcoma virus p21 ras gene and its cellular homologs (c ras) has been studied. Reconstruction experiments between the viral Harvey ras and the rat c Harvey ras 2 gene have revealed a translational defect in the 3' half of the latter gene. A novel ras translational variant, p28 ras, is initiated from the rat c Harvey ras 2 promoter sequences.

Studies of the expression of the c ras genes have revealed that both Harvey and Kirsten c ras genes are expressed by means of vastly differently-sized mRNA species, which apparently differ in the lengths of their 3' untranslated regions. Facilitated by the high levels of c Kirsten ras mRNA in a murine hematopoietic cell line, *in vitro* translation was performed on the two different c Kirsten ras mRNA species: Their p21 translational products were undistinguishable.

Project DescriptionObjectives:

1. Study the molecular biology of ras gene expression in 416B mouse cells, a cell expressing elevated levels of p21 ras.
2. Determine the nature of the biological defectiveness of rat c Ha ras 2 which is incapable of focus formation in transfections of NIH 3T3 mouse cells.
3. Analyze the nucleotide sequence of v Ha ras for eventual comparison with v Ki ras and c Ha ras.

Methods Employed:

1. Recombinant DNA technology: Cloning of viral and cellular DNA sequences in plasmid (pBR322) and phage (Charon 4A) vectors; plasmid DNA purification from bacterial cell lysates by the Birnboim procedure utilizing cesium chloride density gradient ultracentrifugation.
2. Eukaryotic cellular nucleic acid isolation. High molecular weight cellular DNA and nuclear and cytoplasmic mRNA isolated by established procedures.
3. Nucleic acid analysis: restriction endonuclease digestion of DNA; agarose gel electrophoresis; Southern and Northern blot transfers; filter hybridization utilizing nick-translated DNA probes.
4. Protein analysis: An vitro translation of cell lysates by means of a rabbit reticulocyte lysate system; immunoprecipitation of p21 ras with monoclonal antibodies; polyacrylamide gel electrophoresis and autoradiography.

Major findings

1. Mouse cells contain two mRNA species (sized 1.6 and 5.0 kb) homologous to Harvey p21 ras and two mRNA species (sized 2.0 and 5.4 kb) homologous to Kirsten p21 ras. Apparently, there is a difference in the amount of 3' untranslated RNA on these molecules.
2. Each of the two Kirsten ras mRNAs, and by inference, each of the two Harvey ras mRNAs, can be translated in vitro into indistinguishable p21 ras molecules.
3. 416B hemopoietic mouse cells contain elevated levels of Kirsten p21 ras mRNA and p21 protein, apparently as a result of the amplified level of expression of pre-existing mRNA molecules.
4. The nucleotide sequence of Harvey viral p21 ras has been determined.

5. The biological activity of c Harvey ras 2, the rat gene colinear with viral Harvey ras, has been tested by means of in vitro constructions with viral Harvey ras and the viral LTR sequences. Intact c Harvey ras 2 is biologically inactive in transfection assays. Recombinations with the viral ras gene have demonstrated that 1) There is a translational, but not transcriptional, defect in the 3' portion of the gene 2) the sequences up to 200 bp upstream from the p21 AVG codon and downstream through the first 5 codons of p21 support biological activity when spliced onto the viral p21 ras gene, 3) as a result of this spliced construction, there is expressed a novel transational product, p28 ras.
- a. The recently characterized transforming gene (bas) of BALB-MuSV is identical to the p21 ras gene.

Significance to Biomedical Research and the Program of the Institute:

The p21 ras protein is one of a large class of viral transforming proteins derived from normal cellular genes. Our studies of the molecular biology of the viral and cellur genes should lead to a better understanding of factors that influence the expression of these genes. These investigations have taken on added significance as a result of recent observations concerning human transforming genes. Transfection and cloning experiments have identified the transforming gene associated with human bladder cancer as Harvey ras and the gene associated with human lung and colon cancer as Kirsten ras. Therefore, our basic investigations may bear upon the question of mechanisms of human cancer.

Proposed Course:

1. Study of the expression of Kirsten ras genes in mouse cells. Clone both mRNA species from the high "express or" Tine 416B. Clone the Kirsten ras gene(s) from this line. Perform structural/functional studies among these clones.
2. In vitro mutagenesis of viral Harvey and Kirsten ras genes. Based on the nucleotide sequence of these genes, mutant oligonucleotides will be synthesized for creating site-specific mutants in ras genes and their translational products.

Publications:

- Chang, E. H., Gonda, M. A., Ellis, R. W., Scolnick, E. M., and Lowy, D. R.: The human genome contains four genes homologous to the transforming genes of Harvey and Kirsten murine sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. (In press)
- Chattopadhyay, S. K., Chang, E. H., Lander, M. R., Ellis, R. W., Scolnick, E. M., and Lowy, D. R. Amplification and rearrangement of onc genes in mammalian species. Nature 296: 361-363, 1982.

Dhar R., Ellis, R. W., Shih, T. Y., Oroszlan, S., Shapiro, B., Matzel, J., Lowy, D., and Scolnick, E. M.: The nucleotide sequence of the p21 transforming gene of Harvey murine sarcoma virus. Science (In press)

Ellis, R. W., DeFeo, D., Furth, M., and Scolnick, E. M: Mouse cells contain two distinct ras gene messenger RNA species which can be translated into a p21 onc protein. Molecular and Cellular Biology (In press)

Ellis, R. W., Lowy, D. R., Scolnick, E. M. 1982. The Viral and Cellular p21 (ras) Gene Family. In Klein, G. (Ed.): Advances in Viral Oncology (Volume 1): Cell Derived Oncogenes, New York, Raven Press (In press).

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cell Biology of Carcinogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. David Hankins	Expert	LTVG	NCI
OTHER:	Elsbeth Lee	Predoctoral Student	LTVG	NCI
	Joseph Kaminchik	Visiting Fellow	LTVG	NCI
	Paul Lebowitz	IPA (Yale)	LTVG	NCI
	Rachael Huot	Guest Worker	LTVG	NCI
	Claudia Chen	Technician	LTVG	NCI
	Judith Luna	Technician	LTVG	NCI
	Sandra Ruscetti	Sr. Staff Fellow	LTVG	NCI
	Linda Wolff	Guest Worker	LTVG	NCI
	Edward Scolnick	Chief	LTVG	NCI
	Charles Sherr	Senior Surgeon	LTVG	NCI

COOPERATING UNITS (if any) Keith Humphries, NHLBI, NIH; Neil Young, NHLBI, NIH; Arthur Nienhuis, NHLBI, NIH; Michael Hagan, Armed Forces Radiation Research Institute, Bethesda, MD.; Stuart Yuspa, LCCTP, NCI; Ulf Rapp, LVC, NCI; Christine Eastman, NCI

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Viral Biochemistry

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD. 20205

TOTAL MANYEARS:

6.0

PROFESSIONAL:

4.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In August, 1980 the principal investigator was recruited (from Vanderbilt University, Nashville, Tennessee) to take a permanent position at the NCI and establish a cell biology group which would simultaneously perform independent research and collaborate with and provide a resource for the Laboratory of Tumor Virus Genetics in the Division of Cancer Cause & Prevention. During the past year, a group of well trained individuals dedicated to accomplishing these objectives has been assembled. A number of collaborative and independent studies have been initiated and significant findings have already begun to emerge from the efforts of this group. Our approach to the cell biology of carcinogenesis is multifaceted and attempts to integrate information concerning tumor induction by chemicals, viruses, or radiation. Four related studies are described.

Study 1. Effects of environmental carcinogens on hemopoietic stem cells.

Study 2. Characterization of a "new" putative regulatory protein.

Study 3. Mechanism by which retraviruses bring about increased hormone sensitivity and increased cell proliferation.

Study 4. Testing the generality of the "SISTER" hypothesis: A hormonal approach to understanding and treating cancer.

Project DescriptionObjectives:

Our overall objective during the coming year will be to continue our attempts to answer fundamental questions in normal and cancer cell biology and explore any possible application of our findings to the eradication of human disease. The particular objectives of each part of an integrated study 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.
- 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:

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 will be to identify the HSC, its progeny and to optimize growth conditions such that large numbers of HSC will be available for studies in phase II of this study.

In the murine system, it was previously thought that the survival of the HSC required the presence of an adherent monolayer of stromal or supportive cells. However, stromal detachment or overgrowth has often resulted in premature abortion of long-term experiments. Dr. Eastment has recently demonstrated that long-term cultures derived from hamster or mouse marrow continually produce HSC for more than 6 months even in the absence of a stromal monolayer. The ability to grow the pluripotent stem cells in suspension culture will greatly facilitate our investigation of the effects of carcinogens on HSC since one will not have to deal with the effects of the various agents on stromal cells or other adherent cells. Another advance in this research area was the development of an in vitro assay for HSC (the macroburst assay) by Dr. Humphries. The use of the long-term suspension cultures in combination with the macroburst colony assay (which measures the differentiative potential of HSC) enhances our chances of both cloning and growing purified populations of HSC.

Dr. Hagan (AFRRI), who has been interested in the effects of radiation on stem cells and DNA repair mechanisms for several years, recently reported a new stem cell kinetic assay employing BUDR to bring about chromosomal DNA breakage. This in vivo stem cell assay allows the detection of minor qualitative alterations in stem populations which are not possible by classical stem cell assays. Dr. Hagan and his assay will be very beneficial to this project as we attempt to assess the initiation of leukemogenesis by treating HSC with chemicals and radiation.

Finally, by microinjecting human Beta globin genes into the male pronucleus of fertilized mouse eggs, Dr. Humphries has developed "new" strains of mice which contain markers which can be identified unequivocally by molecular hybridization procedures. The HSC from these mice are very useful since the origin (host or recipient) of differentiated progeny can be unequivocally determined by assessing the molecular genotype of the progeny.

As outlined below, HSC from the mutant mouse strains will be exposed to a variety of carcinogens either in the mouse or in culture dishes. These HSC will

be analysed for the ability to self-renew, reconstitute an irradiated mouse, and give rise to differentiated phenotypes or leukemic populations by methods discussed above as well as classical hemotologic methods.

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 hematopoiesis 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 attempts 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. 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 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. However, there were two interesting surprises. First, 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. Therefore, at face value, our observation of the 26 S species in DBA cells would indicate that regulation of transcription of these sequences is under genetic control. It was possible that this RNA represented endogenous virus envelope sequences. However, the second surprise was that no hybridizable RNA was detected in the area corresponding to the size of a virus genome, i.e. 38 S., suggesting that the RNA sequences might be derived from cellular genes rather than from endogenous virus genes. Support for this suggestion was gained when Dr. Kaminchik hybridized an LTR (virus) probe to RNA from DBA fibroblasts and failed to detect the 26 S RNA species.

Using a SFFV-specific monoclonal antibody, Dr. Ruscetti has recently identified a protein on the membrane which is present of DBA cells but not NIH/Swiss cells.

Significance to Biomedical Research and the Program of the Institute:

The identification of a "new" protein which may have a physiologic regulatory role, is under genetic control, and is related to a transforming protein which causes leukemia could open a new area of research: genetic control of the expression of somatic cell regulatory molecules. At the very least, these studies 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.
 (E) Construction of viral vectors for the transfer of normal genes.

Proposed Course:

It is possible that the RNA sequences observed by Drs. Kaminchik and Lee either encodes the protein identified by Dr. Ruscetti or encodes information for a hemopoietic regulatory protein. In either case, identification of these sequences and the chromosomal sequences controlling their transcription would be of obvious significance. The experiments outlined below are designed to provide more information about these interesting SFFV-related sequences.

First, we will identify the SFFV-related RNA expressed in DBA mice. Toward this end we will purify env RNA and translate it in an in vitro translation system. We will then clone SFFV-related sequences by cDNA cloning procedures to permit sequence analysis, heteroduplex analysis, generation of infectious virus containing the sequences, and an analysis of flanking DNA sequences which may influence transcription.

Second, we will examine the relationship of gp52 to normal differentiation and why SFFV is specific for erythroid differentiation. We will accomplish this goal by determining the tissue, cell and strain specificity of expression of SFFV-related sequences. Note: an SFFV-specific, MCF-related probe (Bam-Eco) will be used to detect mRNA sequences in the following preparations in an attempt to examine whether SFFV's specificity is due to differential regulation of transcription of SFFV sequences and to look for "SFFV-like" sequences in normal tissue. We have observed SFFV-related RNA in DBA spleen and established fibroblasts. Other tissues, such as DBA thymus, marrow, muscle, brain kidney, lung, gastric epithelium, primary fibroblasts will also be examined. If there is preferential expression in hemopoietic tissues, individual cell types will be isolated and checked for expression of SFFV-like sequences. Cells will be derived from purified erythroid bursts, megakaryocyte, macrophage, neutrophil, and T lymphocyte colonies. Certain strains (e. g. C57Bl/6) are resistant to SFFV erythroid transformation. This resistance is genetically controlled by the FV-2 locus. By analysing the RNA from resistant and susceptible strains we will examine whether resistance at the FV-2 locus is mediated at the level of virus RNA expression.

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:

During the past year we have investigated 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. Documented a new transforming effect of feline sarcoma virus (FeSV) FeSV was shown to transform spleen cells from fetal and newborn kittens. The transformed cells formed colonies which when picked and replated consistently gave rise to continuous cell lines. Both producer and nonproducer lines were derived and the presence of the transforming gene in these cells was proven by immune precipitation, phosphorylation, and recovery of infectious transforming virus.
2. Developed conditions for routine establishment of hemopoietic cell tumors. Thus, we can predictably (as opposed to magically and spuriously) establish several lines from different leukemic animals or multiple lines from the same animal. This approach will allow examination of transformed clones for unique integration sites, tumor heterogeneity, and identification of factors which control the proliferation and differentiation of the cells in culture. In addition, these methods have already been useful in our recently initiated attempts to study human hemopoietic neoplasms in cultures. Thus, although it is still early, 3 out of 4 human leukemic samples are still growing after 3 months in culture.
3. Identified and developed an assay for a heretofore unidentified type of transformed cell early in the course of F-MuLV induced erythroleukemia. Since it is possible that these represent "pre-leukemic" cells, it is especially intriguing that these cells remain quite sensitive to the hormone, erythropoietin.
4. Described phenotypic differences in the in vitro erythroid effects of the anemia- and polycythemia-inducing strains of the Friend virus complex. The results of these studies have led us to propose a model of transformation which is based on the ability of the transforming agent to bring about an increase in sensitivity of cells for their natural growth regulators.
5. Demonstrated that Harvey and Kirsten sarcoma virus can cause a profound morphological transformation of human skin carcinoma cells (A431). This transformation occurs without a dramatic reduction in the binding of the epidermal growth factor (EGF) to these cells. Further, the transformed cells remain 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 perform further studies in our in vitro transformation system to identify target cells, clarify phenotypes, compare HaSV to SFFV, and determine whether Abelson induced bursts are making embryonic hemoglobin.

Second, we will 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. recombinants between SFFV and Ulf Rapp's virus (induces lung carcinoma), e. a correlation of biological activity with number of glycosylation sites, f. a correlation with membrane localization, g. site-specific mutants, h. 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 its surface, 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.

Fourth, we will characterize the biology of F-MuLV disease. The studies will include a. assessment of the clinical hematology of individual mice to determine if the anemia is due to hemolysis, splenic sequestration, a block in erythroid differentiation, or a defect in non-erythroid components which are normally involved in erythropoiesis. b. Determination of the time course and clinical picture prior to development of erythroleukemia? c. Characterization of Bone Marrow and Splenic Hemopoietic Precursors in F-MuLV Infected Mice. 1. d. examination of the virologic characteristics in the spleen and other organs, and e. identification of the target cells for F-MuLV by in vitro infection of hemopoietic cells, cell separation techniques, in vivo physiologic manipulation.

Finally, we will characterize PRE-LEUKEMIC ERYTHROBLASTS (PLEBS) which appear at 4-6 weeks post-infection and grow only in presence of added EPO.

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:

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 since joining the NCI. 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 which do not share the growth advantage.

Several practical predictions were apparent from this hypothesis, including that tumor cells may serve as hypersensitive substrates for identification of new physiologic regulators. Furthermore, 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 efficacious 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 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, 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 may allow selection and expansion (and purification) of tumor cell population to permit biochemical studies such as: 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 patient may permit a better test for clonality and/or heterogeneity of various tumors.

Publications:

Hankins, W. D.: Increased hormone sensitivity after in vitro infection of hemopoietic precursors with Friend virus complexes. J. Natl. Can. Inst. (In press)

Hankins, W. D., Kost, T. A., and Pragnell, I. B.: The myeloproliferative sarcoma virus causes transformation of erythroid progenitor cells in vitro. Mol. Cell Biol. 2: 138-146, 1982.

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. Advances in comparative leukemia research. Eds. D. Yohn and J. Blakeslee. 1982.

Hankins, W. D. and Scolnick, E. M.: Harvey and Kirsten sarcoma viruses promote the growth and differentiation of erythroid precursor cells in vitro. Cell 26:91-97, 1981.

Kaminchik, J., Hankins, W. D., Linemeyer, D. L., Ruscetti, S. K., and Scolnick, E. M. J. Virol. (In press)

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. 79: 635-639, 1982.

Z01 CP 05183-02 LTVG

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies to p21 ras Proteins and Production of p21 ras in Bacteria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Mark E. Furth	Staff Fellow	LTVG	NCI
Other:	Edward M. Scolnick	Chief	LTVG	NCI
	Barbara J. Fleurdelys	Bio Lab Technician	LTVG	NCI

COOPERATING UNITS (if any)

None.

LAB/BRANCH

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2.0

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1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ras transforming genes of the Harvey and Kirsten strains of murine sarcoma virus and the homologous, evolutionarily conserved germline genes encode a set of related 21,000 dalton proteins. These p21 proteins can exert potent effects on cell proliferation and differentiation. We have produced and characterized monoclonal antibodies to the p21 encoded by Harvey murine sarcoma virus, and have demonstrated that these reagents can be used to study the expression of other viral and cellular forms of p21, both in rodents and in humans. We have cloned the ras gene of Harvey sarcoma virus into a protein expression plasmid vector which allows the synthesis of p21 in Escherichia coli. The bacterial-produced p21 retains its characteristic biochemical activities. The availability of monoclonal antibodies and producer bacterial strains should facilitate the further purification and biochemical characterization of p21.

Project DescriptionObjectives:

The Harvey and Kirsten strains of murine sarcoma virus encode related 21,000 dalton proteins (p21) which cause malignant transformation of infected cells. The transforming genes of these viruses (called v-ras) are related to genes present in the germlines of normal vertebrates and at least some invertebrates. The Harvey-type and Kirsten-type cellular genes (termed c-Ha-ras and c-Ki-ras, respectively) comprise a small family. At least some of these genes have the potential to oncogenically transform cells, and encode distinct p21 species. The major goal of this work has been to further the characterization of the p21 species encoded by the various ras genes.

We have produced monoclonal antibodies specific to p21 to facilitate studies of the viral and cellular-encoded proteins. These reagents can be used to compare the p21 products of a number of ras genes from both rodents and humans, and to study their expression in normal and transformed cells. The aim of this work is to gain insight into the function of p21 in the control of cell proliferation and differentiation, and into the mechanism by which perturbations in p21 expression can lead to malignant transformation.

The functional characterization of p21 ultimately requires purification of the protein and analysis of its biochemical activities. To date the amount of purified material available has been limited by the relatively low level of expression even in transformed cells carrying multiple copies of the ras gene. In order to increase the potential yield of p21, we have sought to insert the v-Ha-ras gene into appropriate plasmid strains to permit the expression of p21 in bacteria. The availability of large amounts of purified p21 would facilitate the structural analysis of the protein, the mapping of antigenic sites, the detection of interactions with other cellular components, and the search for novel biochemical activities.

Methods Employed:

- (1) Growth of lymphocyte-myeloma "hybridoma" cells in serum-free medium for production of specific monoclonal antibodies.
- (2) Immunofluorescence and immunoperoxidase staining and microscopy of fixed cells and tissues.
- (3) Immunoprecipitation and SDS-polyacrylamide gel electrophoresis for analysis of radioactively labeled antigens in cell extracts, and protein "blotting" and detection with ¹²⁵I-labeled monoclonal antibodies for analysis of unlabeled antigens.
- (4) Molecular cloning of DNA fragments into plasmid vectors.

- (5) Agarose gel electrophoresis of DNA, blot transfer to nitrocellulose filters, and hybridization with ³²P-labeled cloned DNA probes.
- (6) Column chromatography of proteins, including immunoaffinity chromatography.

Major Findings:

(1) Eight rat hybridoma cell lines producing monoclonal antibodies to the p21^{ras} transforming protein of Harvey murine sarcoma virus have been established and grown in serum-free medium for production of pure monoclonal antibodies.

(2) The monoclonal antibodies all react with the Ha-ras p21 gene products of two additional rodent sarcoma viruses (BALB sarcoma virus and Rat sarcoma virus). In addition all react with the p21 species encoded by normal cellular Harvey-type (Ha) ras genes, both of rodents and humans. The rat c-Ha-ras1 and c-Ha-ras2 genes and the human c-Ha-ras1 gene have been activated by ligation to retroviral control elements, present in "long terminal repeat" (LTR) sequences, and used to transform murine NIH 3T3 fibroblasts. In every case the transformants express elevated levels of p21 detectable by immunoprecipitation with each of the monoclonal antibodies. Three of the monoclonal antibodies cross-react with the p21 encoded by Kirsten-type (Ki) ras genes in rodents and in man. These monoclonal antibodies therefore can be used to study p21 encoded by ras oncogenes isolated from chemically transformed cells and tumor cells of many species, including human.

(3) Some of the monoclonal antibodies can be used in highly sensitive immunocytochemical and protein blotting assays to determine the relative levels of p21 expression in normal cells and tissues and in tumors. (The immunocytochemical analysis are being carried out with Drs. J. Costa and S.-M. Hsu, Laboratory of Pathology).

(4) The cloned v-Ha-ras gene from Harvey sarcoma virus has been introduced into a "fusion" plasmid for expression of p21 in *E. coli*. Bacterial cells carrying this plasmid produce a hybrid protein which differs from authentic p21 only by a few amino acid substitutions at the aminoterminalus. This protein retains the two characteristic biochemical activities of the viral-encoded p21: it binds of guanine nucleotides; and it undergoes "autophosphorylation" at a specific threonine residue when incubated with GTP as a phosphate donor. Thus, the production of p21 in bacteria provides suitable material for purification and further biochemical analysis of active p21. The protein synthesized in *E. coli* probably corresponds to an precursor of the mature form which accumulates in mammalian cells. Thus, it may provide a substrate for in vitro studies of processing and membrane association of p21.

Significance to Biomedical Research and the Program of the Institute:

The production of potent and specific monoclonal antibody reagents directed against the protein products of well defined viral oncogenes facilitates

studies of the expression and function of these proteins. The demonstration that these monoclonal antibodies react with the proteins encoded by the human genes homologous to the viral oncogenes, and that they can be used in convenient immunocytochemical assays as well as biochemical assays, suggests that these reagents will be useful in studies of human malignancy. It should now be possible to assess whether alterations in the expression of p21 are associated with particular forms of cancer. Recent observations show that active oncogenes can be isolated from cell lines derived from a number of human tumors, that a number of these genes are homologous to the Harvey and Kirsten-type ras genes, and that their protein products can be detected using monoclonal antibodies raised against the viral-encoded transformation proteins. Thus, an appropriate basic research background exists for these studies.

Proposed Course:

- (1) Use monoclonal antibodies to characterize the p21 species of the ras transforming genes of Harvey murine sarcoma virus and Kirsten murine sarcoma virus (v-ras), and of their cellular homologs (c-ras) in rodents and in humans.
- (2) Develop assays utilizing the monoclonal antibodies to study the expression of p21 species in normal tissues and in tumors.
- (3) Produce large amounts of pure monoclonal antibodies by culturing hybridoma cells in serum-free medium.
- (4) Produce large amounts of p21 by expression of the ras gene of Harvey murine sarcoma virus in Escherichia coli.
- (5) Purify the bacterial-produced p21 by conventional and immunoaffinity chromatography and characterize its structure, antigenicity, biochemical activities, and biological activity.
- (6) Utilize the purified p21 to study its processing, association with membranes, and functional interactions with other cellular components.

Publications:

- Anderson, S. J., Furth, M. E., Wolff, L., Ruscetti, S. K., and Sherr, C. J.: Preparation of rat monoclonal antibodies to epitopes encoded by the viral oncogene (v-fms) of McDonough feline sarcoma virus. J. Supramolec. Structure (In press)
- Chang, E. H., Furth, M. E., Scolnick, E. M., and Lowy, D. R.: Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature (In press)

Furth, M. E., Davis, L. J., Fleurdelys, B., and Scolnick, E. M.: Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular ras gene family. J. Virol. (In press)

Furth, M. E., Dove, W. F., and Meyer, B. J.: Specificity determinants for bacteriophage lambda DNA replication. III. Activation of replication in λ ri^C mutants by transcription outside of ori. J. Mol. Biol. 154: 65-80, 1982.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Mechanisms of Oncogenesis by MoMuLV in Rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Peter Strauss	Guest Worker	LTVG	NCI
OTHER:	Li-Fu Hu	Visiting Fellow	LTVG	NCI
	Philip N. Tschlis	Expert	LTVG	NCI

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3

PROFESSIONAL:

3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A 5' LTR-cell DNA junction fragment from a MoMuLV induced rat thymoma has been cloned in bacteriophage lambda. Tumor cell DNA from 16 tumors has been examined for DNA rearrangements within the cellular sequences represented in this clone. We have shown that in 3 out of 16 tumors these cellular sequences have been rearranged due to virus integration. This is the second preferred virus integration event we have identified in these tumors (See Annual Reports by P. N. Tschlis and L. F. Hu). In one tumor, both of the so far identified preferred integration events, appear to be present simultaneously. Analysis of virus related cDNA clones obtained from the same tumors indicates the generation of virus recombinants in this system.

Project DescriptionObjectives:

The overall objectives of this project are to define and analyze biologically important DNA sequences in MoMuLV induced thymomas in rats.

Methods Employed:

Molecular cloning Southern and "Northern", blotting and hybridization, DNA transfection.

Major Findings:

Some but not all of the MoMuLV induced rat thymomas appears to have RNA transcripts that contain both cellular and viral sequences. (For more details see progress report by P. N. Tschlis and L. F. Hu). In order to identify and analyze these cellular sequences we have made an effort to obtain molecular clones of these transcripts. In these studies we have followed two complementary approaches.

1) If the hybrid transcripts are initiated or terminated within the viral LTR as expected, then cloning the virus-cell junction fragments from a tumor that contains these transcripts should allow us to identify the cellular sequences they contain. For this purpose tumor cell DNA from one tumor was digested with Sac I that cleaves within the MoMuLV LTR, the junction fragments were identified and they were cloned in bacteriophage lamda. Ten different junction fragments have been cloned using this approach. We assumed that if these hybrid transcripts are important in oncogenesis then the transcripts from more than one tumor should contain the same cellular sequences. This would happen only if the virus integrates within the same DNA sequence in more than are tumors. Integration of the virus within the same sequence would be detected as a rearrangement of this sequence when the DNA is digested with Sac I that cleaves within the MoMuLV LTR. Using the cellular sequence of one of these clones (Cl 228) as a probe. We identified such DNA rearrangements in three tumors. This indicates that MoMuLV integrates preferentially within this sequence. More important one of the tumors in which this preferred integration event was shown, appears to also have the preferred integration event described in the progress reports by L. F. Hu and P. N. Tschlis. This indicates that two rather than one virus related events may be important in oncogenesis. Preliminary analysis of Cl 228 has shown that it contains nonrepetitive DNA sequences that appear to be evolutionarily conserved. It's expression in normal and tumor tissues as well as its transforming potential are now being investigated.

2) In an attempt to identify the cellular sequences present in the mRNA transcripts we described and we tried to clone cDNA copies of these transcripts from one tumor in PBR322. Several clones were obtained. Two of them were analyzed

more extensively and they were shown to represent transcripts of recombinants generated between MoMuLV and Rat endogenous viruses. (P. G. Strauss, Hu, L. F. and Tschlis P. N. in preparation). The role of these recombinants in oncogenesis is unclear.

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:

1) We will try to understand the significance of the preferred integration event represented by cl 228 in tumor induction. The fact that this sequence is evolutionarily conserved indicates that it may have the characteristics of an oncogene. Therefore, we are going to study its expression at the RNA level and its transforming potential. If this sequence represents an oncogene we will study its relationship to other oncogenes and we will try to identify its protein product. In addition, we will try to identify the possible role of such an oncogene in human malignancy.

2) We will examine the role of viral recombinants in tumor induction. As a first step we will examine the characteristics of the viral sequences present in the two preferred integration events we have identified so far.

Z01-CP 05271-02 LTVG

PERIOD COVERED

September 30, 1981 through June 1, 1982

TITLE OF PROJECT (80 characters or less)

Study of the Molecular Basis of Disease Induced by the Spleen Focus-forming Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: J. Kaminchik	Visiting Fellow	LTVG	NCI
Other : E. M. Scolnick	Chief, LTVG	LTVG	NCI

COOPERATING UNITS (if any)

None.

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Molecular Virology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Friend spleen focus-forming virus (SFFV) a replication defective virus, induces erythroproliferative disease when injected as a pseudotype into susceptible mice. Some of the SFFV isolates will induce polycythemia (SFFV-P) while others will induce anemia (SFFV-A). The molecular basis for the distinctive biological effects of SFFV-P and SFFV-A in vivo is not known. Our approach to this problem was to clone molecularly, the genomes of SFFV-P and SFFV-A, to define the viral genes involved in the induction of disease by each variant, and compare the two genes on the molecular level. The genome of SFFV-P was molecularly cloned in our laboratory by D. Linemeyer. We have molecularly cloned the genome of SFFV-A from unintegrated proviral DNA and subsequently subcloned the env gene from the genomic clone. Both the genomic and env clones were biologically active and caused SFFV-A disease in mice. Since the env genes both of SFFV-A and SFFV-P were proven to be sufficient for induction of characteristic FVA or FVP disease we conclude that the biological differences between the two viruses map within the env gene of each virus.

Project DescriptionObjectives:

The purpose of this project is to identify the gene responsible for the induction of anemia by SFFV_A, and examine the molecular difference between this gene and that responsible for the induction of polycythemia by SFFV_p.

Methods Employed:

1. The entire SFFV_A DNA has been molecularly cloned in E. Coli using the plasmid vector pBR322.
2. Restriction endonuclease map of the cloned viral DNA was established, and the envelope gene was mapped by blot hybridization of digested DNA to env probe derived from SFFV_p.
3. The envelope gene was subcloned into pBR322.
4. The genomic and subgenomic clones were tested for their biological activity by cotransfecting the cloned DNAs into NIH 3T3 cells along with Mo-MuLV DNA. Rescued virus was injected into adult NIH Swiss mice.
5. Sequence homology between the genomic DNA of SFFV_A and that of the parental virus - F-MuLV or SFFV_p were determined by heteroduplexing.

Major Findings:

1. The unintegrated circular form of SFFV_A DNA has been cloned in pBR322 at a unique Cla I site. Clones have been isolated with permuted viral DNA of 5.2 kb. The viral DNA contains two copies of the long terminal repeat at the 5' end and an intact env gene at the 3'.
2. The 3' end of the viral DNA was subcloned in pBR322. The isolated clone contains 3 kb insert of viral sequences, 1 kb of these are the two long terminal repeats and 1.5 kb is the envelope gene.
3. Both the genomic and subgenomic clones are biologically active mice after cotransfection of NIH 3T3 cells with the cloned DNAs and a helper Mo-MuLV DNA. The rescued virus is capable of inducing proliferation of erythroid precursor cells in vitro. Hemoglobin synthesis in both erythro-proliferative disease in mice and the viral induced bursts is erythro-poietin dependent.

Significance to Biomedical Research and the Program of the Institute.

The spleen focus-forming virus as a model system for viral induced leukemia-gensis provides an opportunity for a genetic analysis of the viral oncogenic

gene. Variants of the same gene are present in SFFV_p and SFFV_A, each causes closely related but distinct disease. In vitro recombination between the two molecularly cloned genes will allow us to determine which part of the envelope gene is responsible for the differences on pathogenicity of the two viruses. The significance of differences found in the envelope gene product (gp 52) between SFFV_p and SFFV_A can then be evaluated by correlating changes in the protein with changes in the disease. Experiments of that kind will lead to a better understanding of transformation mechanism by gp 52.

Proposed Course:

1. Recombinants of the env gene of SFFV_A and SFFV_p will be constructed in vitro.
2. The recombinant env gene will be rescued as an infectious virus by cotransfection into NIH 3T3 cells along with helper DNA.
3. The pathogenicity of the rescued virus will be examined by injection into mice. In vitro BFU-E assays will be used to determine the ability of the recombinants to induce hemoglobinized burst formation in the absence and presence of erythropoietin.
4. The protein products of the recombinant env genes will be studied in respect to their size glycosylation patterns and localization in the cells.

Z01 CP 05272-01 LTVG

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Nucleotide Sequence Analysis of the Harvey Virus Related c-ras Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Martin Ruta	Staff Fellow	LTVG, NCI
Other:	Edward M. Scolnick	Lab Chief	LTVG, NCI
	Ronald Ellis	Staff Fellow	LTVG, NCI

COOPERATING UNITS (if any)

Ravi Dahr, Staff Fellow, Laboratory of Molecular Virology

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Molecular Virology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.1

PROFESSIONAL:

1.0

OTHER:

.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Harvey sarcoma virus was derived by recombination between a murine leukemia virus and rat cellular sequences. Two rat genes (designated cRAS-1 and cRAS-2) which are homologous to the Harvey virus transforming gene (RAS) have been identified. However, only the cRAS-1 gene can be activated and will transform NIH 3T3 cells in a DNA transfection assay, whereas the cRAS-2 gene does not transform NIH 3T3 cells. Although both cRAS-1 and the Harvey RAS gene encode similar p21 proteins that induce transformation, these proteins also exhibit differences. For example, a significant proportion of the Harvey p21 is phosphorylated while little if any of the cRAS-1 p21 is phosphorylated. In order to understand the differences between the cRAS-1 and Harvey RAS genes and to determine the nature of the defect in cRAS-2, nucleotide sequencing of the cRAS-1 and cRAS-2 genes was initiated. The cRAS-1 sequence determined thus far shows extensive homology to the known sequence of the Harvey RAS gene. In addition, the metabolism of the cRAS-1 and Harvey p21 proteins was analyzed.

Project DescriptionObjectives:

The purpose of this work is to determine the nucleotide sequence of the rat cellular genes (cRAS-1 and cRAS-2) homologous to the Harvey RAS gene. In addition, the metabolism of the cRAS and Harvey virus encoded p21 proteins were analyzed.

Methods Employed:

1. The rat cellular genes homologous to the Harvey viral transforming gene (cRAS-1 and cRAS-2) have been cloned in E. Coli using the plasmid vector pBR 322.
2. Nucleotide sequencing has been performed using established procedures that chemically modify DNA.
3. The metabolism of membrane associated forms of the Harvey and cRAS-1 p21 proteins was analyzed by labeling infected NIH 3T3 cells with radiolabeled palmitate and immunoprecipitating lysates with monoclonal antibodies. Peptide mapping of the p21 palmitate binding site has been performed using V8 protease.

Major Findings:

1. A partial sequence analysis of the cRAS-1 gene has been determined. A comparison of this sequence with the Harvey viral RAS sequence shows extensive similarity of the cRAS-1 exon sequences with viral sequences. In addition noncoding sequences downstream from the cRAS-1 gene are identical to viral sequences downstream from Harvey RAS.
2. An analysis of Harvey virus infected cells labeled with radiolabeled palmitate detects both p21 and its phosphorylated form pp21 consistent with the idea that both p21 and pp21 are membrane associated. V8 protease mapping of p21 from cells that have been labeled with radiolabeled palmitate indicates that the palmitate binding site is located in the carboxy region of p21.

Significance to Biomedical Research and the Program of the Institute:

An analysis of the molecular properties of the Harvey RAS gene, the cellular RAS genes, and the p21 proteins they encode is important in understanding the origin of this virus and the mechanism by which it mediates transformation. A comparison of the nucleotide sequences of Harvey RAS gene and cRAS-1 will provide insight into the origin of the Harvey virus and elucidate the differences between Harvey p21 and the cRAS-1 encoded p21. An analysis of the cRAS-2 nucleotide sequence will define the apparent defective nature of this gene. An understanding

of the molecular properties of the Harvey RAS gene and the cRAS genes is particularly important because of recent data implicating the human analogs of the Harvey RAS gene in certain human neoplasms.

Proposed Course:

1. The nucleotide sequence analysis of cRAS-1 will be completed.
2. The nucleotide sequence of cRAS-2 will be determined.

Publication:

Ruta, M., Clarke, C., Boswell, B. and Kabot, D.: Heterogeneous metabolism and subcellular localization of a potentially leukemogenic plasma membrane protein encoded by Friend erythroleukemia virus: Isolation of viral and cellular processing mutants. J. Biol. Chem. (In press)

PERIOD COVERED
 October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
 MoMuLV-Induced Rat Thymomas. Mechanisms of Oncogenesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Li Fu Hu	Visiting Fellow	LTVG	NCI
OTHERS:	Peter G. Strauss	Guest Worker	LTVG	NCI
	Philip N. Tsichlis	Expert	LTVG	NCI

COOPERATING UNITS (if any)
 NONE

LAB/BRANCH
 Laboratory of Tumor Virus Genetics

SECTION
 Viral Biochemistry Section

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS
 (b) HUMAN TISSUES
 (c) NEITHER

(a1) MINORS
 (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A 3' LTR- cell DNA junction fragment from a MoMuLV Induced rat thymoma has been cloned in bacteriophage lambda. Tumor cell DNA from 16 tumors has been examined for DNA rearrangements within the cellular sequences represented in this clone. We have shown that in five out of sixteen tumors these cellular sequences have been rearranged due to virus integration. The mechanism by which virus integration operates in tumor induction in this system is other than "promoter insertion".

Project Description

Objectives:

The overall objectives of this project are to isolate and analyze biologically important DNA sequences in MoMuLV induced thymomas in rats.

Methods Employed:

Molecular cloning Southern and Northern, blotting and hybridization, DNA transfection.

Major Findings

Examination of the mRNA in rat thymomas induced by MoMuLV revealed four tumor related RNA transcripts, two of which appear to contain cellular, in addition to viral, sequences. One of these transcripts is 2.4 kb hybridizes only to U5 sequences, and appears in 40% of the tumors. The other transcript is about 7 kb, hybridizes to env-pol but not to U5 sequences and appears in 35% of the tumors. Assuming that the genetic information in these RNA transcripts is important in oncogenesis, we were puzzled by the tumors that appeared to be missing both. Tumor cell high molecular weight DNA from a tumor missing both the RNA transcripts we described was digested with SacI that cuts within the MoMuLV LTR sequences, and the virus-cell junction fragments were identified. Several of these fragments have now been cloned in bacteriophage lamda. One of these clones is 9.5 kilobases long and contains only U5 and cellular sequences.

The cellular sequences of this cloned DNA fragment were used as a probe in order to examine rearrangements in the tumor cell DNA in 16 available tumors. We found that this DNA sequence is rearranged in 5 out of 16 tumors. The DNA rearrangement we detected is due to virus integration. We have conclusively shown this by cloning the same sequence from a different tumor and showing the physical linkage between viral and cellular sequences. Therefore we conclude that MoMuLV integrated preferentially within this cellular DNA sequence. Considering that Retroviruses appears to integrate randomly within the cellular genome it appears that this integration event is important in oncogenesis.

The mechanism by which this virus integration event operates in tumor induction is still unclear. However it does not appear to operate by "promoter insertion" for the following reasons.

1. We could not identify any "promoter insertion" predicted transcripts in the tumor from which we obtained the clone.
2. When the cellular sequences of the clone were used as probes to examine tumor cell mRNA we could not identify any RNA transcripts hybridizing to these sequences.

3. The cellular sequences of this clone do not show the characteristics of an oncogene.
 - a. They are not conserved through evolution.
 - b. They do not transform NIH 3T3 cells in culture.

Significance to Biomedical Research and the Program of the Institute:

Considerable progress has been made in understanding the molecular mechanisms of retrovirus induced oncogenesis in mammals. Our findings indicate that novel mechanisms other than "promoter insertion" are operating in this system. Understanding of these mechanisms will be of major importance in revealing the secrets of human cancer.

Proposed Course:

Our working hypothesis is that genetic elements of major significance to T cell differentiation reside in the vicinity of the site of the preferred integration we have identified. Virus integration could operate by altering the expression of these elements by mechanism (s) other than promoter insertion. Therefore, our first priority is to clone the sequences on either site of the preferred integration event and examine their conservation through evolution and their expression in the thymomas as well as in normal thymocytes. In addition we are planning to chromosomally map this sequence in an attempt to obtain clues as to what genes known to be important in T cell function may be located nearby.

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cellular Mutants Which are Resistant to Transformation by RNA Tumor Viruses.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	M. Noda	Visiting Fellow	LTVG	NCI
Other:	R. H. Bassin	Head, Viral Biochemistry Section	LTVG	NCI
	E. M. Scolnick	Chief	LTVG	NCI

COOPERATING UNITS (if any)

Z. Selinger, Hebrew University, Jerusalem, Israel

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Viral Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Flat revertants of Kirsten sarcoma virus-transformed cells have been isolated which appears to represent mutants in one of the cellular genes involved in transformation by Kirsten sarcoma virus. The revertants were isolated following selection in ouabain, which appears to be much less toxic for these mutants than for their parental transformed cells.

Revertants have been fused to a number of transformed cell lines, and the properties of the resulting heterokaryons have been examined in monolayer cultures and in agar suspension. The results show that the revertant phenotype is dominant in heterokaryons involving cells transformed by Kirsten and Harvey sarcoma viruses. On the other hand, fusion of revertant cells with cells transformed by other agents, including other RNA and DNA tumor viruses as well as chemicals results in heterokaryons which are morphologically transformed and grow in soft agar. These results indicate that the revertants described here represent mutants in a cellular gene or genes involved in transformation by Harvey and Kirsten sarcoma viruses. The differential toxic effect of ouabain on Kirsten sarcoma virus-transformed NIH/3T3 cells may indicate the mechanism for transformation by these viruses.

Project Description

Objectives:

We have been concentrating our efforts on the isolation and characterization of mutant mouse cells which are resistant to transformation by Kirsten murine sarcoma virus (Ki-MSV). Our objective is to elucidate the mechanism of action of the transforming gene product (p21) of Ki-MSV using these cellular mutants.

Methods Employed:

Morphologically flat revertants of NIH/3T3 cells originally transformed by Ki-MSV were isolated by taking advantage of the observation that such transformed cells are more sensitive to a Na/K ATPase inhibitor, ouabain, than are the parental untransformed NIH/3T3 cells. Growth in soft agar was used to test the anchorage dependency of various cell mutants and transformants, while other cell properties were assessed by routine cell culture techniques. Somatic cell hybridization using polyethylene glycol was employed to investigate the genetic properties of mutant cell lines. ^{86}Rb was used as a tracer for K^+ -flux to test whether alteration in cation transport can be correlated with morphological changes in revertant cells.

Major Findings:

Flat morphological revertants were isolated in this laboratory from Ki-MSV transformed nonproducer NIH/3T3 cells and were characterized. The possibility that a mutation in the viral transforming gene (v-ras) was responsible for the morphological reversion was deemed to be unlikely for the following reasons: 1) The DNA of the revertants still contains the two copies of exogenous v-ras sequences, which are present in the parental transformed cells; 2) Infectious MSV can be rescued from the revertants by superinfecting with competent murine leukemia viruses; 3) Approximately the same levels of v-ras gene product p21 were detected in the revertants as in the parental transformant.

The genetic properties of two revertants were investigated by somatic cell hybridization techniques. The results indicate that the revertant phenotype is dominant over transformed phenotypes exhibited by Ki-MSV or Harvey MSV-transformed cells but is recessive in hybrids with cells transformed by SV40, polyoma, or chemically transformed cells. These data suggest that our revertants can be used to elucidate some of the biochemical pathways of transformation resulting from infection of cells with Ki-MSV or related transforming agents.

Since these revertants seem to represent cellular mutants able to function normally in the presence of the v-ras gene product, a search for the altered functions (s) in these revertants may provide some insight into the mechanism of transformation.

Proposed Course:

Since our revertants were obtained by mutagenization followed by ouabain selection, ouabain resistance might behave co-dominantly with the revertant phenotype. If we can establish this point, isolation of the gene responsible for the reversion should be possible by using the ouabain resistant phenotype as a selective marker. Studies to confirm this idea will include the transfection of DNA from appropriate cells into normal and virus transformed cells as well as further cell hybridization experiments. The role of Na/K ATPase in transformation by Kirsten sarcoma virus will be examined.

Publications:

M. Noda, Kurihara, M. and Takano, T.: Retrovirus-related sequences in human DNA: detection and cloning of sequences which hybridize with the long terminal repeat of baboon endogenous virus. Nucl. Acids Res. (In press).

Tamura, T., Noda, M., and Takano, T.: Structure of the baboon endogenous virus genome: nucleotide sequences of the long terminal repeat. Nucl. Acids Res. 9: 6615-6626, 1981.

Takano, T., Noda, M., and Tamura T.: Transfection of cells from a xeroderma pigmentosum patient with normal human DNA confers UV resistance. Nature 296: 269-270, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05283-01 LTVG
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of the Expression of Mouse Mammary Tumor Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	M. Rousset	Fogarty Fellow	LTVG	NCI
Other:	D. S. Berard	Microbiologist	LTVG	NCI
	A. Huang	Chemist	LTVG	NCI
	A. Lichtler	Postdoctoral Fellow	LTVG	NCI
	G. L. Hager	Head, Viral Immunogenetics Sec.	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: 0.0	PROFESSIONAL: 0.0	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We previously demonstrated that the Mouse Mammary Tumor Virus Long Terminal Repeat (MMTV LTR) contains a target for glucocorticoids. Chimeric molecules containing the MMTV LTR ligated to the transforming gene v-ras of the Harvey murine sarcoma virus (Ha MuSV) have been constructed thereby placing the v-ras gene under hormonal control. Because the MMTV LTR unit was relatively inefficient upon transfection, a transcriptional enhancer sequence was ligated upstream from the LTR increasing 1,000 fold the efficiency of foci formation. We, therefore, propose that the MMTV LTR is very inefficient by itself and requires a cis-acting element for its efficient functions as a transcriptional unit. Deletions within the U3 region of the MMTV LTR have been made and tested by transfection on NIH 3T3 for their ability to give foci in the presence or absence of hormone. The results permit us to locate the hormone target site in the U3 region between 215 bp and (105) bp 5' proximal to the cap site and to conclude that the open reading frame within the U3 region is not necessary for hormone inducibility.

Project DescriptionObjectives:

- (1) Hybrids between MMTV LTRs, viral transforming gene and transcriptional activators will be constructed by molecular cloning. These hybrids will be transfected into NIH 3T3 cells. Transformed cells will be subcloned and studied for the expression of the v-ras gene in the presence or absence of a synthetic glucocorticoid, the dexamethasone.
- (2) Deletion mutants of the MMTV LTR will be made in the chimeric constructions described above. The deleted molecules will be tested by transfection into NIH 3T3 for their ability to give foci in the presence or absence of dexamethasone. These experiments should allow us to locate the hormone target site within the MMTV LTR.

Methods Employed:

- (1) Molecular hybrids are constructed from purified restriction fragments of cloned retroviral elements in the bacterial cloning vector PBR 322 and characterized by restriction endonucleases mapping.
- (2) Transfection of chimeric molecules will be performed using the calcium phosphate technique. Transformed cells will be subcloned and their DNA analyzed by Southern blot analysis.
- (3) The initiation sites for transcription will be determined by S1 mapping of the RNA 5' ends.
- (4) The expression of the viral transforming protein under the hormonal control will be used using the Western blot technique. Protein extracted from cells, subjected to electrophoresis on polyacrylamide gels, are transferred to nitrocellulose sheets. The sheets are reacted with ¹²⁵I labeled monoclonal antibodies to detect the position and quantities of the transforming protein.
- (5) Deletions are constructed by specific restriction endonuclease digestion within the MMTV LTR using appropriate chimera in PBR 322.
- (6) Sequencing analysis of cloned fragments have been made using the Sanger technique.

Major Findings:

The transfection efficiency of MMTV LTR v-ras hybrids is 100 - 1,000 fold lower than when v-ras is joined to its normal Ha MuSV C LTR. By adding sequences from the Ha MuSV LTR upstream to the MMTV LTR-v-ras chimeras we increased the transfection efficiency 1,000 fold compared to the original chimeras in the presence

of glucocorticoids. By sequencing we show that these enhancer sequences possess the 72 base pair direct repeats sequences which have been implicated as transcriptional "activators".

The level of v-ras mRNA and protein in these new foci remain responsive to regulation by glucocorticoids, we then propose that the MMTV LTR requires a cis-acting element for its efficient use as a transcriptional promoter or that a transcriptional host-range exists in different cell types for the utilization of specific promoters.

Transfection of deleted molecules within the U3 region of the MMTV LTR allowed us to locate the hormonal target site in the LTR. Deletions up to -365 from the 5' cap site in the LTR gave chimera which give as many foci as the chimera containing the entire LTR only in the presence of dexamethasone, therefore, deletions up to -365 do not affect glucocorticoid inducibility. When we delete everything up to the position -105 from the cap site, the number of foci obtained by transfection of these molecules containing a 1,222 base pairs deletion is very high in the presence or in the absence of dexamethasone. These results show that the expression of the v-ras transforming gene is no longer under the control of the glucocorticoid hormone.

Moreover, the Unit V of MMTV was cloned and sequenced and showed that a 100 base pairs sequence is rearranged in the U3 region between position -325 and -215 from the cap site. This rearranged region does not affect the regulation of transcription. We propose that the hormone target site is located between position -215 and -105 from the cap site and that the pLTR is not necessary for hormone induction. Single point mutations within the hormone target site region within the LTR should allow us to precise the exact site of interaction between the LTR and the hormone receptor complex.

Significance to Biomedical Research and the Program of the Institute:

Mouse mammary tumor virus has served as a model to study the etiology of neoplastic disease and to investigate the mechanisms of eukaryotic gene expression regulated by hormones. Molecular cloning of the hormonal target site will allow us to examine in details the interactions between the DNA and the hormone-receptor complex.

Proposed Course:

Given the availability of our chimera, single point mutations will be made to locate more precisely the hormonal target within the LTR.

The gene pLTR will be cloned into a prokaryotic vector to secrete pLTR protein. Antibodies will be raised against pLTR to study the role of such a protein in the process of mammary tumor formation.

Z01 CP 05284-01 LTVG

PERIOD COVERED

October 1, 1982 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies to Oncogene Products of McDonough Feline Sarcoma Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. J. Anderson	Staff Fellow	LTVG	NCI
OTHERS:	C. J. Sherr	Head, Viral Pathology	LTVG	NCI
	Mark Furth	Staff Fellow	LTVG	NCI
	Sandra Ruscetti	Senior Staff Fellow	LTVG	NCI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Tumor Virus Genetics

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies reactive with epitopes encoded by the viral oncogene, v-fms, of McDonough feline sarcoma virus (SM FeSV) have been prepared. The classes and reactive specificities of these antibodies have been characterized. Biochemical analysis and subcellular localization studies of the proteins encoded by v-fms have been facilitated by use of these reagents. Such studies have indicated the presence of detectable levels of a potential normal cellular analog of the v-fms encoded proteins in various cell lines of human and other species origin.

Project Description

Objectives:

- (i) To generate and characterize monoclonal antibodies reactive with v-fms encoded proteins.
- (ii) To analyze the biochemical features of v-fms encoded proteins.
- (iii) To identify the cellular location of v-fms encoded proteins.
- (iv) To isolate and analyze candidate normal cellular analogs of v-fms encoded proteins.

Methods Employed:

G-2/mink, a nonproducer clone of CCL64 mink lung cells transformed by SM-FeSV, was used as a source of v-fms-encoded proteins. In this cell line, v-fms translation products are gp180gag-fms, which contains part of the feline leukemia virus gag protein as well as the v-fms encoded peptides, and gp120fms, which is entirely the translation product of v-fms. Hyperimmune sera from rats bearing SM-FeSV-induced tumors were assayed for reactivity against these proteins. A single animal which had the highest titer of immunoprecipitating antibody against gp120fms was used as spleen donor. Splenic lymphocytes were fused to rat myeloma cells, and hybrid cells were selected by growth in the presence of hypoxanthine, aminopterin, and thymidine. Hybrid cells producing antibodies specifically reactive with gp120fms were identified by assaying the cell culture media for immunoprecipitating activity. These cells were subcloned twice in soft agar, established as cell lines, and grown in serum-free medium. Monoclonal immunoglobulins were precipitated from the medium by addition of ammonium sulfate, and purified by ion exchange chromatography. The class and subclass of the immunoglobulins were identified by Ouchterlony immunodiffusion assays, and by electrophoresis on polyacrylamide gels.

The isolated, characterized monoclonal antibodies were used, either directly to immunoprecipitate gp120fms from SM-FeSV transformed cells, or to assay fms encoded proteins by immunoblotting. Direct immunoprecipitation of fms encoded proteins from metabolically labeled cells allowed studies of amino acid and sugar incorporation, as well as preparation of the proteins for tryptic and V-8 protease peptide mapping. For immunoblotting assays, the monoclonal antibodies were labeled with ¹²⁵I and reacted with cellular lysates which had been electrophoresed on polyacrylamide gels and transferred to nitrocellulose. Total cellular pools of proteins were assayed by immunoblotting, or alternatively, subcellular fractions were isolated prior to electrophoresis, transfer to nitrocellulose, and exposure to ¹²⁵I-labeled monoclonal antibodies.

Major Findings:

Of nine hybrid cell clones producing monoclonal antibodies specific for fms encoded proteins, four were chosen for characterization and further use by virtue

of superior growth characteristics and specific antibody titers. Of these four clones, one proved to be an IgM secretor, and three produced antibodies of the IgG class, subclass γ 1. Of the three IgG monoclonals, two reacted both in immunoprecipitation and in immunoblotting assays, and one reacted only in immunoprecipitation assays. Direct immunoprecipitation of proteins from metabolically labelled, SM-FeSV transformed cells demonstrated that gp120fms, in contrast to other characterized oncogene products, is not phosphorylated, and is heavily glycosylated. Immunoprecipitation of v-fms encoded proteins labeled in the presence of tunicamycin indicated that the unglycosylated forms of gp180gag-fms and gp120fms are 155 kilodaltons and 95 kilodaltons, respectively. Immunoblotting of SM-FeSV-transformed cells has revealed the proportion of v-fms encoded proteins in total cellular pools. Considering the results of both metabolic labelling and immunoblotting studies, a model of processing of v-fms encoded proteins was formulated: a primary translation product of 155 kilodaltons is rapidly glycosylated, yielding the gp180gag-fms protein, and rapidly cleaved between the gag and fms encoded portions of the polyprotein, yielding an unglycosylated gag encoded protein of 60 kilodaltons, and the glycosylated gp120fms. Further glycosylation of gp120fms yields a final product of 140 kilodaltons, which is readily detected in immunoblotted protein pools of SM-FeSV transformed cells.

One of the two monoclonal IgGs used in the immunoblotting assay reacted with a protein of 85 kilodaltons (ncp85) which was detected, not only in SM-FeSV transformed cells, but also in CCL64 mink lung cells, feline embryonic fibroblasts, dog and monkey kidney cells, and in some, but not all, lines of normal and transformed human cellular origin.

Subcellular fractionation studies have shown that the v-fms encoded proteins and the potential c-fms product, ncp85, co-purify in association with the sedimentable cytoplasmic organelles.

Significance to Biomedical Research and the Program of the Institute:

The present study has focused on the products of a single oncogene whose proto-oncogene has been identified and cloned from human cellular DNA. Finding the protein(s) encoded by the proto-oncogene and comparing these products to those of the oncogene is essential to comprehending the process of transformation evoked in this system.

Proposed Course:

In order to address the question of comparative functions of the proteins encoded by c-fms and v-fms, biochemical analysis of the proteins is required. V-8 protease and tryptic peptide mapping of gp180gag-fms, gp120fms, and ncp85 are in progress. Further subcellular fractionation experiments are aimed at pinpointing the specific organelle(s) with which the fms encoded proteins are associated. Such experiments will be coordinated with collaborative efforts involving immunoelectron microscopy using the gp120fms-specific monoclonal antibodies. The combination of biochemical characterization and subcellular localization of the transforming and putative normal fms encoded proteins should elucidate their functions in normal and transformed cellular metabolism.

CONTRACT NARRATIVE
CARCINOGENESIS INTRAMURAL PROGRAM
DIVISION OF CANCER CAUSE AND PREVENTION
Fiscal Year 1983

Laboratory of Tumor Virus Genetics

MELDY LABORATORIES (N01-CP-01013)

Title: Immunological and Biochemical Studies of Mammalian Viral Oncology

Contractor's Project Director: Dr. Richard Howk

Project Officers (NCI): Dr. Edward M. Scolnick and Dr. Robert J. Goldberg

Objectives: The purpose of this contract is to provide resource support and technical services to the Laboratory of Tumor Virus Genetics, Carcinogenesis Intramural Program, Division of Cancer Cause and Prevention, National Cancer Institute. The areas of support include cell culture virology, biochemistry, immunology and recombinant DNA Technology.

Methods Employed: Various mammalian cell lines were propagated using standard, and when specified specialized tissue culture procedures including cell cloning and culture in defined, serum-free media. Virus stocks were grown and titrated using XC, S⁺L- and reverse transcriptase assays. Nucleic acids and proteins were isolated and purified using various biochemical techniques including differential centrifugation, affinity, ion-exchange and gel chromatography, immunoprecipitation and gel electrophoresis. The contractor maintained the capability to perform multiple RIA's when needed. Hybridoma cell lines for the production of monoclonal antibodies to retroviral determinants were prepared and characterized. The recombinant DNA's support service was responsible for the growth, harvest, purification and analysis of bacteria containing recombinant DNA's using biological and molecular techniques specified by NCI investigators. In addition the recombinant DNA lab carried out isolation of DNA inserts via agarose gel electrophoresis and electroelution, ligation of inserts to vectors, transfection or *in vitro* packaging of DNA into suitable hosts and the screening of the clones thus generated via the Benton and Davis filter hybridization method. For plasmid screening a small-scale Birnboim extraction was done.

Major Findings: Twenty to thirty cell lines were maintained and passaged on a bi-weekly basis. Normal and transformed cells were radioactively labeled with ³²P and/or ³⁵S-methionine weekly. Defined, serum-free media was designed to study the effect of various factors on normal and transformed cells. One system was applied to the study of dexamethasone on cells transfected with recombinant DNA consisting of MMTV long terminal repeat joined to the Ha-SV genome. Monoclonal antibodies to Friend murine leukemia virus and to Friend MCF virus proteins were prepared, maintained, titered and characterized by immunoprecipitation. Over 474 mls of Ascites fluid were obtained. In addition, use of these monoclonals for the affinity column purification of their respective proteins was explored. Biochemistry conducted the purification of high molecular weight DNA from six cell lines. Cell membrane fractions were from Ha-SV infected cells obtained by the biphasic polyethylene glycol-dextran system of Burnett and Till. Fourteen preparations each from 200 roller bottles were made. The membranes were used for the purification of Ha-SV p21 protein. The major emphasis of the

Major Findings Continued:

contract was as support service for the molecular cloning of RNA tumor virus DNA and the use of these molecules to probe the structure, expression and function of the tumor virus genome. During the report period 90 plasmid preparation (1-5 liters each, generally 4 liters) were grown. Plasmid DNA was isolated according to the alkaline-SDS procedure described by Birnboim and Doly. All DNA's were digested with the appropriate restriction endonuclease and analyzed by agarose gel electrophoresis. Bacteriophage lambda DNA (25 preparations) was purified from twice CsCl banded phage from lysates of 1 to 4 liters. Other work included ten recombinant DNA clone constructions and subsequent screenings.

Significance to Biomedical Research and the Program of the Institute: Tumor viruses are recognized as valuable reagents for defining the molecular pathways leading to malignant transformation. The isolation of viral transforming proteins and the identification of the genetic sequences that code for these proteins have provided reproducible means by which the molecular mechanisms of cancer can be studied. It is now clear that retrovirus transforming genes are derived by recombination with normal cellular genes which are highly conserved in all vertebrate species including man. These studies are the mainstay of the research program of the Laboratory of Tumor Virus Genetics and this contract supplies indispensable resource support and technical services not presently available on the NIH reservation to this critically important area of cancer research.

Proposed Course: The present contract is expected to continue through November 19, 1984. Primary emphasis will be placed on those resource support activities related to LTVG research using DNA recombinant and hybridoma technology. No major changes in the workscope of the contract are presently planned.

Date Contract Initiated: May 1980

Current Annual Level: \$550,000

Manyears: 7.4

Annual Report of the Laboratory of Viral Carcinogenesis

National Cancer Institute

October 1, 1981 to September 30, 1982

The Laboratory of Viral Carcinogenesis (LVC) is charged with the planning, development, implementation, and coordination of the Institute's research programs in carcinogenesis with special emphasis on delineating the role of oncogenic viruses, virus-related genetic sequences in human cancer, and the general mechanisms of cellular gene control. Research efforts are conducted on virus-host relationships in virus-induced cancers with emphasis on the detection and characterization of oncogenic viruses, their integrating genetic tracts, and their mode of transmission in animals and man. The host immune mechanism is studied in relationship to its genetic control, especially in virus-related cancers. Investigations are conducted on the molecular processes in carcinogenesis and cocarcinogenesis. Viruses are used as the most precise tools for the analysis of gene control and malignant transformation. Activities of the Laboratory are conducted by in-house research and collaborative agreements with other research organizations.

During the last month of the previous year the LVC was site visited by a team of distinguished non-government scientists that included members and non-members of the Division of Cancer Cause and Prevention Board of Scientific Counselors. This team reviewed all scientific activities of the Laboratory including details on personnel, space, and financial resources allocated to each section, working unit, and the Laboratory as a whole. Their findings included such statements as: "The LVC has been extremely productive, ... is responsible for a number of key advances in the concepts and development of new knowledge in the general area of viral carcinogenesis. (It) is a good laboratory which has and is playing an important role ... which brings credit to the NCI." The Site Visitors and the Board made constructive and specific recommendations concerning each Section. Their report was received in February, 1982 and its suggestions have been seriously considered and implemented. Changes have been organized in an orderly manner leading to essential compliance by the completion of this fiscal year. The physical plant changes required to accommodate all units of the LVC at the Frederick Cancer Research Facility (FCRF) were mostly completed during the first quarter. Facilities for animal holding and experimentation were at contract sites for most of this year with a phased transition to the FCRF from June through September.

Results described in this report strongly support the concept that malignant transformation in humans is most probably a multistage, multifactorial progression of internal and external events which follow well-established pathways governed by molecular, enzymatic and genetic controls as well as mechanisms mediated by definable, biologically active molecules. It appears further that the capacity to start, promote, and accelerate such progression depends strongly on the mosaic genetic constitution intrinsic to a given host; some genetic information naturally conferring protection while another increasing risk. Among these endogenous host factors are the presence, absence, control and gene-dose of certain DNA sequences, integrated in the host's chromosomes, which are related to known oncogenic mammalian and primate viruses and other, possibly nonviral (cellular), information that can express intermediate molecules, hormone-like substances

which can induce phenotypic changes related to malignancy in cells. Some exogenous factors may include chemical carcinogens, tumor promoters and oncogenic viruses. This Laboratory seeks to elucidate the basic mechanisms of cancer induction and progression in order to harness them in ways useful in prevention or modification of a cell's progression towards cancer. The most significant findings obtained during this year can be clustered as follows: (A) the identity, nature and function of hormone-like control and signal factors that mediate the genetic and phenotypic expression of malignancy in mammalian cells; (B) the pathways and intermediate products involved in endogenous transformation, tumor promotion and cocarcinogenesis; (C) the identity, origin, transmission, structure, function and virulence of proviral and cellular DNA sequences in mammals, primates and possibly humans; and (D) the results of epidemiological studies on human cancers with associated viral etiological components.

A. Identity, Nature and Function of Hormone-like Control and Signal Factors that Mediate the Genetic and Phenotypic Expression of Malignancy in Mammalian Cells.

Perhaps one of the most exciting and promising areas in cancer research at present concerns the discovery of an ever-increasing number of factors or families of hormone-like cellular substances that have profound influences on the phenotypic, and probably genotypic, behavior of mammalian cells. Many of these peptides stimulate growth and mitogenic activity (e.g., epidermal growth factor, EGF); and others (transforming growth factors, TGFs) stimulate cells to phenotypic malignant behavior. These factors act by interacting with specific receptor sites on the cell membrane and may sustain the malignant state by secretion and autostimulation by the malignant cell.

A low molecular weight TGF was isolated from serum-free medium conditioned by the human metastatic melanoma tumor line A2058 and purified. Only one major single band, with an apparent molecular weight of 7,400, was observed. Reduction with 2-mercaptoethanol did not change the mobility of TGF, which is evidence for a single chain polypeptide. Sarcoma growth factor (SGF) isolated from Moloney murine sarcoma virus (MSV)-transformed mouse 3T3 cells, and rat TGFs, from either Abelson murine leukemia virus (MuLV)-transformed or Snyder-Theilen feline sarcoma virus (FeSV)-transformed Fisher rat embryo (FRE) fibroblasts, were purified to apparent homogeneity using the same methodology developed for the purification of human TGF. The amino acid compositions of human melanoma-derived TGF, murine SGF and rat TGFs were then determined. TGF/SGF is a single chain polypeptide of 67 residues with three intrachain disulfide bridges and no free sulphydryl groups. The amino acid compositions of TGF/SGF are similar, but not identical. Human TGF lacks tyrosine, whereas both rat TGF and murine SGF have two tyrosine residues. The amino acid compositions of TGF/SGF are unique and show a number of important differences when compared with the amino acid compositions of either human or mouse EGF, indicating that TGF/SGF are distinct from EGF. Both human and mouse EGF have one methionine, whereas TGF/SGF lacks methionine. Human and mouse EGF have 5 tyrosine and no phenylalanine residues. In contrast, human TGF has no tyrosine, but 3 phenylalanine residues; murine SGF and rat TGF both have 2 tyrosine and 3 phenylalanine residues. TGF/SGF is a less hydrophobic polypeptide than EGF. The amino terminal sequences of human TGF, murine SGF and rat TGF were determined. The sequence homology of the first 41 residues of rat TGF and murine SGF is 100%. The homology of the first 34 residues of human TGF compared with rat TGF and murine SGF is 91%. Thus, human, rat and murine TGF/SGF are structurally closely related and are more conserved than are mouse and human EGF (68% sequence homology for residues 1 through 34). Alignment of the amino acid sequences

of TGF/SGF with the sequence of mouse or human EGF revealed statistically significant sequence homology. The alignment results in 12 common or conserved amino acid residues out of 42 possible comparisons. To obtain such an alignment, the introduction of only a single break was necessary in the TGF/SGF sequence. Thus, the conserved amino terminal sequences of TGF/SGF and EGF could represent the receptor binding site common to both TGF/SGF and EGF.

In addition to the production of TGFs which confer the transformed phenotype on untransformed fibroblasts, some human tumor cells have been found to produce another class of factors, designated tumor inhibitory factors (TIFs), which inhibit the growth of some human tumors (rhabdomyosarcomas, melanomas and carcinomas) and mink lung epithelial cells *in vitro*. The major TIF activities fractionate into three molecular weight classes (5,000, 10,000-12,000 and 20,000) by gel permeation chromatography. TIFs stimulate normal human and rat fibroblasts to proliferate in monolayer cultures. Both growth-inhibiting and growth-promoting activities were found to be concentration-dependent. The concentration of the 5,000 molecular weight species of TIF required for inhibition of tumor cells or stimulation of normal human fibroblasts was 10-120 ng/ml. This is the concentration at which potent mitogens, such as EGF, stimulate mitogenesis. TIFs do not possess the antiviral activity found in interferon.

TGFs were detected in the urine of normal, pregnant and tumor-bearing humans. All urine samples contained an 8,000 M_r soft agar growth-stimulating activity which is chromatographically separable from EGF. The urine from pregnant females contains TGF activities with apparent molecular weights of 10,000 and 17,000-20,000. In addition, the urine from an 18-year old female with metastatic alveolar cell soft tissue sarcoma contained a 30,000-35,000 M_r EGF-competing activity which was not present in urine derived from healthy individuals or mid- to late-term pregnant donors. To determine if the high molecular weight TGF activity observed in the urine of the above-described sarcoma patient was also present in other cancer patients, a rapid screening assay using smaller urine volumes was designed. The 30,000-35,000 M_r TGF activity was detected in the urine of 18 of 22 cancer patients with diagnoses including carcinoma of the lung, breast, colon, several sarcomas and melanomas and in only 5 of 22 nonmalignant controls. Control urine specimens were collected from a population in which half of the donors had nonmalignant infections and inflammatory conditions, including bronchitis, pneumonia, colitis and diverticulitis. The patterns of expression of the high molecular weight TGF in the urine of cancer patients has led to the exploration of diagnostic, prognostic and therapeutic applications. For example, it provides an indication of tumor burden that may be useful for evaluation of the effectiveness of therapy. In addition, the detection of a lower molecular weight species of TGF in the urine of mid- to late-term pregnant females suggests a normal role for these factors in embryogenesis. The relationship of the TGFs and TIFs to the activation (or inactivation) of genetic information exhibited by malignant cells will significantly contribute to the definition of the mechanisms and progressive nature of carcinogenesis by either endogenous or environmental factors.

Cells transformed by acute transforming viruses of feline origin which differ in cellular acquired sequences were used to examine the effect of v-fes and v-fms genes on both the production and biological activity of TGFs. Cell cultures nonproductively transformed by the Snyder-Theilen and Gardner strains of FeSV, both of which contain the v-fms gene product and release a small (10,000 M_r) and large (20,000 M_r) form of TGF. Both size classes of TGF compete with EGF for EGF membrane receptors and stimulate anchorage-independent cell growth and thus

resemble SGF and TGFs released by human tumor cells. Snyder-Theilen FeSV-transformed cultures which contain a polyprotein-associated, tyrosine-specific protein kinase consistently release the highest titers of TGF relative to all transformants examined. In contrast, cells transformed by McDonough FeSV (v-fms gene) which lack detectable kinase activity produce low levels of TGF. Large scale production of Snyder-Theilen transformed cell culture supernatants currently provide a rich source of TGF for use in primary sequence studies.

Membrane components that interact with EGF and TGFs have been identified by covalent crosslinking to their respective ^{125}I -labeled ligands. Under appropriate conditions, disuccinimidyl suberate or hydroxysuccinimidyl azidobenzoate cross-link receptor-bound ^{125}I -EGF to a 140-170 kilodalton receptor species in membranes from both A431 human carcinoma cells and normal rat kidney cells. ^{125}I -SGF, a TGF from virally-transformed mouse 3T3 cells, can also be affinity cross-linked to the 140-170 kilodalton EGF receptor species in membranes from A431 and rat kidney cells. The labeling of this receptor is inhibited when either excess unlabeled EGF or SGF is present during incubation of membranes with ^{125}I -EGF or ^{125}I -SGF. In contrast, a second receptor species of 60 kilodaltons is affinity-labeled with ^{125}I -SGF but not ^{125}I -EGF in membranes from both A431 and rat kidney cells. SGF and a TGF from virally-transformed rat embryo cells inhibit the labeling of the 60 kilodalton species when present in excess during incubation of membranes with ^{125}I -SGF, while EGF is completely ineffective in inhibiting the labeling of this receptor. The data suggest that a specific 60 kilodalton receptor that displays high affinity for TGFs but not EGF may mediate induction of the transformed phenotype. In addition, SGF and other TGFs interact with the 140-170 kilodalton EGF receptor that appears to mediate normal cell growth effects.

Current evidence suggests that the genes which code for SGF obtained from the supernatant fluid of cells transformed by murine sarcoma virus are of host cell, rather than viral, origin. Normal rat kidney cells were transformed by either the wild type Kirsten sarcoma virus or a temperature sensitive (ts) variant of this virus. The growth factors released by these cells were isolated and compared for temperature sensitivity. Both factors were resistant to heat treatment and the growth factor obtained from the cells transformed by the ts variant stimulated phenotypic transformation and anchorage-independent cell growth in soft agar at the nonpermissive temperature. If SGF is a direct viral gene product, it would be expected that the SGF obtained from the cells transformed by the ts variant would itself be temperature sensitive, as compared to the SGF released by the cells transformed by the wild type virus. Another line of evidence is the observation that spontaneous murine tumors from nonvirally transformed cells produce similar SGFs. Also, human melanomas, which have no known viral etiology, produce factors similar to SGF, with the amino acid sequence data showing greater than 90% homology.

Rabbit antisera, produced against purified EGF receptors from A431 cells (human epidermoid carcinoma), block the binding of radiolabeled EGF to the EGF receptors on A431 cells and to the EGF receptors of several other species. The antisera also block the mitogenic effect of EGF and purified SGF when tested on human skin fibroblasts. This suggests that SGF exerts its effect through the EGF receptor system on these cells. This does not eliminate the possibility that SGF has separate receptors, but rather suggests that, on these cells, it either acts directly through the EGF receptor system or with receptors that cross-react with the antibody to the EGF receptor.

TGFs confer a malignant phenotype on non-neoplastic cells. The possibility was examined that TGFs play a role during embryogenesis and takes advantage of the fact that embryonal carcinoma (EC) cells represent a model system for the study of early mammalian development. The EC cell lines used were induced to differentiate into parietal and visceral endoderm, which are two of the first cell types to form during mammalian development. The differentiated cells from EC cells but not the parental cells themselves, were found to respond by increased growth to TGFs released by virus-transformed cells. Furthermore, EC cells which did not respond to exogenously added TGFs were shown to release their own factors with TGF activity. Based on the close relationship between mouse EC cells and the cells of early embryos, the possibility that TGFs play a role during the early stages of mammalian development must be seriously considered.

Hormone-supplemented defined media able to support the growth and differentiation of several EC cell lines are being developed in order to identify and study the extracellular signals that induce differentiation. Previous studies have demonstrated that the nullipotent EC cell line F9 can be grown in a defined medium (EM-3) containing fibronectin, insulin and transferrin. The F9 cells do not differentiate in EM-3 unless exposed to an inducer such as retinoic acid. Current studies indicate that multipotent EC cells do not proliferate in EM-3. In direct contrast to F9, three different multipotent EC cell lines rapidly differentiate when cultured in EM-3 and the differentiated cells that form continue to proliferate for several generations provided EM-3 is supplemented with high density lipoprotein. In each case the entire EC cell population differentiates in the defined medium and one major cell type forms; greater than 85% of the cells exhibit the properties of parietal extraembryonic endoderm. Attempts to understand these results led to the finding that fibronectin and laminin induce multipotent EC cells to differentiate and provide the first evidence that these molecules may directly influence the appearance of extraembryonic endoderm during early mammalian development.

B. Pathways and Intermediate Products Involved in Endogenous Transformation, Tumor Promotion and Cocarcinogenesis.

Single tyrosine phosphorylation sites identified within the amino terminal domains of transformation-specific polyproteins encoded by the Gardner and Snyder-Theilen strains of FeSV were shown to represent major *in vitro* acceptors for the polyprotein-associated protein kinases. By two dimensional tryptic peptide analysis, these acceptor sites are highly related and by analytical high performance liquid chromatography, all three elute at approximately the same acetonitrile concentration (22%). The tyrosine acceptor sites in these FeSV-encoded polyproteins were localized by sequential Edman degradation at a position seven amino acid residues distal to their trypsin cleavage sites. By a similar approach two separate tyrosine acceptors were identified with the 120,000 M_r polyprotein gene product of the Abelson strain of MuLV. These were localized at six and seven residues, respectively, distal to a trypsin cleavage site. The peptide acceptor of P120^{gag-ab1} mapping at position seven represents the major tyrosine site phosphorylated *in vivo*.

A series of hybridomas were derived from spleen cells of Fisher rats immunized with syngeneic Gardner (GA), Snyder-Theilen (ST) and McDonough FeSV-transformed cells. These include two hybridomas producing IgG₁ antibody against FeLV gag gene structural components, one of which is directed against FeLV p15 and the second, specific for FeLV p30. The FeLV p15 determinants recognized by the first

of these clones are shared by polyprotein gene products of all three strains of FeSV while the p30 determinants recognized by the second are unique to the P170^{gag-fms}. The three remaining hybridomas, one initially isolated from spleen cells of Gardner FeSV-immunized rats and two from spleen cells of rats immunized with Snyder-Theilen FeSV, lack detectable reactivity with FeLV structural proteins. Of these latter monoclonal antibody reagents, one is an IgG_{2a}, one an IgG_{2b}, and the remaining one, an IgM. The Gardner and Snyder-Theilen FeSV-encoded polyproteins exhibit tyrosine-specific protein kinase activity following immunoprecipitation by F72 hybridoma antibody, supporting the possibility that the observed reactivities represent intrinsic properties of the polyproteins themselves rather than associated cellular reactivities. In contrast, P170^{gag-fms}, when assayed under similar conditions, lacked detectable protein kinase activity, but was phosphorylated in tyrosine when coimmunoprecipitated with either the Gardner or Snyder-Theilen FeSV-encoded polyproteins. These findings are consistent with our previous results indicating P170^{gag-fms} to lack intrinsic protein kinase enzymatic activity and may account for a reported low level of P170^{gag-fms} phosphorylation in immunoprecipitates obtained with conventional hyperimmune antisera. Monoclonal antibodies produced by hybridomas F113, F115 and F123 are specific for the acquired sequence (v-fes) components of the Gardner- and Snyder-Theilen-encoded polyproteins. None of these three reagents exhibit detectable cross-reactivity with FeLV Pr65^{gag}, or with any of the FeLV gag and env gene-encoded structural proteins. In contrast to polyproteins immunoprecipitated by F72 antibody, the F113, F115 and F123 precipitated polyproteins lack detectable enzymatic activity, and in appropriate mixing experiments direct inhibition of the protein kinase by these latter monoclonal reagents can be demonstrated. Immunoprecipitation of ST P85^{gag-fes} by antibody secreted by F123, the one hybridoma originally isolated from spleen cells of a Gardner FeSV-transformed cell immunized rat argues that it is specific for immunologic determinants mapping within the region of GA P110^{gag-fes} shared with ST P85^{gag-fes}. Finally the lack of detectable immunoprecipitation of P170^{gag-fms} by any of these three v-fes specific reagents is consistent with previous studies indicating the translational products of the v-fes and v-fms genes to be structurally and immunologically unrelated.

To define the human homolog(s) of transforming sequences (v-fes gene) common to the Gardner and Snyder-Theilen isolates of FeSV independent virus isolates, a representative library of human lung carcinoma DNA in a cosmid vector system was constructed. Two cosmids containing the entire cellular homolog of the GA/ST FeSV-acquired cellular sequences, and a third cosmid containing a major portion of these sequences were isolated. Cellular inserts within these three cosmids range from between 32 to 42 kb in length and represent overlapping regions corresponding to a total of 56 kb. Sequences, both homologous to and colinear with, GA/ST FeSV v-fes are distributed discontinuously over a region of up to 9.5 kb and contain a minimum of 3 regions of nonhomology representing probable introns. Upon transfection to RAT-2 cells, using a thymidine kinase selection system, the human c-fes sequence lacked detectable transforming activity. Using a similar approach the human lung cosmid library has also been utilized for the molecular cloning of human cellular sequences homologous to v-abl, an oncogenic sequence with specificity for lymphoid transformation. The human c-abl gene is distributed over a much more extensive region of the human genome than c-fes, thus requiring several overlapping cosmid clones for its complete representation.

The Gardner and Snyder-Theilen isolates of FeSV represent genetic recombinants between feline leukemia virus (FeLV) and transformation-specific sequences of cat

cellular origin (v-fes gene). A related transforming gene (v-fps) common to the Fujinami (FSV) and PRC II strains of avian sarcoma virus has also been described. Polyprotein gene products of each of these virus isolates exhibit tyrosine-specific protein kinase activity. By restriction endonuclease and molecular hybridization analysis, the v-fes and v-fps genes have been found to contain highly related sequences. Moreover, molecular probes corresponding to each hybridize to a single well-resolved 12 kb EcoRI restriction fragment of human cellular DNA.

To accurately define regions of sequence homology of the v-fps gene with the human cellular homolog of v-fes, a series of three cosmid clones containing v-fes homologous sequences within a total of 56 kb of contiguous human DNA sequences were examined. Hybridization with FSV v-fps was restricted to a single 12.0 EcoRI restriction fragment containing v-fes homologous sequences. Following subcloning, the 12.0 EcoRI fragment was digested with a series of restriction endonucleases and analyzed for hybridization to v-fes- and v-fps-specific probes. FSV v-fps homology to human DNA most closely resembled that of GA FeSV v-fes. However, the 3' terminal v-fes homologous region of human DNA common to ST and GA v-fes did not hybridize to detectable extent with the FSV v-fps probe. ST v-fes homologous sequences differ from those of both GA v-fes and FSV v-fps in that they are not represented within the 5' terminal region hybridizing to the other two. Finally, two regions of human DNA homology unique to FSV v-fps were identified in the 5' half of the 12.0 kbp human EcoRI DNA fragment. Thus, although all three viral "oncogenes" appear to be entirely represented by homologous sequences within a single c-fes/c-fps human genetic locus, the exact positions at which regions of homology map differ among the individual viral transforming genes. The lack of detectable hybridization of v-fps with the 3' region of the c-fes/c-fps locus could reflect evolutionary divergence. Alternatively, a comparison of the 5' terminal position of ST v-fes, and 3' position of FSV v-fps homologous sequences within the human c-fes/c-fps locus, may provide an estimate of human DNA sequences required for transformation.

Research on the mechanism of tumor promotion led to the following conclusions: (a) Mitogenic stimulation can be dissociated from promotion of transformation. If mitogenic stimulation by the promoter were required for promotion of transformation, then selection of cells for mitogen resistance should coselect for promotion resistance. Selection of JB6 mouse epidermal cells for resistance to plateau density mitogenic stimulation by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) yielded several clonal cell lines that remained sensitive to promotion, thus ruling out mitogenesis as a required event. Hence TPA, although mitogenic, must be inducing other required events on the promotion pathway. (b) Interaction with phorbol ester receptors cannot be dissociated from promoting activity. All promotable cells tested show 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 have receptors with number and affinity comparable to their sensitive counterparts. (c) EGF receptors are not required for promotion of transformation by phorbol esters or various TGFs but may be required for phorbol ester mitogenesis. TPA and TGF promotable cells have been found to lack EGF receptors. However, all of the TPA mitogen-resistant cells lack EGF receptors, thus suggesting a mediator role for EGF or EGF receptors in TPA mitogenesis. (d) A specific decrease in cell surface trisialoganglioside may be required for promotion of transformation. Tumor-promoting, but not nonpromoting, phorbol esters produce a 90% decrease in surface trisialoganglioside (G_{T-}) levels. This is completely blocked by antipromoting retinoic acid. The G_{T-} decrease occurs consistently in promotable cells but not in nonpromotable variants.

This is the first defect in biochemical response that has been found in promotion-resistant cells. This suggests that the G_T switch in response to TPA may be one of a few critical required events in tumor promotion. This suggestion has been further substantiated by the observation that inhibiting the G_T loss by G_T reconstitution inhibits promotion of transformation by TPA. (e) A specific collagen loss is pretranslationally regulated by phorbol esters and may be necessary but not sufficient for promotion. In vitro translation experiments have indicated that phorbol esters produce a rapid loss of translatable message for collagen. This collagen loss occurs in response to a variety of promoters in all JB6 mouse epidermal cells tested, both promotable ones and their nonpromotable counterparts, 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 probably not at the level of transcription. (f) Promotability behaves as a dominant trait which can be transferred 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-independent growth in response to TPA.

Cell culture models developed to study the action of tumor promoters are often limited by the requirement for serum in the culture medium. This problem can be overcome by the use of defined media selectively supplemented with highly purified and well-characterized attachment factors, growth factors and hormones. A defined medium has been developed for the mouse epithelial cell line JB6 Cl 41, which has previously been shown to respond to tumor promoters, such as TPA, in serum-containing media. In place of serum, the defined medium (referred to as 4F) contains fibronectin, insulin, transferrin and EGF. JB6 Cl 41 was grown in 4F for over 35 generations without any noticeable change in cell behavior. In contrast, JB6 Cl 41 cells that had been induced to grow in soft agar by treatment with TPA were unable to grow in 4F, even though their growth in serum-containing media was unaffected. These results demonstrate that the use of defined media offers the advantage of exposing differences in the growth requirements of cells before and after treatment with tumor promoters.

C. Identity, Origin, Transmission, Structure, Function and Virulence of Proviral and Cellular DNA Sequences in Mammals, Primates and Possibly Humans.

The coordinant development of technological advances in molecular virology and cell biology on one hand and in somatic cell genetics on the other have permitted the identification and characterization of mammalian cellular loci which participate in neoplastic transformation. This Laboratory has emphasized the domestic cat and man as model systems of biological and environmental mutagenesis. A preliminary requirement for this effort is the availability of a substantial biochemical genetic map of the domestic cat.

Briefly, some 36 different cell hybridization crosses between fresh diploid cat tissue or feline cell lines have generated over 1000 hybrid clones. A genetic map of 35 biochemical loci located on sixteen feline syntenic (linkage) groups was derived. The majority of these linkage groups have been assigned karyologically to one of 19 feline chromosomes. A population survey of feral cats revealed twelve (12) polymorphic biochemical loci. A colony of feral and specific breeds of domestic cat was established. The colony has approximately 25 biochemical and/or morphological loci segregating which are amenable to genetic analysis. Colony cats have been successfully bred into the third generation in a program of

mapping a number of genes. Genetic characterization of two other cat colonies also is in progress for further sources of genetic variation and for pedigree analyses. The sum of these biochemical genetic characteristics render the domestic cat now a feasible model for genetic analysis.

In the process of gene mapping of the cat, it was discovered that the feline genetic map shares a striking conservation of linkage associations with that of homologous loci previously mapped in man. Of the 31 loci placed on 16 groups in the cat, the human linkages are identical with only three exceptions. Certain of the homologous feline and human chromosomes also have apparent G-banding homologies in portions of the homologous chromosomes. 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. As the feline (and human) genetic map was being developed, a number of examples of cancer-associated loci were 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 EGF is modulated in transformed cells and has been shown to be controlled by two cellular genes both required for expression of the EGF receptor. The two genes map to respectively feline A3 and C2; (3) endogenous viral sequences: over twenty molecular clones of feline cellular sequences homologous to RD-114 cDNA probes have been selected from EcoRI library of feline cellular DNA. Restriction mapping of these clones has revealed marked differences between both endogenous RD-114 virogenes as well as between their cellular flanks. A molecular clone of RD-114 from infected human DNA is being used to genetically map endogenous RD-114 virogenes of the cat. Preliminary results reveal that the sequences distribute on multiple chromosomal positions of the cat; (4) cell surface antigens: allogeneic feline antisera has been derived from over 20 reciprocal surgical skin grafts of colony cats. Sexual and parasexual genetic analysis is in progress. Monoclonal antibodies were prepared against feline lymphocytes and 40+ stable hybridomas were derived and are being characterized both as chromosome markers as well as for specific cell surface determinants defined in human and murine systems; (5) restriction genes: BVRI 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; (6) transforming genes: a nick translated probe of Snyder-Theilen FeSV (kindly provided by Dr. C. Sherr, NCI) was used to locate the feline cellular homologue of FeSV.

A human locus, BEVI (for baboon endogenous virus integration), has been mapped to human chromosome six and is required for successful infection of rodent x human hybrids with baboon endogenous virus. Molecular hybridization experiments with cDNA probes prepared with baboon virus to cellular DNA of BEVI⁺ and BEVI⁻ hybrids of infected human x hamster crosses have demonstrated that BEVI is a high affinity

integration site for baboon endogenous virus in the human genome. Restriction enzyme analyses of BEV-infected cells and their derived cellular clones revealed heterogeneous flanking cellular DNA fragments about the proviral integrations. However, one enzyme, Pst I, revealed a common cellular restriction site in all infected cells at a position at or adjacent to the 3' virus-host cell DNA junction. We have proposed that a short cellular DNA sequence (defined by the Pst I site) is redundant on chromosome 6, separated by heterogeneous DNA sequences, and presents a high affinity target for the integration of baboon endogenous virus in human cells.

The allozyme genetic signature of human cells (composite allozyme phenotype at eight gene-enzyme systems that are polymorphic in human populations) has found human cell contaminants that have been identified from materials submitted for fetal versus maternal cells for purposes of genetic counseling. The triploid and androgenetic origin of partial and complete hydatidiform moles, respectively, has been achieved. These carcinomas develop in pregnant women and represent abnormal fetal development. Twelve isozyme systems have been resolved in cultured mycoplasmas and in mycoplasma-infected cells. The systems are useful in identification and discrimination of different mycoplasmas as well as indicators of mycoplasma contamination of cultured cells.

Alveologenic lung carcinoma-inducing virus which had a latency 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 virus (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. The unintegrated circular provirus was cloned from productively transformed mink lung cells. The genome was cut once by EcoRI and the permuted proviral DNA was inserted into λ WES. λ B and subcloned in pBR322. A comparison of restriction maps from this new virus, CI-2, with that from AKR/MuLV showed nonidentity in the entire 3' half of the genome. Comparison by heteroduplex mapping showed a substitution and a deletion loop in the envelope gene. The 5' proximal substitution loop spanned 400 bases and was separated from the second deletion loop of 220 bases by a common region that was 150 bases long. An additional difference from previously isolated transforming viruses showed no relationship to the tumor gene of Moloney or Harvey sarcoma virus, however, the substitution present in CI-4 was also contained in the genome of spleen focus-forming virus (SFFV). The recombinant genome of CI-4 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. In order to definitively establish whether the viral genome molecularly cloned from *in vitro* transformed mink lung cells was indeed the causative agent in the induction of lung tumors the following lines of research are being pursued: 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 virus CI-2,3 and CI-4 described above and b) cellular DNA from the induced lung carcinoma as well as lung carcinoma cell lines. In attempts to understand the mechanism of transformation of this new virus the growth requirement of transformed mink epithelial lung cells was determined. 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.

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 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 polypeptide 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 polypeptides 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. By Southern blot hybridization analysis of the cellular DNA of 3611-MSV nonproductively transformed rat cells, an EcoRI restriction fragment was identified containing the entire 3611-MSV genome. This DNA fragment hybridized strongly to MuLV gag gene-specific probes but lacked detectable homology with molecular probes corresponding to the mos, ras, abl or fes mammalian oncogenes. These findings suggest that the transformation-specific sequences represented within the 3611-MSV genome may represent a new, as yet unidentified, mammalian cellular oncogene corresponding to sequences originally involved in transformation of C3H/MCA-5 cells by methylcholanthrene.

A cell line has been 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 to 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 dibutylryl 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.

The experiments that led to the isolation of virus which induced lung carcinomas also yielded a virus associated with induction of ovarian carcinoma. Some of these ovarian adenocarcinomas had the characteristics of teratocarcinomas. Differentiating cell lines were established from one such tumor in culture epithelial stem cells were obtained which form embryonal bodies in vivo. This is the first time that such a tumor was induced with 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.

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 derived from *Colobus polykomos* 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_t 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 promoter 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 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 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 fractionality will subsequently be determined at the nucleotide sequence level.

CPC-1's endogenous parent 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 suggested that its repression was the result of multiple factors. This is supported by the following observations: (1) both cloned endogenous proviral promoters are transcriptionally inactive; and (2) recent 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 inconsistent with the high proviral copy number being the result of super-infections (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. Comparison of the genes at the extremities of a retrovirus of fowl (REV), colobus and baboon families of retroviruses shows 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.

Retrovirus LTRs hold a significant promise of being 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 stimulate 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.

Twenty-six kilobases (kb) of a single human locus which contains sequences with a clear retroviral structure were cloned. It has clearly significant homology to the gag p15 and p30 genes of Moloney MuLV as well as to regions spanning 2 kb of the polymerase gene of Moloney MuLV. It appears to be missing the 5' copy of the LTR sequence, but an apparent LTR sequence was identified at the appropriate position for the 3' LTR. This sequence is present in only one or two closely related copies in the human genome, but a number of more distantly related copies are present. From the fragments detected in restriction digests of human DNA, it is clear that these sequences are not closely related to the retrovirus-related human sequences reported by Martin et al. (Proc. Natl. Acad. Sci. USA, 78, 4892-4896, 1981). The homologous chimpanzee sequence is 1-2% divergent and has at least 4 kb of identical 3' flanking sequence. This result leads us to conclude that this locus represents an ancient integration event in which the common ancestor of man and chimpanzee was infected.

Using a murine viral sequence as the probe a number of human clones which hybridize to the gag and pol genes of Moloney MuLV have been isolated. The exact nature of the homologies is being determined by nucleotide sequencing. Three of the clones have been extensively characterized. They have very different restriction maps and cross-hybridization experiments at various stringencies indicate that they are 15-20% divergent. However, similar multiple copy restriction fragments are detected in human DNA using the three clones as probes. From the pattern of these fragments we conclude that this family of sequences is not closely related to the sequences reported by Martin et al., nor to the sequences described above.

D. Results of Epidemiological Studies on Human Cancers with Associated Viral Etiologies.

In applying laboratory techniques to studies on the etiology and control of human cancer, the following observations were made: 1) a serum factor inhibiting the lymphocyte response to Epstein-Barr virus (EBV) was detected in patients with active nasopharyngeal carcinoma (NPC) but not controls; 2) elevated complement fixation antibody titers to EBV were found in Greenland Eskimos, a group at high risk to NPC; 3) hormone receptors and pathologic studies demonstrated that the earliest form of rapidly progressing breast cancer was an entity more closely related to the more advanced forms than to the more commonly identified forms of breast cancer in the United States. In addition, Burkitt's lymphoma (BL) in the Western Hemisphere was compared with African BL by a number of parameters and was found to be comparable in most respects. Time-space clustering, a relationship to EBV and an excellent response to chemotherapy was found in BL patients from both areas. A study of 256 pathologically confirmed American BL patients demonstrated that target organs for the disease were related to age and sex, suggesting a predilection of BL for rapidly dividing cells. Laboratory and epidemiologic studies provided evidence for environmental as well as genetically determined predisposing factors in both American and African BL. Serologic assays for EBV antibodies correlated with prognosis in both groups.

In vivo and in vitro studies showed a heightened immune response to a variety of antigens in rapidly progressing breast cancer (RPBC) patients, and breast cancer specimens from Tunisia proved to have a higher frequency and amount of RNA virus-related proteins than those obtained from the United States. In vitro studies of cellular immunity showed that Tunisians generally reacted more strongly than Americans to mouse mammary tumor virus antigens. Epidemiologic studies associated premenopausal RPBC with rural residence, recent pregnancy, and blood group A, while late menarche, older age, rural residence, blood group A and delay in diagnosis were risk factors for post-menopausal RPBC. Hormone receptor patterns did not differ significantly from those reported for breast cancer in the United States. A chemotherapy trial documented the responsiveness of RPBC to systemic therapy and increased the metastases-free interval, apparently improving the cure rate for RPBC.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Identification of Cellular and Viral Hormone-Like Factors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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PROFESSIONAL:

1.2

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1.9

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A low molecular weight transforming growth factor (TGF) was isolated and purified to homogeneity. The amino acid compositions of human melanoma-derived TGF, murine sarcoma growth factor (SGF) and rat TGFs were then determined. In addition to the production of TGFs some human tumor cells release tumor inhibitory factors (TIFs) which inhibit the growth of some human tumors. Different molecular weight species of TGFs have also been detected in the urine of normal, pregnant and tumor-bearing humans. These TGFs can serve as markers with potential clinical applications. TGFs produced by cells transformed by different strains of feline sarcoma virus isolates have been isolated and compared. Membrane receptors on A431 human carcinoma cells have been characterized. There is now evidence that the genes which code for SGF obtained from murine sarcoma virus-transformed cells are of host cell, rather than viral, origin. Rabbit antisera has been produced against purified EGF receptors from A431 cells which block the binding of SGF, suggesting that SGF exerts its effect through the EGF receptor system on fibroblast cells.

Project Description

Objectives:

To investigate the mechanisms of carcinogenesis with emphases on the role of oncogenic viruses and virally-related genetic sequences in human cancer. Research is conducted on virus-host relationships in viral-induced, chemically-induced and spontaneous cancers. Investigations are conducted on the molecular processes of carcinogenesis and cocarcinogenesis with the goal of control of malignancy at the molecular level. This includes: 1) detection and characterization of hormone-like factors that may serve as molecular mediators of phenotypic or genotypic malignant expression or that may act to suppress tumor growth; and 2) identification and characterization of the nature of the receptors with which these factors interact.

Methods Employed:

Standard cell culture, virological, molecular biological and biochemical methodologies were employed. To characterize peptides produced by cell cultures, conditioned medium from cultures maintained in serum-free medium was collected. Gel permeation chromatography, carboxymethyl cellulose and reverse phase high pressure liquid chromatography (rpHPLC) were used for purification and characterization of growth-promoting polypeptides. Radioreceptor assays were used to determine competing activities. Soft agar growth assays were set up in tissue culture dishes containing indicator cells and growth factors. Amino acid sequences of growth-promoting polypeptides were determined by semi-automated Edman degradation. Ion exchange chromatography, gel filtration, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and other specialized methodologies were used.

Major Findings:

1. Isolation, purification and characterization of transforming growth factors (TGFs). A low molecular weight TGF was isolated from serum-free medium conditioned by the human metastatic melanoma tumor line A2058. The purification of TGF was achieved by gel permeation chromatography of the acid-soluble growth-promoting activity, followed by rpHPLC using sequentially acetonitrile and 1-propanol as the mobile phase and trifluoroacetic acid as the ionic modifier in the organic solvent. The purification of TGF was approximately 7,000-fold after gel permeation chromatography with an overall recovery of 75%. The purity of the final preparation was established by SDS-PAGE. Only one major single band, with an apparent molecular weight of 7,400, was observed. Reduction with 2-mercaptoethanol did not change the mobility of TGF, which is evidence for a single chain polypeptide. Sarcoma growth factor (SGF) isolated from Moloney murine sarcoma virus (MSV)-transformed mouse 3T3 cells, and rat TGFs, from either Abelson murine leukemia virus (MuLV)-transformed or Snyder-Theilen feline sarcoma virus (FeSV)-transformed Fisher rat embryo (FRE) fibroblasts, were purified to apparent homogeneity using the same methodology developed for the purification of human TGF. The amino acid compositions of human melanoma-derived TGF, murine SGF and rat TGFs were then determined. TGF/SGF is a single chain polypeptide of 67 residues with three intrachain disulfide bridges and no free sulfhydryl groups. The amino acid compositions of TGF/SGF are similar, but not identical. Human TGF lacks

tyrosine, whereas both rat TGF and murine SGF have two tyrosine residues. The amino acid compositions of TGF/SGF are unique and show a number of important differences when compared with the amino acid compositions of either human or mouse epidermal growth factor (EGF), indicating that TGF/SGF are distinctly different from EGF. Both human and mouse EGF have one methionine, whereas TGF/SGF lacks methionine. Human and mouse EGF have 5 tyrosine and no phenylalanine residues. In contrast, human TGF has no tyrosine, but 3 phenylalanine residues; murine SGF and rat TGF both have 2 tyrosine and 3 phenylalanine residues. TGF/SGF is a less hydrophobic polypeptide than EGF, in agreement with its lower retention time relative to EGF on reverse phase columns. The amino terminal sequences of human TGF, murine SGF and rat TGF have been determined in collaboration with Dr. Leroy E. Hood. The sequence homology of the first 41 residues of rat TGF and murine SGF is 100%. The homology of the first 34 residues of human TGF compared with rat TGF and murine SGF is 91%. Thus, human, rat and murine TGF/SGF are structurally closely related and are more conserved than are mouse and human EGF (68% sequence homology for residues 1 through 34). Alignment of the amino acid sequences of TGF/SGF with the sequence of mouse or human EGF reveals statistically significant sequence homology. The alignment results in 12 common or conserved amino acid residues out of 42 possible comparisons. To obtain such an alignment, the introduction of only a single break was necessary in the TGF/SGF sequence. Thus, the conserved amino terminal sequences of TGF/SGF and EGF could represent the receptor binding site common to both TGF/SGF and EGF.

2. Isolation and characterization of tumor inhibitory factors (TIFs). In addition to the production of TGFs which confer the transformed phenotype on untransformed fibroblasts, some human tumor cells have been found to produce another class of factors (TIFs) which inhibit the growth of some human tumors (rhabdomyosarcomas, melanomas and carcinomas) and mink lung epithelial cells in vitro. The major TIF activities fractionate into three molecular weight classes (5,000, 10,000-12,000 and 20,000) by gel permeation chromatography. TIFs stimulate normal human and rat fibroblasts to proliferate in monolayer cultures. Both growth-inhibiting and growth-promoting activities were found to be concentration-dependent. The concentration of the 5,000 molecular weight species of TIF required for inhibition of tumor cells or stimulation of normal human fibroblasts was 10-120 ng/ml. This is the concentration at which potent mitogens, such as epidermal growth factor, stimulate mitogenesis. TIFs do not possess the antiviral activity found in interferon.

3. Detection of TGFs in the urine of normal, pregnant and tumor-bearing humans. Liter volumes of urine from selected cancer patients and normal control pools were extracted with acidified ethanol and chromatographed on Bio-Gel P-100 columns. Aliquots of each fraction were tested for EGF-competing and soft agar growth-stimulating activities. All urine samples contained an 8,000 M_r soft agar growth-stimulating activity which is chromatographically separable from EGF. The urine from pregnant females contains TGF activities with apparent molecular weights of 10,000 and 17,000-20,000. In addition, the urine from an 18-year old female with metastatic alveolar cell soft tissue sarcoma contained a 30,000-35,000 M_r EGF-competing activity which was not present in urine derived from healthy individuals or mid- to late-term pregnant donors. This high molecular weight TGF activity demonstrated a differential elution profile on HPLC. The EGF-competing and soft agar growth-stimulating activities coeluted as a major

doublet from C_{18} μ Bondapak columns at an acetonitrile concentration of approximately 40%; a minor TGF activity was also detected which eluted at 33% acetonitrile. Human EGF eluted under similar conditions from these columns at 28% acetonitrile. The 30,000-35,000 M_r TGF from the urine of the cancer patients also exhibited a more basic charge relative to EGF using carboxymethyl cellulose chromatography. EGF does not adsorb to this resin at a pH of 5.0, whereas the urine-derived TGF activity demonstrated a high affinity, eluting at a high salt concentration. To determine if the high molecular weight TGF activity observed in the urine of the above-described sarcoma patient was also present in other cancer patients, a rapid screening assay using smaller urine volumes was designed. Urine specimens were acidified, subjected to chromatography on Bio-Gel P-30 columns and fractions were tested for soft agar growth-stimulating activity. In collaboration with Dr. Stephen Sherwin the 30,000-35,000 M_r TGF activity was detected in the urine of 18 of 22 cancer patients with diagnoses including carcinoma of the lung, breast, colon, several sarcomas and melanomas and in only 5 of 22 nonmalignant controls. Control urine specimens were collected from a population in which half of the donors had nonmalignant infections and inflammatory conditions, including bronchitis, pneumonia, colitis and diverticulitis.

4. Production of TGFs produced by cells transformed by FeSV isolates. Conditioned media harvested at 12-hour intervals for four days from monolayer (T150 flasks) cultures of Snyder-Theilen virus-transformed rodent cells contained predominantly a small molecular weight form of TGF. Concentration by lyophilization was found to be a more efficient method of concentration than filtration. Yields ranged from approximately 120-200 ng of TGF per liter of culture supernatant following acid/ethanol extraction and chromatography on acidified Bio-Gel P-10 columns. Although yields were high, the presence of extraneous protein in large volumes of supernatants (>100 l) facilitated the acid/ethanol extraction of lyophilized conditioned media prior to gel filtration chromatography. Large scale production of conditioned media harvested at 24-hour intervals from confluent roller bottle cultures of Snyder-Theilen FeSV transformants resulted in the production of μ g quantities of the small 10,000 M_r rodent TGF for subsequent purification and primary sequence analysis. Early harvests of these cultures also gave high TGF yields, however, unlike monolayers, more of the high molecular weight (20,000 M_r) TGF was produced relative to the smaller form. Unlike supernatants harvested from monolayers, however, less contaminating protein is found in conditioned media from roller bottle cultures.

5. Comparison of TGFs produced by cell cultures transformed by FeSV isolates containing v-fes and v-fms acquired cellular sequences. Cells transformed by acute transforming viruses of feline origin which differ in cellular acquired sequences were used to examine the effect of v-fes and v-fms genes on both the production and biological activity of TGFs. Cell cultures nonproductively transformed by the Snyder-Theilen and Gardner strains of FeSV, both of which contain the v-fms gene product and release a small (10,000 M_r) and large (20,000 M_r) form of TGF. Both size classes of TGF compete with EGF for EGF membrane receptors and stimulate anchorage-independent cell growth and thus resemble SGF and TGFs released by human tumor cells. Snyder-Theilen FeSV-transformed cultures which contain a polyprotein-associated, tyrosine-specific protein kinase consistently release the highest liters of TGF (approximately 200 ng/l of cell culture) relative to all transformants examined. In contrast, cells transformed by McDonough FeSV (v-fms gene) which lack detectable kinase activity produce low levels of TGF (less than 10 ng/l).

6. Comparison of TGFs released from cells transformed by different strains of FeSV. Fisher rat embryo cells nonproductively transformed by isolates of FeSV were shown to produce TGFs. Bio-Gel P-100 column chromatography of processed conditioned media from Snyder-Theilen-transformed FRE cells contains a major peak of EGF-competing activity eluting in the region of 10,000 M_r . Additional minor peaks of EGF-competing activity eluting in the 20,000 and 35,000 M_r range were also seen. Both the 10,000 and 20,000 M_r activity stimulate the growth of non-transformed rat kidney cells in soft agar. The smaller TGF promotes the growth of large, compact, round colonies in agar containing hundreds of cells and concomitant acid production in these cultures. The larger (20,000 M_r) TGF promotes smaller and slower colony growth, whereas no anchorage-independent cell growth is observed in agar plates to which the 35,000 M_r EGF-competing activity was added. This 35,000 M_r minor component is the only EGF-competing activity found in processed conditioned media from highly contact-inhibited normal Fisher rat embryo cell cultures and no transforming activity was found in any column fraction tested. A chromatographic profile similar to Snyder-Theilen transformants was also seen in conditioned media processed from Gardner FeSV-transformed cells. A major EGF-competing and soft agar growth-promoting activity was found eluting in the 10,000 M_r range as is an additional minor 20,000 M_r TGF activity. Conditioned media harvested from equivalent cultures of McDonough FeSV also contain, albeit low, two peaks of EGF-competing activity eluting in the same relative positions as Snyder-Theilen and Gardner FeSV TGFs with apparent molecular weights of 10,000 and 20,000. When tested on an equivalent ng basis TGFs released by all FeSV transformants exhibit similar transforming activity in soft agar growth assays. Nontransformed FRE cells have available EGF membrane receptors and bind ^{125}I -EGF, whereas these same cells transformed by various FeSV isolates or by Abelson murine leukemia virus do not bind EGF. Concordant with unoccupied EGF receptors, highly contact-inhibited FRE cells release no detectable TGFs into culture supernatants as determined by either EGF competition or by the more sensitive NRK soft agar assays. In contrast, the highest amounts of 10,000 M_r TGF (200 ng equivalents of EGF/liter of conditioned media) are released by Snyder-Theilen-FeSV transformants, whereas Gardner FeSV-transformed cells release 3-fold less of the smaller TGF. In contrast, McDonough FeSV-transformed cells produce very low amounts of 10,000 M_r TGF (<10 ng/l). The 10,000 M_r TGFs derived from Snyder-Theilen and McDonough FeSV-transformed FRE cells were further compared by rpHPLC. The small 10,000 M_r TGF from Snyder-Theilen and McDonough FeSV-transformed cell culture supernatants elute from C_{18} μ Bondapak columns at approximately 20.0% acetonitrile. Mouse submaxillary gland EGF elutes from these columns at 28% acetonitrile and does not stimulate the growth of nontransformed cells in soft agar. Both the soft agar growth-promoting and EGF-competing activities of the 10,000 M_r TGF from Snyder-Theilen-transformed FRE conditioned media were susceptible to treatment with trypsin and dithiothreitol as previously reported for SGF and TGFs from human tumor cells. In addition, the small TGF activity was also stable in 1 M acetic acid at various temperatures. As previously reported for other TGFs, the rat small TGF does not show any detectable immunological cross-reactivity when tested in a sensitive homologous radioimmunoassay for mouse EGF.

7. Characterization of a receptor for TGFs. Membrane components that interact with EGF and TGFs have been identified by covalent crosslinking to their respective ^{125}I -labeled ligands. Under appropriate conditions, disuccinimidyl suberate or hydroxysuccinimidyl azidobenzoate crosslink receptor-bound ^{125}I -EGF to a

140-170 kilodalton receptor species in membranes from both A431 human carcinoma cells and normal rat kidney cells. ^{125}I -sarcoma growth factor (SGF), a TGF from virally-transformed mouse 3T3 cells, can also be affinity crosslinked to the 140-170 kilodalton EGF receptor species in membranes from A431 and rat kidney cells. The labeling of this receptor is inhibited when either excess unlabeled EGF or SGF is present during incubation of membranes with ^{125}I -EGF or ^{125}I -SGF. In contrast, a second receptor species of 60 kilodaltons is affinity-labeled with ^{125}I -SGF but not ^{125}I -EGF in membranes from both A431 and rat kidney cells. SGF and a TGF from virally-transformed rat embryo cells inhibit the labeling of the 60 kilodalton species when present in excess during incubation of membranes with ^{125}I -SGF, while EGF is completely ineffective in inhibiting the labeling of this receptor. The data suggest that a specific 60 kilodalton receptor that displays high affinity for TGFs but not EGF may mediate induction of the transformed phenotype. In addition, SGF and other TGFs interact with the 140-170 kilodalton EGF receptor that appears to mediate normal cell growth effects.

8. Origin of SGF and production of antibodies to its receptor. Current evidence suggests that the genes which code for SGF obtained from the supernatant fluid of cells transformed by MSV is host cell, rather than viral, in origin. Normal rat kidney cells were transformed by either the wild type Kirsten sarcoma virus or a temperature sensitive (ts) variant of this virus. The growth factors released by these cells were isolated and compared for temperature sensitivity. Both factors were resistant to heat treatment and the growth factor obtained from the cells transformed by the ts variant stimulated phenotypic transformation and anchorage-independent cell growth in soft agar at the nonpermissive temperature. If SGF is a direct viral gene product, it would be expected that the SGF obtained from the cells transformed by the ts variant would itself be temperature sensitive, as compared to the SGF released by the cells transformed by the wild type virus. Another line of evidence is the observation that spontaneous murine tumors from non-virally transformed cells produce similar SGFs. Also, human melanomas, which have no known viral etiology, produce factors similar to SGF, with the amino acid sequence data showing greater than 90% homology. Rabbit antisera has been produced against purified EGF receptors from A431 cells (human epidermoid carcinoma) which block the binding of radiolabeled EGF to the EGF receptors on A431 cells and to the EGF receptors of several other species. The antisera also block the mitogenic effect of EGF and purified SGF when tested on human skin fibroblasts. This suggests that SGF exerts its effect through the EGF receptor system on these cells. This does not eliminate the possibility that SGF has separate receptors, but rather suggests that on these cells, it either acts directly through the EGF receptor system or with receptors that cross-react with the antibody to the EGF receptor.

9. Characterization and classification of the major internal proteins of endogenous type C viruses of two distantly related species of Old World monkeys. The major internal protein (p30) of MAC-1, an endogenous type C virus of Macaca arcoides and the p30 of CPC-1, an endogenous type C virus of Colobus polykomos were purified and subjected to primary structure analysis. Despite the distant evolutionary relationship between these two species of Old World monkeys, the amino acid compositions of the viral p30s were very similar and their COOH-terminal sequences were found to be identical. Moreover, the NH₂-terminal sequences differed only in three positions. On the basis of these p30 relationships MAC-1 and CPC-1 together with MMC-1, an endogenous virus of Macaca mulatta, can be classified into

a new subgroup. Avian reticuloendotheliosis virus and its relatives, transforming viruses of birds, are more related to this primate virus subgroup than to any of the three other subgroups of mammalian type C viruses.

Significance to Biomedical Research and the Program of the Institute:

These investigations seek to define the mechanisms and factors responsible for the uncontrolled proliferation and the transformed morphologies exhibited by malignant cells. The isolation and preliminary characterization of factors derived from human tumor cells which inhibit tumor growth provides new tools with which to study the control of tumor growth as well as a diagnostic marker for the presence of the tumor. The isolation and characterization of TGFs and TIFs suggest that malignant transformation may be controlled by means of specific inhibitors. The patterns of expression of the high molecular weight TGF in the urine of cancer patients promises significant prognostic and therapeutic applications. For example, it provides a basis for an indication of tumor burden and evaluation of the effectiveness of therapy. In addition, the detection of a lower molecular weight species of TGF in the urine of mid- to late-term pregnant females suggests a normal role for these factors in embryogenesis. The relationship of the TGFs and tumor inhibitory factors to the activation (or inactivation) of genetic information exhibited by malignant cells will significantly contribute to the definition of the mechanisms and progressive nature of carcinogenesis by either endogenous or environmental factors. The demonstration that a family of highly virulent avian tumor viruses are close relatives of endogenous primate viruses represents another example of transmission of viruses between distantly related species that has occurred under natural conditions.

Proposed Course:

Expansion of current work in the purification and concentration of TGFs, TIFs and other factors to allow for the collection of sufficient quantities to permit further characterization studies and sequence analysis, and the production of monoclonal antibodies and further studies into their mode of action. Eventually, DNA recombinant technology will be used to produce quantities of these factors for further studies. Inhibitors to the polypeptide growth hormones will be sought for application to the control of carcinogenesis and the host response to the onset and progression of cancer. The high molecular weight TGF will be purified from the urine of patients with pathologically distinct tumors and compared for specificity and similarity with TGFs produced by tumor cells in vitro. Urine from cancer patients will be monitored prior to, during and after therapy for levels of the 30,000-35,000 M_r TGF activity as an indication of tumor burden and response to treatment to verify its applicability as a diagnostic tool. In addition, the possible normal roles of these growth factors (for example, in normal embryogenesis) will be investigated with emphasis on the mechanism(s) of abnormal production and its possible relationship to the uncontrolled growth of malignant cells. Various new virus isolates will be further characterized particularly by nucleic acid and protein sequencing and the use of monoclonal antibodies. Viral gene sequences from human and higher ape cellular DNA will be characterized. New approaches, for example DNA transfection, will be used to isolate new ape and/or human virus-related genes.

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U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04822-12 LVC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Multi-Disciplinary Studies on EBV-Associated Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Clinical Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

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PROFESSIONAL:

0.7

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS

(a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project integrates several disciplines in an attempt to understand the pathogenesis and improve the control of Epstein-Barr virus-associated tumors, particularly nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL). Current projects include the evaluation of data from the American Burkitt Lymphoma Registry and the Surveillance, Epidemiology and End Results Program to characterize BL and NPC in the U.S.; the study of EBV serology in the diagnosis and monitoring of patients with NPC, BL and other EBV-related diseases; development of a primate model for BL, and in vivo and in vitro tests for compounds with activity against EBV.

Project Description

Objectives:

To utilize immunologic and epidemiologic techniques in studies on the etiology and control of Epstein-Barr virus (EBV)-associated diseases, with particular emphasis on nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL).

Methods Employed:

Pathology based registries were maintained for American NPC and BL as a source of cases for epidemiologic and laboratory studies. A battery of serologic tests were used to monitor patients with BL, NPC and other EBV-associated diseases. Marmosets and rhesus monkeys were inoculated with EBV and Herpesvirus saimiri (HVS) and monitored by humoral and cell-mediated immunity (CMI) assays. Transfer factor was prepared from immune leukocytes and the transfer of specific immunity to EBV and HVS antigens was evaluated. The effect of steroids and other materials on EBV replication was monitored in vitro.

Major Findings:

1. Serum inhibitory factor(s) in NPC patients. A serum protein with activity inhibiting the ability of EBV to stimulate sensitized lymphocytes was discovered in the serum of NPC patients with active disease. This serum factor proved to be more reliable than any EBV-related antibody in the monitoring of NPC patients.
2. Epidemiologic studies of BL and NPC. Studies on Burkitt's lymphoma (BL) in the United States and Ghana revealed a correlation between age at diagnosis and the target organs involved with tumor. BL appears to affect the most rapidly growing tissues and there is a higher frequency of abdominal involvement in older patients than in younger patients. The analysis of data from the Burkitt Tumor Project in Ghana indicates a time trend with increasing rates of abdominal tumor and an increasing age distribution over the past 8 years; these findings correlate with an apparent decrease in the incidence of BL in Ghana. Evaluation of data from the SEER Program and the Connecticut Tumor Registry demonstrated an interrelationship between age, race and pathology in more than 1000 American NPC patients. Well-differentiated tumors were restricted to adult white NPC patients whereas undifferentiated tumors were the rule in black and Chinese American NPC patients as well as children of all races.
3. Seroepidemiologic studies of EBV. Serologic assays applied to a migrant study comparing EBV immunity in Greenland Eskimos, Danes migrating to Greenland, and Danes living in Denmark indicated an intense exposure to EBV in migrating Danes and suggested that environment plays a particularly important role in determining the EBV pattern of native Greenlanders, who are known to have more than 30-fold higher risk of NPC than Danes. A case-control comparison between immunofluorescence assays detecting viral capsid antigen (VCA) and early antigen (EA) antibody and the complement fixation (CF) assay detecting antibody to soluble antigen indicated the CF assay was the most sensitive indicator of differences in the three populations. For all assays and at all ages, females had higher antibody titers to EBV than males.

4. EBV assays as clinical tools. The IgA antibody to EBV viral capsid antigen (VCA) appeared to provide a useful diagnostic test for NPC. Either high titers or absence of IgA antibody to EBV VCA were of great help in assisting in the diagnosis of patients with head and neck cancer and, on occasion, determined the diagnostic workup of the patient. The radiocomplement-fixation assay detecting antibody to EBV soluble antigen proved to be more useful than the IgA VCA test in the monitoring of NPC patients. Serologic studies for EBV-associated antigens indicated that American BL patients with high EBV titers had a better survival rate than patients with low titers; in African BL, antibodies to the early antigen appeared to be useful in predicting late relapse in patients with long periods of remission. The finding of elevated EBV antibodies and/or oligoclonal antibodies in the cerebrospinal fluid of African BL patients indicated the presence of active disease in the central nervous system.

5. Laboratory studies on the control of EBV. Studies on EBV and HVS in vitro and in vivo were implemented to improve the systems available for the evaluation of antiviral agents in the treatment of BL and NPC. Transfer factor was prepared from rhesus monkeys immunized with HVS. The transfer factor was replicated in vitro using the LDV/7 lymphoblastoid cell line. Successful transfer of immunity to HVS was observed in vivo in both rhesus and owl monkeys. Steroids were shown to inhibit the induction of EBV early antigen by tumor-promoting agents.

Significance to Biomedical Research and the Program of the Institute:

The availability of laboratory assays measuring immunity to EBV provides an opportunity to define the factors modifying the effect of EBV in different individuals. Additional diagnostic tools and predictors of morbidity may be developed from these new assays. The detection of a serum factor present in NPC patients with active disease may have great importance in the monitoring and treatment of NPC patients, while the detection of immunologic markers of active CNS disease in African BL patients may facilitate the treatment and improve the survival of patients with this disease. The IgA antibody to EBV has proven to be an effective diagnostic test for NPC and is now being used frequently for patients in the Washington area. Studies on the interrelationship of pathology, age, racial/ethnic status and EBV serology will help to distinguish among cofactors in the studies on the etiology of NPC. The population based serologic studies in Greenland Eskimos, Greenland Danes and Danish Danes may provide information on the relative importance of environment and genetics as related to the high incidence of NPC in Eskimos. The American BL Registry is providing important information on BL in a non-endemic area, the United States, thus permitting studies on the pathogenesis of the disease without the complicating effect of malaria. Progress on the in vitro and in vivo studies of EBV and HVS will permit the testing of materials potentially useful in the control of EBV-associated tumors.

Proposed Course:

A closer collaboration with participants in the SEER program is expected to provide an expansion of the integrated studies on the pathogenesis and control of NPC in the United States. Serologic techniques will continue to be applied to populations of normal individuals at high and low risk to NPC. Additional longitudinal studies on patients with BL and NPC will be performed to increase our understanding of which tests will be of value in the monitoring of patients with these

tumors. The American Burkitt's Lymphoma Registry will work with the X-Linked Recessive Lymphoproliferative Disease Registry to determine if there is any relationship between these two EBV-associated diseases.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04825-09 LVC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies of the Nature and Control of Endogenous (RD-114-like) Viruses in Cats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Daniel K. Haapala Microbiologist LVC NCI
OTHER: William G. Robey Research Chemist LVC NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

1.1

PROFESSIONAL:

1.0

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies with numerous domestic cat embryo lines demonstrated no absolute restriction of RD-114-like virus replication. However, embryos could be characterized as "restrictive" or "permissive" based on whether they were transformed by the RD-114 pseudotype of Moloney MSV. The kinetics of productive infection of both cell types was one hit. Clones and subclones of "restrictive" cells behaved quantitatively like their parental populations, and showed normal diploid karyotypes. S+L- cell lines, generated from both "restrictive" and "permissive" embryonic cat cells were exploited to study the nature of intracellular RD-114-like virus restriction. These new S+L- cell lines were useful as assay lines for other type C viruses including murine xenotropic and MCF isolates and certain primate viruses. Several FeLV virus-producing cat cells also produced RD-114-like sequences. The sequences were heterogeneous; comprised of those common to all FeLV preparations and those produced only by transformed cells. An RD-114-like virus with a novel coat was further characterized. Preliminary restriction enzyme mapping of its proviral DNA showed no differences from RD-114 DNA other than the 3' "env" region which was 500 bp longer and contained two novel enzyme sites.

Project Description

Objectives:

To characterize and utilize viral nucleic acids and proteins as diagnostic and analytical tools for probing the nature and mechanism 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. Growth of feline endogenous viruses in embryonic cat cells. As reported last year, we have searched for different phenotypes of cat cells and RD-114-like feline endogenous viruses (FEV) based on other models for intracellular restriction of type C viruses. Examination of additional embryos has again failed to reveal an absolute feline embryo fibroblast (FEF) intracellular restriction of FEV. Additional cell lines have been tested by the infectious center assay and also show a one hit curve for productive infection by several FEV isolates. Although the efficiency of virus infection differed widely in various embryonic cells, no absolute intracellular restriction was seen.
2. The nature of intracellular FEV restriction. FEV pseudotypes of murine sarcoma virus (MSV) were used to study the nature of FEV restriction. Like the FV-1 restriction system of mice, the FEV phenotype is transferred to MSV, making it susceptible to restriction. Unlike FV-1 restriction, this phenotypic change can only be overcome by a replicating helper virus other than FEV (e.g., murine xenotropic, or mink cell focus-forming or feline leukemia virus). Embryos could be characterized as "restrictive" or "permissive" depending on whether or not they allowed MSV/FEV focus formation without heterologous helper virus. Biological and molecular studies revealed that, in addition to the lack of transformation by MSV/FEV, most restrictive cells rapidly lose the MSV genome. However, the MSV can persist in a small proportion of the cells in the absence of transformation. Another biological approach involved the infection of S^+L^- cells derived from restrictive and permissive cells with FEV. Kinetic studies revealed: a) productive infection by FEV with concomitant rescue of MSV in the absence of focus induction; b) rapid loss of FEV- and MSV-producing cells within 1-2 days; c) persistence of a small but stable population (0.3%) of FEV and MSV producer cells still measurable after several months. This population was found to be negative for RD-114-specific gp70 as measured by fluorescent antibody technique. This result was explored and confirmed by fluorescent antibody cell sorting (FACS) using RD-114 anti-gp70. A FACS selection of 10% of the cells resulted in a 10-fold enrichment of the MSV/FEV producer cells. A second FACS selection further increased to 20% of the population producing MSV/FEV. These cells were of comparable morphology to the parental restrictive S^+L^- line and could still serve as an assay line for focus-inducing FeLV. These experiments show: a) virus coat expression resulting in an

"antigenic conversion" and that surface interference is not the cause of minimal FEV spread in the cell population (this suggests an alternative, intracellular control of FEV replication); and b) a population wherein approximately 20% of the cells produce MSV/FEV still maintain the nonproducer phenotype. This interesting result suggests the possibility of using this system to separately examine the replicative and "oncogenic" functions of these viruses.

3. Heterogeneity of FEV. A putative coat variant of FEV, called L2, has been further characterized. Overall RNA-cDNA homology to RD-114 is relative to, for example, the homology measured between the Moloney and Rauscher MuLV isolates. The L2 variant is not neutralized by anti-RD-114 tween-ether-disrupted virus, anti-RD-114 gp70, anti-FeLV, or murine xenotropic or recombinant virus neutralizing sera. Interference studies using different pseudotypes of MSV show no viral interference between L2 and RD-114 or FeLV A, B, and C. Preliminary studies of the viral proteins show considerable peptide similarity between the p28 proteins of L2 and RD-114. By contrast, the gp70 molecules are quite dissimilar.

Restriction enzyme maps of the L2 and L1 (an RD-114-like virus isolated from the L2 containing stock) are identical up to 6.5 kb from the 5' (gag) terminus of the two viruses. The 3' ends are dissimilar, with the L2 virus being approximately 500 base pairs longer and having two enzyme sites not present in L1, RD-114, and a virus biologically cloned from the Crandall cat cell line CRFK.

Attempts to isolate additional FEV biological variants have continued. It now appears that we have succeeded in isolating a virus with an RD-like coat but which is capable of inducing foci in "restrictive" FEF S⁺L⁻ cells. We are beginning biochemical characterization of this virus in an attempt to analyze the variation which allows its phenotypic expression in restrictive cells.

4. Utility of FEF S⁺L⁻ cells for assay of heterologous type C viruses. We reported last year that our S⁺L⁻ lines had utility for the assay of numerous viruses including certain primate viruses. Focus formation is both more rapid (4-5 days in many cases compared to 10-14 days for other assay lines) and more distinct than other lines. We have extended the useful application of these assay lines to the MCF viruses of mice. All of the isolates tested to date show the same titer on the FEF S⁺L⁻ cell line PG-4 as on Bassin's D-56 mouse line whereas some of these isolates titer 2-3 logs lower on mink S⁺L⁻ cells. This line proved useful in our studies of the nature of the DBA/2 mouse restriction of MCF infection and leukemogenesis (see report # Z01 CP0 4848-09 LTVG).

5. Molecular nature of FEV control. Studies of the organization of endogenous virus information in the cat genome have not yet been informative. Neither have studies of the quantitative expression of endogenous viral RNA revealed differences which could indicate their possible role in intracellular virus control. However, we have found sequences homologous to RD-114 cDNA in the 50-70S RNA purified from FeLV viruses. These sequences appear to comprise 5% of the total RNA in these preparations and their specificity was found to differ in the FeLV-producing cell lines. One set of RD-like sequences was present in all FeLV preparations, another set was found only in FeLV produced by transformed cat cells. Both sets of sequences are found in normal cat DNA and are expressed in non-FeLV-producing FEF cells. Attempts to correlate these data with oncogenic processes will be carried out with our newly developed virus reagents.

Significance to Biomedical Research and the Program of the Institute:

The use of viral proteins and nucleic acids as markers in the study of oncogenesis is a basic essential. Increasing the number and availability of such markers has obvious utility in increasing our ability to understand the oncogenic process. The question of how a cell organizes vast amounts of genetic material has been and is of fundamental importance. Cancer-causing viruses provide one of several tools which can be used to examine the control of a few specific gene products and so become of general interest. In addition, these studies may prove useful in determining how to control certain cancers.

Proposed Course:

Studies on the control of RD-114 expression in cat cells will be continued. Moloney MSV will be included in these studies to provide the additional capabilities of detecting both early and late viral expression in a clean genetic background. The rescue of MSV from "restrictive" S⁺L⁻ cells in the absence of transformation provides a powerful tool for studying the mechanism of transformation by m1MSV. Other cat embryo cells and RD-114 virus isolates will be studied to determine if more than three subtypes exist. Additionally, biochemical methods will be employed to further characterize and distinguish the subtypes isolated to date.

We are presently comparing peptide maps of the major virion proteins, p28 and gp70 of the L1 and L2 isolates. We will include other virion proteins in these studies as the necessary biological reagents are obtained. Antiserum has been prepared against the virion coat proteins of type-2 FEV and will be used along with DNA-RNA hybridization to determine the possible involvement of type-2 FEV in feline cancer. Molecular studies of the intracellular control of FEV will be continued. These will include the fate of endogenous and exogenous FEV DNA after de novo infection of "permissive" and "restrictive" cells as well as expression of FEV RNA in each cell type. Types 1 and 2 FEV will be cloned and various restriction fragments, including coat-specific DNA sequences, will be prepared for the above studies.

Publications:

None

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Etiology and Control of Human Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Paul H. Levine	Medical Director	LVC	NCI
	Alberto Faggioni	Visiting Fellow	LCMB	NCI
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Laboratory of Viral Carcinogenesis

SECTION

Clinical Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

0.95

PROFESSIONAL:

0.9

OTHER:

0.05

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project seeks to study the pathogenesis of virus-associated tumors in man, particularly lymphoma, with an attempt to utilize animal models whenever possible. The studies are designed to provide information as to the possible viral etiology and means of controlling the tumors under evaluation.

Project DescriptionObjectives:

To evaluate nonhuman primates as models for studies on the etiology and control of human cancer.

Methods Employed:

Six owl monkeys and six white-lipped marmosets were studied to determine the effect of tumor promoters on the oncogenicity of Epstein-Barr virus (EBV). These twelve animals and eight rhesus monkeys were studied by the leukocyte migration inhibition (LMI) assay for the effect of virus and transfer factor on the immune response to herpesvirus antigens.

Major Findings:

1. Changes in cellular immunity in non-human primates. Immune suppression was noted to precede the appearance of leukocytosis in the single owl monkey developing a virus-induced lymphoproliferative disease. Of six white-lipped marmosets inoculated with EBV, one developed immunosuppression and a malignant lymphoma. Administration of specific transfer factor was shown to produce specific immunity against viral and cellular antigens.

Significance to Biomedical Research and the Program of the Institute:

The development of a useful animal model for EBV infection in man continues to have a high priority for the Institute. The relative availability of rhesus monkeys and their phylogenetic proximity to humans make this animal a potentially valuable model for EBV infection in man. The production of lymphoma in a white-lipped marmoset is important because of the relative availability of this animal for experimental studies. The successful passive transfer of immunity may be important in the control of virus-associated human tumors.

Proposed Course:

These studies will be completed and prepared for publication during this fiscal year.

Publications:

Lvovsky, E., Levine, P.H., Bengali, Z., Leiseca, S.A., Cicmanec, J.L., Robinson, J.E., Bautro, N., Levy, H.B., and Scott, R.M.: Stimulation of hematopoietic stem cells by interferon inducers in nonhuman primates receiving fractionated total body irradiation. Int. J. Radiat. Oncol. Biol. Phys., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04868-07 LVC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Generation of New Transforming Mouse Type C Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Cha-Mer Wei	Expert	LVC	NCI
	Angie Rizzino	Expert	LVC	NCI

COOPERATING UNITS (if any) F. H. Reynolds, Jr., Litton Bionetics, Inc. (FCRF), Frederick, MD; D. Hankins, Laboratory of Tumor Virus Genetics, NCI, Bethesda, MD; T. Ward, Laboratory of Comparative Carcinogenesis NCI/FCRF, Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

1.2

PROFESSIONAL:

0.6

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

New transforming murine type C viruses were isolated and characterized with respect to target cell specificity in vivo, ability to transform cells in culture and their genome structure: (a) A lung carcinoma-associated virus (LCAV) 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 EGF suggesting that the mechanism of transformation by LCAV may involve sensitization of alveologenic lung cells to EGF. A virus-induced lung carcinoma cell line was characterized and shown to be derived from alveolar type I cells; (b) A new sarcoma virus was isolated and purified by the derivation of rescuable non-producer transformed mouse and rat cells. The translational products of this virus were P90 and P75 polyproteins which contained viral p15 and p12 structural proteins fused to a presumptive tumor gene product lacking tyrosine-specific phosphokinase activity. The genome of this virus, which does not contain the mammalian viral onc genes Ha-ras, v-abl, v-fes or v-mos, was molecularly cloned; (c) Several differentiating clonal lines were derived from a cell line established from a virus-induced ovarian carcinoma.

Project DescriptionObjectives:

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 transforming genes.

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 MuLV after induction of endogenous type C virus with 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.

Major Findings:

1. Derivation of directly transforming type C virus from cell cultures. Alveolo-genic lung carcinoma-inducing virus which had a latency 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 virus (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 genome was cut once by EcoRI and the permuted proviral DNA was inserted into λ WES. λ B and subcloned in pBR322. A comparison of restriction maps from this new virus, CI-2, with that from AKR/MuLV showed nonidentity in the entire 3' half of the genome. Comparison by heteroduplex mapping showed a substitution and a deletion loop in the envelope gene. The 5' proximal substitution loop spanned 400 bases and was separated from the second deletion loop of 220 bases by a common region that was 150 bases long. An additional difference from previously isolated transforming viruses showed no relationship to the tumor gene of Moloney or Harvey sarcoma virus, however, the substitution present in CI-4 was also contained in the genome of spleen focus-forming virus (SFFV). The recombinant genome of CI-4 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.

In order to definitively establish whether the viral genome that we have molecularly cloned from in vitro transformed mink lung cells was indeed the causative agent in the induction of lung tumors we are pursuing 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.

In attempts to understand the mechanism of transformation of this new virus we have 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 epidermal growth factor (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.

2. 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 non-productively 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) polypeptide 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 polypeptides 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. By Southern blot hybridization analysis of the cellular DNA of 3611-MSV nonproductively transformed rat cells, an EcoRI restriction fragment was identified containing the entire 3611-MSV genome. This DNA fragment hybridized strongly to MuLV gag gene-specific probes but lacked detectable homology with molecular probes corresponding to the mos, ras, abl or fes mammalian oncogenes. These findings suggest that the transformation-specific sequences represented within the 3611-MSV genome may represent a new, as yet unidentified, mammalian cellular oncogene corresponding to sequences originally involved in transformation of C3H/MCA-5 cells by methylcholanthrene.

3. Characterization of lung carcinoma tumor cells. A cell line has been 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 dibutyl 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 virus which induced lung carcinomas had also yielded 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 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: a) analyzing the biological properties of virus recovered from this tumor, including tumorigenicity and *in vitro* transforming properties; b) determining the genome structure of the virus after molecular cloning; and c) examining the integrated viral genome in the tumor cells.

5. Cloning of tumor genes from MuLV-transformed cells. This project was performed in collaboration with Dr. Wei. The cell line used was Mus musculus castaneus epithelial (MMCE) MB46, which is a MuLV-transformed MMCE cell line. DNA from these cells was previously shown to transmit foci upon transfection to NIH 3T3 cells. Work aimed at the molecular cloning in cosmid phage of transforming DNA from these cells is currently underway.

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. Characterization of the genome of one of the new histiocytoma/fibrosarcoma-inducing isolates will be accomplished after cloning integrated provirus from nonproducer transformed cells in bacteriophage lambda. The question of whether a virus-coded transformation-specific protein kinase may be present in the transformed cells will be examined.

Proposed Course:

The present emphasis is on the detailed characterization of viral genomes of non-producer 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 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 carcinoma-associated virus will be used to isolate integrated provirus of mouse origin by molecular cloning.

The new histiocytoma/fibrosarcoma-inducing virus is a much easier virus to work with since it is a readily rescued nonproducer, and transformed mouse and rat cells were easily obtained. These express a candidate gag-x polyprotein. Consequently, this virus will be characterized along established experimental pathways. The analysis of viral gene products is being pursued in collaboration with Dr. John Stephenson, Laboratory of Viral Carcinogenesis. The molecular cloning of the viral genome, as well as isolation of an expected homolog from a human gene library will be done collaboratively with Dr. Tom Bonner, Litton Bionetics, Inc. (FCRF). Studies on the expression of transformation by the histiocytoma/fibrosarcoma-inducing virus as well as search for expression of the cellular homolog of the tumor gene in normal and malignant tissues will be pursued by Dr. George Mark, Laboratory of Viral Carcinogenesis.

Publications:

Rapp, U.R., and Keski-Oja, J.: Murine leukemia virus-mediated transformation of a mouse epithelial cell line MMC-E. Cancer Res., in press.

Rapp, U.R., Reynolds, F.H., Jr., and Stephenson, J.R.: Isolation of New Mammalian Type C Transforming Virus. In Pearson, M.L., and Sternberg, N.L. (Eds.): Gene Transfer and Cancer. 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., in press.

Rizzino, A., Gonda, M.A., and Rapp, U.R.: Dome formation by a retrovirus-induced lung adenocarcinoma cell line. Cancer Res. 42: 1881-1887, 1982.

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Analysis of Gene Controlled Events in Neoplastic Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Stephen J. O'Brien	Geneticist	LVC	NCI
OTHER:	Mitchell Gail	Medical Statistical Investigator	BB	NCI
	Charles J. Sherr	Senior Surgeon	LTVG	NCI
	Malcolm Martin	Medical Officer	LMM	NIAID

COOPERATING UNITS (if any) W. Nash, Litton Bionetics, Inc. (FCRF), Frederick, MD; M.B. Gardner, Univ. Southern Calif., Los Angeles, CA; W. Nelson-Rees, Univ. Calif., Oakland, CA; M. Barile, Food and Drug Administration, Bethesda, MD; U. Surti, Univ. Pittsburg, Pittsburg, PA; J.C. Cohen, Tulane Univ., New Orleans, LA

LAB/BRANCH
Laboratory of Viral Carcinogenesis
SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	0.9	4.6

CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
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. Somatic cell genetic procedures have been used to derive a biochemical genetic map of the domestic cat of 35 genes distributed on 19 cat chromosomes. An extraordinary degree of linkage conservation between the cat and man has been demonstrated. The cat, which has previously been shown to be an excellent model for leukemia, sarcoma and a variety of inborn human errors, now is the third major mammal (after man and mouse) suitable for genetic analysis.

Project Description

Objectives:

1. The construction of a biochemical genetic map of domestic cat (*Felis catus*) with emphasis on genes which relate to the extraordinary 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, leuk, src, etc.) 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 or 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; and (6) molecular biology techniques including ³H-cDNA transcription in vitro, solution hybridization, HAP chromatography, visualization of restricted DNA by the techniques of Southern following agarose electrophoresis, molecular cloning of eukaryotic genes.

Major Findings:

1. Development of genetic analysis of the domestic cat. A preliminary requirement for this effort is the availability of a substantial biochemical genetic map of the domestic cat. Briefly, some 36 different cell hybridization crosses between fresh diploid cat tissue or feline cell lines have generated over 1000 hybrid clones. A genetic map of 35 biochemical loci located on sixteen feline syntenic (linkage) groups was derived. The majority of these linkage groups have been assigned karyologically to one of 19 feline chromosomes. A population survey of feral cats revealed twelve (12) polymorphic biochemical loci. A colony of feral and specific breeds of domestic cat has been established at the NIH Animal Center in Poolesville. The colony has approximately 25 biochemical and/or morphological loci segregating which are amenable to genetic analysis. Colony cats have been successfully bred into the third generation in a program of mapping a number of genes. We have also begun a genetic characterization of the cat colonies at the University of Pennsylvania and Cornell University veterinary schools for further sources of genetic variation and for pedigree analyses. The sum of these biochemical genetic characteristics render the domestic cat now a feasible model for genetic analysis.

2. Conservation of chromosome linkage homologies between the primates and the Felidae. Comparison of the feline genetic map with the human and murine maps of homologous loci revealed a striking similarity of the human and feline genomes. Of the seventeen linkage groups described in the cat, the linkage associations of the 31 included loci correspond to those seen in man with only three exceptions. One of the exceptions, the placement of HK and PP on separate feline chromosomes (D2 and D4, respectively), are apparently Felidae-specific since both mouse and man retain this linkage. The other two exceptions (human chromosome 2, and human chromosome 14/15) are apparently recent primate divergences from the "primitive prototype linkages" by similar logic. This striking linkage homology between cat and human is not retained in the mouse-human or mouse-cat homologies where over half of these same homologies have been reassorted.

Certain of the homologous feline and human chromosomes also have apparent G-banding homologies in portions of the homologous chromosomes. 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.

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 A3 and C2, respectively; (3) endogenous viral sequences: over twenty molecular clones of feline cellular sequences homologous to endogenous feline retrovirus (RD-114) cDNA probes have been selected from an EcoRI library of feline cellular DNA. Restriction mapping of these clones has revealed marked differences between both endogenous RD-114 virogenes as well as between their cellular flanks. A molecular clone of RD-114 from infected human DNA is being used to genetically map endogenous RD-114 virogenes of the cat. Preliminary results reveal that the sequences distribute on multiple chromosomal positions of the cat; (4) cell surface antigens: allogeneic feline antisera were derived from over 20 reciprocal surgical skin grafts of colony cats. Sexual and parasexual genetic analyses are in progress. Monoclonal antibodies were prepared against feline lymphocytes and 40+ stable hybridomas were derived and are being characterized, both as chromosome markers and for specific cell surface determinants defined in human and murine systems; (5) restriction genes: BVRI 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; and (6) transforming genes: a nick translated probe of Snyder-Theilen feline sarcoma virus (FeSV) (kindly provided by Dr. C. Sherr, NCI) was used to locate the feline cellular homolog of FeSV.

4. The genetic analysis of the human BEVI locus. A human locus, BEVI (for baboon endogenous virus integration), has been mapped to human chromosome six and is required for successful infection of rodent x human hybrids with baboon endogenous virus. Molecular hybridization experiments with cDNA probes prepared with baboon virus to cellular DNA of BEVI⁺ and BEVI⁻ hybrids of infected human x hamster crosses have demonstrated that BEVI is a high affinity integration site for baboon endogenous virus in the human genome. Restriction enzyme analysis of BEV-infected cells and their derived cellular clones revealed heterogeneous flanking cellular DNA fragments about the proviral integrations. However, one enzyme, Pst I, revealed a common cellular restriction site in all infected cells at a position at or adjacent to the 3' virus-host cell DNA junction. We have proposed that a short cellular DNA sequence (defined by the Pst I site) is redundant on chromosome 6, separated by heterogeneous DNA sequences, and presents a high affinity target for the integration of baboon endogenous virus in human cells.

5. Isozyme and allozyme genetic signatures as tools for human genetic analysis. The allozyme genetic signature of human cells (composite allozyme phenotype at eight gene-enzyme systems that are polymorphic in human populations) has found human cell contaminants that have been identified from materials submitted for fetal versus maternal cells for purposes of genetic counseling. The triploid and androgenetic origin of partial and complete hydatidiform moles, respectively, has been achieved. These carcinomas develop in pregnant women and represent abnormal fetal development. Twelve isozyme systems have been resolved in cultured mycoplasmas and in mycoplasma-infected cells. The systems are useful in identification and discrimination of different mycoplasmas as well as indicators of mycoplasma contamination of cultured cells.

6. Determination of the origins of ecotropic retrovirus in inbred mice. In order to study the origin of AKR ecotropic endogenous virus (with respect to its identity with molossinus virus), the polymorphic allozyme (allelic isozyme) genotype of 51 nonvirus-related loci in 17 strains of mice including AKR, C58, BALB/c, Swiss and molossinus has been examined. By comparing the composite allozyme genotype of different inbred and outbred mouse strains, the "genetic distance" statistic was derived. Genetic distance measures the degree of allelic substitution between populations and increased proportionately with the amount of time the populations have been reproductively isolated. The genetic distance computed between molossinus and AKR is large, nearly 5-10x the distance between known related populations and strains (e.g., C57L vs. C57BL/6). Molossinus had a similarly large distance from AKR murine endogenous retrovirus (AKV)-negative strains (Swiss, C57L) as it did from AKV-positive strains. These data effectively exclude the interpretation of consanguinity of AKR and molossinus. The restriction flanks of endogenous virus from AKR and molossinus have also been examined and common integration flanks have not been detected. The genetic distance data and the restriction enzyme data both effectively excluded the notion of a common ancestor and support the notion of acquisition of the virus in AKR by horizontal infection.

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 cascade 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 a source 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 mammalian evolution: The cat and man. Proc. Natl. Acad. Sci. USA, in press.

O'Brien, S.J. (Ed.): Genetic Maps, Vol. II. Frederick, Maryland, National Cancer Institute, 1982.

- O'Brien, S.J., and Barile, M.F.: Isozyme Resolution in Mycoplasmas. In Tully, J.G. and Razin, S. (Eds.): Methods of Mycoplasmology. New York, Academic Press, 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., and Nash, W.G.: Comparative genetic mapping in mammals: Chromosome map of the domestic cat. Science 216: 257-265, 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, in press.
- O'Brien, S.J., Nash, W.G., Winkler, C.A., Simonson, J.M., and Lemons, R.S.: Control of integration of baboon endogenous virus in human chromosomes by the BEVI locus. In Yohn, D.S., Lapin, B.A., and Blakeslee, J.R. (Eds.): Comparative Leukemia Research. New York, Elsevier/North Holland, in press.
- O'Brien, S.J., Simonson, J.M., and Eichelberger, M.A.: Genetic Analysis of Hybrid Cells Using Isozyme Markers as Monitors of Chromosome Segregation. In Shay, J.W. (Ed.): Techniques in Somatic Cell Genetics. New York, Plenum Press, in press.
- O'Brien, S.J., Simonson, J.M., Grabowski, M.W., and Barile, M.R.: A survey of isozyme expression in mycoplasmas and mycoplasma infected mammalian cell cultures. J. Bacteriol. 146: 222-232, 1981.
- O'Brien, S.J., Wildt, D.E., Simonson, J.M., Brand, D.J., Ebedese, H., van Dyk, A., Meltzer, D., Simmons, L.G., and Bush, M.: On the extent of genetic variation of the African cheetah, Acinonyx jubatus. Proceedings of the American Association of Zoo Veterinarians, in press.
- 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., in press.
- Surti, U., Szulman, A.E., and O'Brien, S.J.: Dispermin origin and clinical outcome of three complete hydatidiform moles with 46,XY karyotype. J. Obs. Gyn., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 04916-06 LVC

PERIOD COVERED

October 1, 1981 to February 16, 1982

TITLE OF PROJECT (80 characters or less)

Organization and Evolution of Endogenous Mammalian Type C Viral Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Tom I. Bonner

Expert

LVC

NCI

COOPERATING UNITS (if any)

M. Cohen and C. O'Connell, Litton Bionetics, Inc. (FCRF), Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

0.5

PROFESSIONAL:

0.4

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Retrovirus-related DNA sequences have been cloned from human DNA and characterized by comparing their nucleotide sequences with the published sequence of Moloney murine leukemia virus (MoLV). One set of clones represents a retrovirus which infected the common ancestor of man and chimpanzee. A second set of clones contains sequences with homology to both gag and pol genes of MoLV and is present as a family of related but not identical sequences in the human genome. Since the two sets of sequences are very distantly related, the results suggest that human DNA contains a number of families of integrated retroviral genomes.

Project Description

Objectives:

To characterize the distribution within primates of nucleic acid sequences related to endogenous retroviruses, their sequence organization and its relationship to their biological activity. In particular, to isolate endogenous retroviral sequences from human DNA.

Methods Employed:

We have screened a human DNA sequence library using two different viral sequences as probes. One probe was a chimpanzee sequence with very distant homology to the polymerase gene of the endogenous baboon virus. The second probe was a portion of a murine mink cell focus-forming (MCF)-type virus containing gag and polymerase sequences closely related to the AKR ecotropic virus. In order to clearly define the viral nature of the human clones, partial nucleotide sequences were obtained and compared to the complete sequence of Moloney leukemia virus (MoLV). The best sequence homology was found using the ALIGN computer program which also provided an estimate of the statistical significance of the homology. Retroviral organization of the sequences was demonstrated by showing that the spacing between homologous sequences in the human clones is the same as the spacing in MoLV.

Major Findings:

1. An ancient endogenous retroviral genome in human DNA. We have cloned about 26 kb of a single human locus which contains sequences with a clear retroviral structure. It has clearly significant homology to the gag p15 and p30 genes of MoLV as well as to regions spanning 2 kb of the polymerase gene of MoLV. It appears to be missing the 5' copy of the long terminal repeat (LTR) sequence, but an apparent LTR sequence was identified at the appropriate position for the 3' LTR. This sequence is present in only one or two closely related copies in the human genome, but a number of more distantly related copies are present. From the fragments detected in restriction digests of human DNA, it is clear that these sequences are not closely related to the retrovirus-related human sequences reported by Martin et al. (Proc. Natl. Acad. Sci. USA, 78, 4892-4896, 1981). The homologous chimpanzee sequence is 1-2% divergent and has at least 4 kb of identical 3' flanking sequence. This result leads us to conclude that this locus represents an ancient integration event in which the common ancestor of man and chimpanzee was infected.

2. A second set of retrovirus-related sequences in human DNA. Using a murine viral sequence as the probe we have isolated a number of human clones which hybridize to the gag and pol genes of Moloney murine leukemia virus. The exact nature of the homologies is being determined by nucleotide sequencing. Three of the clones have been extensively characterized. They have very different restriction maps and cross-hybridization experiments at various stringencies indicate that they are 15-20% divergent. However, similar multiple copy restriction fragments are detected in human DNA using the three clones as probes. From the pattern of these fragments we conclude that this family of sequences is not closely related to the sequences reported by Martin et al., nor to the sequences described above.

Significance to Biomedical Research and the Program of the Institute:

Our data clearly show that human DNA contains endogenous retroviral genomes. These clones therefore provide the means for testing the long-standing hypothesis that the activation of endogenous retroviruses is involved in human carcinogenesis.

Proposed Course:

This project terminated February 16, 1982 and is being continued under different auspices.

Publications:

Birkenmeier, E.H., Bonner, T.I., Reynolds, K., Searfoss, G.H., and Todaro, G.J.: Colobus type C virus: Molecular cloning of unintegrated viral DNA and characterization of the endogenous viral genomes of colobus. J. Virol. 41: 842-854, 1982.

Bonner, T.I., Birkenmeier, E.H., Gonda, M.A., Mark, G.E., Searfoss, G.H., and Todaro, G.J.: Molecular cloning of a family of retroviral sequences found in chimpanzee but not human DNA. J. Virol., in press.

Bonner, T.I., O'Connell, C., and Cohen, M.: Cloned endogenous retroviral sequences from human DNA. Proc. Natl. Acad. Sci. USA, in press.

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Analysis of Cellular and Viral Transforming Proteins with Monoclonal Antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gary J. Kelloff	Senior Surgeon	LVC	NCI
OTHER:	Fulvia Veronese	Visiting Fellow	LVC	NCI
	John R. Stephenson	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has defined the host's immune response to endogenous viral antigens present in spontaneous and chemically induced tumors and are active as transplantation antigens. These studies have resulted in conceptual advances of how the immune responses of the host interact with each other and the transplantation antigens in determining the outcome of the tumor-host relationship. The endogenous viral structural proteins have been shown to act as significant transplantation antigens. The transforming proteins encoded by the virus, but clearly of host cell origin, provide potential transplantation antigens of great importance. The oncogenic sequences coding for these transforming proteins and variants of the transforming proteins themselves have in many cases been found in normal cells in several species including humans, and there now seems little doubt that they will have significance in the understanding and treatment of human cancer. The project is now focusing on the production of monoclonal antibodies to these proteins for their evaluation as transplantation antigens, their presence in tumors throughout the phylogenetic scale and their mechanisms of transformation and thus to define the means to biochemically or immunologically alter the events that lead to cell transformation.

Project Description

Objectives:

Production of hybridomas from immune lymphocytes obtained from rats immune to syngeneic tumor cell lines containing the polyprotein of the feline sarcoma virus(es) or the Abelson leukemia virus. The hybridomas are to provide a library of high titered monospecific antibodies to the different antigenic determinants of the transforming proteins. Immunoepidemiologic characterization of rats bearing tumors produced by various etiologic agents for evidence of antigens reactive with monospecific antibodies to the different antigenic determinants of the transforming proteins. Extension of this survey to tumors obtained from species throughout the phylogenetic scale. Examination of the cell surface reactivity of the monoclonal antibodies with transformed cells and use of such antibody in immunotherapeutic experimentation. Use of rat cells transfected with the c-fes or c-abl homologs from human cells to allow the production of monoclonal antibodies to the proteins encoded by potential onc genes (c-fes or c-abl) of human origin.

Methods Employed:

Cell culture techniques included maintenance of myeloma lines, cell fusions of myeloma cells with immune lymphocytes, cell cloning, and production of monoclonal hybridomas. Immunological procedures included humoral and cell-mediated cytotoxicity assays, blocking assays, immunoprecipitation and gel electrophoresis.

Major Findings:

1. Production of monoclonal antibodies specific to transforming polyproteins encoded by isolates of feline sarcoma virus. Monospecific antibodies were produced against the polyproteins encoded by the three strains of feline sarcoma virus (FeSV). To date, monoclonal antibodies to the v-fes- and v-fms-encoded transformation-specific components and the feline leukemia virus (FeLV) gag gene structural components have been produced. A cleavage product of the major polyprotein encoded by McDonough FeSV that is devoid of p15 and p30 structural components is precipitated by monoclonal antibodies that are directed against the transforming (v-fms) specific components. Additional hybridomas secrete antibody directed against acquired sequence (v-fes) components common to the Gardner and Snyder-Theilen FeSV-encoded polyproteins but lack detectable reactivity with the McDonough FeSV-encoded polyprotein. The Gardner and Snyder-Theilen polyproteins immunoprecipitated by antibody directed against their p15 structural components exhibit readily detectable levels of autophosphorylation using $\gamma^{32}\text{P}$ -ATP as substrate but lack such activity when precipitated by antibody to their acquired sequence (v-fes) components. Moreover, in mixing experiments, monoclonal antibodies specific for the v-fes gene product inhibit the Gardner and Snyder-Theilen FeSV-associated protein kinases. The McDonough-encoded polyprotein immunoprecipitated by monoclonal antibody to either p15 or p30, lacks detectable levels of autophosphorylation but represents an efficient substrate for the Gardner and Snyder-Theilen polyprotein enzymatic activities. These findings indicate that the v-fes-associated protein kinase represents an intrinsic property of the v-fes gene product and recognizes tyrosine acceptor sites within polyprotein gene products of all three strains of FeSV.

2. Production of monoclonal antibodies to the transforming proteins encoded for by v-fms and v-abl and to the proteins encoded for by transfected human c-fes and c-abl. Since the onc genes naturally cloned by type C viruses (about 18 isolates) probably fall into a few discrete families, we feel that it is important to produce highly specific antibodies to proteins encoded by onc genes that are represented in several of these families. Since v-fes, v-fms and v-abl are quite distinct from each other, monoclonal antibodies to the proteins encoded by each is a primary objective. As reported here, antibodies to v-fes and v-fms already have been produced and immunization, hybridoma production and antibody screening is in process for the transforming proteins encoded for by v-abl. Further, antibody production to the human homolog of c-fes and c-abl transfected into syngeneic rat cells is in progress.

3. Use of monoclonal antibodies to v-fes for immunotherapy. The transforming proteins encoded for by the prototype onc gene v-src, pp60^{src} has been found to be immunologically active at the cell surface by fluorescent antibody techniques using polyvalent antisera. Investigation with the monoclonal antibodies available to the transforming protein of v-fes have shown that these antibodies can induce cytotoxic lysis of v-fes-transformed cells. Premixing of transformed cells with specific antibody in a Winn assay has been shown to significantly reduce the tumor incidence in challenged animals, compared to controls challenged with tumor cells premixed with several control antisera.

Significance to Biomedical Research and the Program of the Institute:

This effort has contributed to our understanding of the host's natural immune response to spontaneous and chemically-induced tumors and to the viral gene products ubiquitously expressed in these tumors. Independently of etiologic relationships, these viral gene products act as transplantation antigens in the tumors. The availability of transplantation antigens whose structural specificities are defined have heretofore not generally been available which has slowed the progress of tumor transplantation research. These studies have established that the host responds to high dose, chronic exposure of an endogenous antigen with an IgM response which by blocking the host's cell mediated immune activity enhances the growth of naturally occurring tumors. This mechanism of immune enhancement of naturally occurring tumors is likely to be operative against any tumor-specific transplantation antigen to which the host is chronically exposed, and an understanding of these immunological mechanisms has led to an ability to immunoprevent these tumors. The transforming proteins encoded by the viruses will also most likely be shown to be transplantation antigens which should be examined immunologically and in immunobiology experimentation as has been done for the viral structural proteins. The genes encoding these transforming proteins have been detected in normal cells and in many cases has been shown to be present in species throughout the phylogenetic scale. The viral vectors carrying the transforming genes have made it possible to isolate, mutagenize and insert these oncogenic gene sequences from many species into different cells. In addition to genetic characterization this has provided the opportunity to define and characterize the transforming proteins. The production of a library of monoclonal antibodies reactive with single antigenic determinants of these proteins will make possible the search for these proteins in primates; the evaluation of these proteins as potential transplantation antigens; the fine structure analysis of these proteins which will lead to a better understanding of their mechanism of transformation and how to

biochemically alter it; and finally, these reagents will provide indispensable tools to more rapid progress in one of the most promising areas in basic cancer research.

Proposed Course:

The human homologs of the viral onc genes have been transfected to rat cells. Immunization of syngeneic hosts with these transfected rat cells (prototypes under study include c-fes, c-fms and c-abl) will produce antibody to the proteins encoded by these human genes that are homologous to the viral onc genes. Monoclonal antibodies to these proteins will allow the analysis of their structure and transforming function. Monoclonal antibodies to these proteins and the viral prototype proteins encoded by v-fes, v-fms, v-abl and v-fps will be evaluated for their cytotoxic activity against tumor cells expressing these proteins.

Publications:

Veronese, F., Kelloff, G.J., Reynolds, F.H. Jr., Hill, R.W., and Stephenson, J.R.: Monoclonal antibodies specific to transforming polyproteins encoded by independent isolates of feline sarcoma virus. J. Virol., in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Characterization of Cellular Gene Products with Transforming Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Gary J. Kelloff	Senior Surgeon	LVC	NCI
	John Groffen	Visiting Fellow	LVC	NCI
	Nora Heisterkamp	Visiting Fellow	LVC	NCI
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COOPERATING UNITS (if any)

F.H. Reynolds, Jr., and W.J.M. Van de Ven, Litton Bionetics, Inc. (FCRF),
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Laboratory of Viral Carcinogenesis

SECTION

Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

3.1

PROFESSIONAL:

1.1

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A human lung carcinoma cosmid library has been constructed and a series of clones corresponding to the complete human c-fes and c-abl genes identified, subcloned in appropriate plasmid vectors, and subjected to detailed restriction endonuclease analysis. Neither gene exhibits detectable transforming activity even when linked to SV40 promoter sequences. The avian v-fps gene was found to map at the same position in the human genome as the mammalian c-fes gene and the chromosomal location of each of these genes has been determined. Tyrosine phosphorylation acceptor sites within v-fes and v-abl have been localized with respect to trypsin cleavage sites. High level production of transforming growth factor was found in v-abl- and v-fes-transformed cells. These factors have been purified to homogeneity and subjected to partial amino acid sequence analysis. Monoclonal antibodies with specificity for various domains of gag-fes- and gag-fms- encoded polyproteins have been isolated. A major 90,000 Mr polyprotein gene product of a new transforming retrovirus isolate, 3611-MSV, has been identified and shown to lack protein kinase activity.

Project Description

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 are also employed.

Major Findings:

1. The Gardner and Snyder-Theilen isolates of feline sarcoma virus (FeSV) represent genetic recombinants between feline leukemia virus (FeLV) and transformation-specific sequences of cat cellular origin (v-fes gene). An independent transforming gene (v-fps) common to the Fujinami and PRC II strains of avian sarcoma virus (ASV) has also been described. By restriction endonuclease and molecular hybridization analysis, we have shown the v-fes and v-fps genes to contain highly related sequences. Moreover, molecular probes corresponding to each hybridize to a single well-resolved 12 kb EcoRI restriction fragment of human cellular DNA. DNA clones containing v-fes and v-fps homologous sequences were isolated from a representative cosmid library of human lung carcinoma DNA. Cellular inserts within these cosmids, ranging from 32 to 42 kb in length, represent overlapping regions corresponding to 56 kb of contiguous human DNA sequences. Sequences both homologous to, and colinear with the Gardner/Snyder-Theilen FeSV v-fes and Fujinami ASV v-fps genes are distributed discontinuously over a region of up to 9.5 kb and contain a minimum of 3 distinct noncoding regions (introns). A 12 kb EcoRI restriction fragment representing the entire v-fes/v-fps human homolog has been subcloned in pBR328 and subjected to fine structure mapping. By this means the arrangement of human DNA sequences homologous to acquired sequences of each virus isolate were accurately defined. Upon transfection to RAT-2 cells, using the thymidine kinase gene as a selective marker, the human v-fes/v-fps homolog lacked transforming activity.

More recently, the above-described human lung cosmid library has been utilized for the molecular cloning of human cellular sequences homologous to v-abl, an oncogenic sequence with specificity for lymphoid transformation, represented within the Abelson strain of murine leukemia virus. The human c-abl gene is distributed over a much more extensive region of the human genome than c-fes, thus requiring several overlapping cosmid clones for its complete representation.

2. Single tyrosine phosphorylation sites identified within the amino terminal domains of transformation-specific polyproteins encoded by the Gardner and Snyder-Theilen strains of FeSV are shown to represent major *in vitro* acceptors for the

polyprotein-associated protein kinases. By two dimensional tryptic peptide analysis, these acceptor sites are highly related and by analytical high performance liquid chromatography, all three elute at approximately the same acetonitrile concentration (22%). 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 sites. By a similar approach two separate tyrosine acceptors have been identified with the 120,000 M_r polyprotein gene product of the Abelson strain of MuLV. These have been localized at six and seven residues, respectively, distal to a trypsin cleavage site. The peptide acceptor of P120^{gag-abl} mapping at position seven represents the major tyrosine site phosphorylated *in vivo*.

3. Transformation of cultured rat embryo fibroblasts by virus isolates containing the *v-fes* and *v-abl* transforming sequences results in a marked reduction in binding of mouse epidermal growth factor (EGF) and in the synthesis of low molecular weight polypeptide transforming growth factors (TGFs). Production of TGFs is dependent upon a functionally active tyrosine-specific protein kinase activity associated with the *v-fes* and *v-abl* gene products. Cells nonproductively infected with transformation-defective viral mutants bind EGF at levels comparable to uninfected cells, do not have their virus-coded transforming gene product phosphorylated in tyrosine and do not induce TGF production. Both *v-fes*- and *v-abl*-induced TGF(s) promote anchorage-independent growth of nontransformed fibroblasts, compete with ¹²⁵I-labeled EGF for binding cell receptors, and induce phosphorylation of tyrosine acceptor sites in the 160,000 molecular weight EGF membrane receptor. Following purification to homogeneity, TGFs induced in both systems migrate as single polypeptides with apparent molecular weights of 7,400 daltons as determined by SDS-PAGE. By primary sequence analysis these viral-induced factors have been shown to be distinct from EGF but highly related to TGFs produced by certain human tumor cells.

4. Hybridomas secreting monoclonal antibodies directed against polyprotein gene products of the Gardner, Snyder-Theilen and McDonough strains of FeSV have been isolated. Within these are representatives of several immunoglobulin subclasses including IgG₁, IgG_{2a}, IgG_{2b} and IgM. High titered antibodies produced by each have been obtained *in vivo* passage of ascites tumors. Antibody produced by one class of hybridomas recognizes immunologic determinants localized within an FeLV *gag* gene structural component (p15) common to polyproteins encoded by each FeSV isolate, while antibody produced by a second is specific for p30 determinants unique to P170^{gag-fms}. A 120,000 M_r cleavage product of P170^{gag-fms} is efficiently precipitated by antibody against its transforming (*v-fms*)-specific components, but is not recognized by monoclonal antibodies directed against either p15 or p30. Additional hybridomas secrete antibody directed against acquired sequence (*v-fes*) components common to the Gardner and Snyder-Theilen FeSV-encoded polyproteins but lack detectable reactivity with P170^{gag-fms}. Gardner- and Snyder-Theilen-encoded polyproteins, immunoprecipitated by antibody directed against their p15 structural components, exhibit readily detectable levels of autophosphorylation using α³²P-ATP as substrate but lack detectable activity when precipitated by antibody to their acquired sequence (*v-fes*) components. P170^{gag-fms}, immunoprecipitated by monoclonal antibody to either p15 or p30, lacks detectable levels of autophosphorylation but represents an efficient substrate for the Gardner (GA) P110^{gag-fes} and Snyder-Theilen (ST) P85^{gag-fes} enzymatic activities. These findings indicate that

the v-fes-associated protein kinase represents an intrinsic property of the v-fes gene product and recognizes tyrosine acceptor sites within polyprotein gene products of all three strains of FeSV.

5. The major translational product of a newly isolated replication-defective transforming retrovirus of mouse cell origin, 3611-MSV, has been identified as a 90,000 (P90) molecular weight 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 polyprotein lacks 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 elevated levels of phosphotyrosine. By Southern blot hybridization analysis of the cellular DNA of 3611-MSV nonproductively transformed rat cells, an EcoRI restriction fragment was identified containing the entire 3611-MSV genome. This DNA fragment hybridized strongly to MuLV gag gene-specific probes but lacked detectable homology with molecular probes corresponding to the mos, ras, abl or fes mammalian oncogenes. These findings suggest that the transformation-specific sequences represented within the 3611-MSV genome may represent a new, as yet unidentified, mammalian cellular oncogene corresponding to sequences originally involved in transformation of C3H/MCA-5 cells by methylcholanthrene.

Significance to Biomedical Research and the Program of the Institute:

A determination of the role of cellular homologs of prototype viral oncogenes (v-fes, v-fms and v-abl) 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. The degree of polymorphism of these genes and its possible correlation with extent of gene expression will be examined. Attempts will be made 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 active 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 producing monoclonal antibody directed against human c-fes- and c-abl-encoded proteins and utilized for initial screening of a diverse range of human tumors. The c-fes and c-abl gene will be molecularly cloned from tumors in which their gene products are expressed at high levels as active protein kinases, and subsequently analyzed for transforming activity.

Publications:

Blomberg, J., Van de Ven, W.J.M., Reynolds, F.H., Jr., Nalewaik, R.P., and Stephenson, J.R.: Snyder-Theilen FeSV P85 contains a single phosphotyrosine acceptor site recognized by its associated protein kinase. J. Virol. 38: 886-894, 1981.

Groffen, J., Heisterkamp, N., Grosveld, F., Van de Ven, W.J.M., and Stephenson, J.R.: Isolation of human oncogene sequences (v-fes homolog) from a cosmid library. Science 216: 1136-1138, 1982.

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.): Gene Transfer and Cancer. New York, Raven Press, 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.): Gene Transfer and Cancer. 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., in press.

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, in press.

Reynolds, F.H., Jr., Todaro, G.J., Fryling, C., and Stephenson, J.R.: Human transforming growth factors induce tyrosine phosphorylation of EGF receptors. Nature 292: 259-262, 1981.

Reynolds, F.H., Jr., Van de Ven, W.J.M., Blomberg, J., and Stephenson, J.R.: Differences in mechanisms of transformation by independent feline sarcoma virus isolates. J. Virol. 38: 1084-1089, 1981.

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, Vol. 1, in press.

Todaro, G.J., Marquardt, H., De Larco, J.E., Fryling, C.M., Reynolds, F.H., Jr., and Stephenson, J.R.: Transforming Growth Factors Produced by Human Tumor Cells: Interactions with Epidermal Growth Factor (EGF) Membrane Receptors. In Mozes, L.W., Schultz, J., Scott, W.A., and Werner, R. (Eds.): Cellular Responses to Molecular Modulators. New York, Academic Press, 1981, Vol. 18, pp. 183-204.

Twardzik, D.R., Todaro, G.J., Marquardt, H., Reynolds, F.H., Jr., and Stephenson, J.R.: Abelson MuLV induced transformation involves production of a polypeptide growth factor. Science 216: 894-897, 1982.

Veronese, F., Kelloff, G.J., Reynolds, F.H., Jr., Hill, R.W., and Stephenson, J.R.: Monoclonal antibodies specific to transforming polyroteins encoded by independent isolates of feline sarcoma virus. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05108-04 LVC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of Sarcoma Growth Factor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	George J. Todaro	Medical Officer	LVC	NCI
	Hans Marquardt	Visiting Scientist	LVC	NCI
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LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

1.3

PROFESSIONAL:

1.1

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sarcoma growth factor (SGF) is an EGF-like peptide released by murine sarcoma virus-transformed cells. It can reversibly induce the transformed phenotype in untransformed indicator cells. SGF is a potent mitogen that can interact with the EGF receptor system. Antibodies to the EGF receptor block the mitogenic effects of both EGF and SGF; similarly, cells lacking EGF receptors are unable to respond to either of these mitogens. SGF from serum-free conditioned media is purified to homogeneity using 5 column chromatography steps. The purified peptide is used to determine the relationship between SGF and EGF. The amino acid composition shows SGF is distinctly different from EGF. SGF has alanines, phenylalanines and lysines; whereas EGF is missing these residues. The EGF molecule has methionine and isoleucine residues which are missing in SGF. Amino acid sequencing of this peptide has identified residues as far as residue 42. If the first disulfide bonds of each molecule are aligned and a single space is introduced in the SGF molecule between residues 18 and 19 then 6 of the 34 known residues are identical for a 17.6% homology. If SGF is compared to human EGF (urogastrone) in a similar manner there are 10 identities for 29.4% homology.

Project Description

Objectives:

To purify a peptide growth factor released by transformed cells, sarcoma growth factor (SGF), that is partially responsible for the transformed phenotype expressed by the cells producing it. SGF is also capable of reversibly inducing the transformed phenotype in untransformed indicator cells. When sufficient quantities of this homogeneous product have been accumulated, the remainder of the amino acid sequence will be determined as well as the secondary structure. With the information from the amino acid sequence, peptides will be synthesized and coupled to the carrier protein keyhole limpet hemocyanin for the production of antibodies which, hopefully, will cross-react with the same sequence in the native peptide and thereby yield the reagents for a radioimmune assay. 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). The antibodies against these factors will be examined to determine if they can be used as diagnostic tools in the detection of either a qualitative or quantitative difference of 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. With these clones the expression of these factors can be determined both *in vivo* and *in vitro*. The *in vivo* data will allow us to examine 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. It will also be used to determine if there are drugs which can affect the expression of these sequences. Are there drugs that can turn on or increase the level of expression in normal cells and are there substances which will either decrease or eliminate the expression of these factors in transformed cells?

The purified SGF will be used to examine the kinetic interactions of this growth factor with responsive cells and these will be contrasted with those of epidermal growth factor (EGF). The kinetic interactions of radiolabeled SGF and EGF with cell surface membrane receptors will be examined using unlabeled peptides to compete both in direct competitions and in cross-competitions. The results will indicate if SGF is interacting predominantly with the EGF receptor system or predominantly with an independent receptor system and only cross-reacting with the EGF receptors. These experiments will determine if there are differential affinities within the same or separate receptor systems. The direct biological responses to this homogeneous peptide will be examined. It will also be used to determine if there are synergistic effects on the cellular level when acting with components from either serum or serum-free media conditioned by transformed cells. If there are components in either serum or conditioned media that act synergistically with purified SGF the mechanisms of these interactions will be examined.

Methods Employed:

Tissue culture methods are used for the production and assay of growth factors from transformed cells. For the production of SGF, transformed cells are grown to confluency in serum-containing media; they are then washed twice in serum-free media, which is discarded, and additional 48-hour harvests of conditioned serum-free media

are collected. These conditioned media are the source of the growth factors released by the transformed cells. The conditioned media is clarified using centrifugation and concentrated 25-fold using a hollow fiber concentrator. The concentrate is desalted by dialyzing against 1% acetic acid and the retentate further concentrated by lyophilization. The acid-soluble peptides are extracted with one molar acetic acid. The initial step in the purification is performed using Bio-Gel P-60 gel filtration chromatography. The activities present in these columns are monitored using *in vitro* assay methods. Mitogenic activities are measured by either ³H-thymidine incorporation or cell number increase. Phenotypic transformation is measured by morphological changes in monolayers or anchorage-independent growth in soft agar. Ligand bindings and competition are measured on tissue culture cells using trace amounts of radiolabeled ligands. The amount of EGF-competing activity in a sample is 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 ¹²⁵I-EGF. The final three steps in the purification of SGF are 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 the native sequence that is located in the SGF molecule.

Major Findings:

1. Host origin of EGF-like growth factors. Evidence from several sources indicate the genes for these SGF-like growth factors released by murine sarcoma virus-transformed cells are not viral in origin, but rather host cell in origin. The first evidence suggesting that SGF is not of sarcoma viral gene origin came from studies that examined the SGF-like growth factors from normal rat kidney (NRK) cells transformed by either the wild type Kirsten sarcoma virus (KiSV) or a variant of this virus that is temperature sensitive (ts) with respect to transformation (ts-371). If the SGF-like growth factor is a direct viral gene product and capable of producing the transformed phenotype, one would expect the growth factor from a cell transformed by a temperature sensitive virus to be itself temperature sensitive compared to the product obtained from the cell transformed by KiSV. The growth factors from these two transformed cells were isolated and compared for temperature sensitivity. They were both resistant to heat treatment (65°C x 120 min) and the growth factor from the cell transformed by the ts-virus was able to stimulate phenotypic transformation and anchorage-independent growth at the non-permissive temperature (39.5°C). These results are consistent with the model in which the growth factor is not a direct viral gene product but rather a host cell product whose expression is controlled by the sarcoma gene product. Another piece of evidence consistent with the growth factors being host products is that spontaneous murine tumors from nonvirally transformed cells produce similar growth factors. Human tumors, with no known viral etiology (melanomas), also produce factors that are quite similar to SGF, with the amino acid sequence data showing greater than 90% homology.

2. Antibodies for EGF receptors. Rabbit antisera raised against purified EGF receptors from a human epidermoid carcinoma cell line (A431), block the binding of radiolabeled EGF to the EGF receptors present on A431 cells and to the EGF receptors from several other species. This antisera is also able to block the mitogenic effect of EGF. This same antisera also block the mitogenic effect of purified SGF when tested on human skin fibroblasts. This suggests that SGF is acting through the EGF receptors on these cells. This does not eliminate the possibility that the SGF has a separate receptor system, but suggests that, on these fibroblastic cells, it is either directly acting through the EGF receptor system or a receptor system that cross-reacts with the antibody to the EGF receptor.

3. Purification of SGF. The SGF from serum-free media conditioned by Moloney sarcoma virus-transformed 3T3 cells was purified to homogeneity using rpHPLC. A portion of the purified product was used to determine amino acid composition and another portion was used for partial amino acid sequence analysis. Both the amino acid composition and sequence analysis indicated that SGF is distinctly different from EGF. The sequence data, however, showed some sequence homology with EGF. There is approximately 30% homology between SGF and EGF; whereas human and mouse EGFs are approximately 65% conserved. The transforming growth factors (TGFs), of which SGF was the first described, are much more conserved than the EGFs. The mouse SGF and the rat analog are identical for the first 30 residues and the human and mouse differ in only three residues. The conservation of these molecules suggests these factors must play an important role other than the maintenance of the transformed phenotype of malignant cells. This tight conservation suggests these peptides serve a critical function either during development or for the maintenance of life.

Significance to Biomedical Research and the Program of the Institute:

This work is significant and of high priority because it seeks to define the mechanisms and factors responsible for the uncontrolled proliferation and the transformed morphologies expressed by malignant cells. The data collected from the murine system appears to be directly applicable to certain human malignancies as seen in patients. 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 class consists of those agents that will suppress the expression of the transformed phenotype through any of several mechanisms. The second class of therapeutic agents would be immunological in origin and would be designed to destroy the cells producing these "ectopic" growth factors. These goals are consistent with the main goals and mission of the section, the laboratory, 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 project will continue in two phases. The first phase will consist of the purification of SGF from conditioned media. This purified product will be used: (a) to further characterize the structure of this peptide including the elucidation of the entire primary and secondary structures; (b) to immunize with, and hopefully

raise antibodies against the native molecule; (c) to determine, in biological studies, its mode of action in stimulating morphological changes and anchorage-independent growth of untransformed cells in soft agar. The action of the purified product will be investigated both alone and while interacting with other components released by transformed cells. The second phase will use the data from the sequence analysis to prepare reagents that will contribute to our basic knowledge of these factors. With the primary sequence known, synthetic peptides containing portions of the sequence will be coupled to proteins for the production of antisera and monoclonal antibodies against the SGF peptide. These antibodies will be used for: (a) affinity columns for rapid purification of SGF and its closely related TGFs; (b) determining the normal pattern of expression of these peptides either during development or in vivo in the mature organism; (c) determining if there is a correlation with the expression of these factors and the transformed phenotype; (d) studies on the control of the expression of these factors in vitro; and (e) patient care either as diagnostic tools (radioimmune assays) or therapeutic reagents to seek out and destroy the cells producing these peptides. The cloned SGF gene will be used to study the expression under normal physiological conditions as well as in malignancies and in the transformed states in vitro. The clone of the gene, hopefully, will be used for the production of large quantities of these factors.

Publications:

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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Expression of Cancer-Associated Proteins in Primate Placenta

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Dennis A. Pigott	Visiting Fellow	LVC	NCI
	Daniel R. Twardzik	Research Chemist	LVC	NCI
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COOPERATING UNITS (if any)

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SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

1.2

PROFESSIONAL:

1.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

As an extension of a study (Virology, 112, 365-369, 1981) documenting the preferential expression of p26 antigen from Macaca arctoides type C virus (MAC-1) in Macaca mulatta trophoblast, cocultivation of isolated trophoblasts from rhesus placentas with three indicator cell lines (A549, FEC, and CF2Th) were carried out which led to rapid isolation (3 to 4 weeks) in FEC of type C rhesus retrovirus in 4 of 10 cases. Melting curves of DNA-DNA hybridization indicated a 2 degree difference (83oC vs. 81oC) between these M. mulatta isolates and the single M. arctoides isolate. Previous isolates from Macaca were obtained in single, long term experiments (over 7 months). Consequently, primary trophoblasts represent a useful differentiated cell source for isolation of infectious retrovirus from a "low producer" primate species. Growth factors of apparent molecular weights of 6,000, 10,000, 20,000, and one in excess of 30,000 can be isolated from acid ethanol/extracts of human term placentas. Each size class of polypeptide has the defining properties of transforming growth factors (TGFs). By homologous radioimmunoassay, neither the 6,000 nor the 10,000 dalton polypeptide is related to human epidermal growth factor. The presence of these TGFs in ample concentrations indicates the usefulness of this tissue source for the study of human TGFs.

Project Description

Objectives:

First, to characterize 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. Second, to study type C viral expression in rhesus placenta so as 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 red and African green monkeys and chimpanzees. And third, to document the expression of transforming growth factors (TGFs) in human placenta, and to use this tissue as an in vitro model system to study the biochemistry of TGF synthesis. The use of human tumors growing in nude rats will be exploited to attempt to isolate sufficient TGF to develop reagents to study the mechanism of TGF action.

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 ³H-DNA transcript prepared from the MAC-1 isolate, and then melting curves performed. An RIA for alpha and beta subunit secretion of hCG is an additional assay procedure. Growth factor secretion from placenta was assessed by stimulation of growth of normal rat kidney cells (NRK) in soft agar, and competition with ¹²⁵I-labeled epidermal growth factor (EGF) for binding to membrane receptors.

Major Findings:

1. In vitro growth of primate placenta. Improved methods have been developed for growth of first trimester human placenta in organ culture and for isolation of trophoblast cells for growth in monolayer culture. HCG expression in human placental organ culture is highest under Gelfoam support conditions, incubation in 95% air and 5% CO₂ in a chamber on a platform rocker and use of CMRL 1066 medium with FCS, insulin, hydrocortisone, putrescine and retinyl acetate. The kinetics of hCG secretion are in the manner of a bell-shaped curve with peak levels around day 6 in organ culture. Addition of theophylline and dibutyryl cyclic AMP elevated hCG levels by about 4-fold over untreated controls. The amount of hCG expression with this organ culture system was approximately ten thousand times higher on a per gram basis than those obtained with term placenta in conventional explant culture.

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. The usefulness of this approach in order to generate continuous cell strains or cell strains with greatly increased division potential had been previously established from species (sea mammals) in which no

continuous cell lines were currently available (Stromberg et al., Proc. Soc. Exp. Biol. Med., 164, 439-444, 1980). The resultant rhesus cell strain (RheTro) maintained an epithelioid morphology with desmosomes by electron microscopy (EM), and a monolayer growth pattern. In addition, these trophoblast-like cells expressed SV40 T-antigen, heat-stable placental alkaline phosphatase (which could be induced by sodium butyrate, iododeoxyuridine, cyclic AMP and theophylline), type IV basement membrane collagen, fibronectin, and laminin. Cytogenetic study of this RheTro cell strain revealed that all the cells had a female karyotype and, in addition, contained numerous numerical and structural abnormalities involving all of the chromosomes. A wide range of chromosome numbers was observed (courtesy of Jacqueline Whang-Pang, NIH), with 30% of cells in the diploid (2n) range, 54% of the cells in the tetraploid (4n) range, and 16% of cells between 6n and 16n ranges. However, no retroviral p26 antigen expression could be detected. In contrast, freshly isolated rhesus trophoblasts expressed MAC-1 p26 at levels equivalent to the placental tissue from which they were isolated, and p26 antigen expression was maintained during placental organ culture after up to 12 days in culture.

2. Biochemistry and modulation of hCG in normal and malignant trophoblast. The biosynthesis and secretion of chorionic gonadotropin (hCG) have been studied by pulse-chase labeling techniques in organ cultures of normal first trimester placenta. First trimester placental tissue produces 18,000 and 15,000 M.W. intracellular forms of alpha subunit and 24,000 and 18,000 M.W. forms of beta subunit. These forms have a long intracellular half-life (>1 h) and appear to be slowly processed to mature forms that are detectable intracellularly prior to secretion. A high-mannose, (Man)₈(GlcNAc)₂, oligosaccharide-containing form of alpha subunit accumulates intracellularly, suggesting that the rate-limiting step in alpha subunit processing involves an α -mannosidase activity. Placental tissue secretes both a large free alpha subunit and an hCG- α subunit that is part of complete hCG. There is no evidence for the synthesis of high molecular weight prohormone forms that might be precursors to the secreted forms of hCG subunits. These findings are consistent with what has previously been reported for the biosynthesis and secretion of hCG subunits by cultured human malignant trophoblastic cell lines.

A second concern in regard to hCG biochemistry has been to determine the kinetics of carbohydrate synthesis and processing using tunicamycin and endoglycosidase H (endo H). Following labeling of placental organ culture specimens with ³H-mannose, chasing for various time periods, and immunoprecipitation, the oligosaccharides were prepared by endo H digestion and the carbohydrate size (Bio-Gel P-4 columns) and composition were determined. The data suggest that, as in chorio-carcinoma cell lines, the 18,000 M.W. form of alpha contains one high mannose core, and that the 12,000 M.W. form is the apoprotein of the α -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 α and β 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 various metabolic factors (methotrexate, iododeoxyuridine, hydroxyurea, sodium butyrate, dibutyryl cyclic AMP, and epidermal growth factors). These compounds were chosen because of their demonstrated inductive effect on hCG in malignantly transformed

trophoblastic cells (choriocarcinoma cell lines). For example, in both the JAr and BeWo lines of choriocarcinoma, inhibition of DNA synthesis by methotrexate had markedly increased secretion of hCG. However, of those modulating factors above, only dibutyryl cyclic AMP enhanced hCG expression in normal trophoblasts in organ and cell culture. Interestingly, exposure to dibutyryl cyclic AMP at 1 mM during placental organ culture resulted in a several fold stimulation of the alpha subunit secretion while the beta subunit expression of hCG was only slightly increased.

The comparative effect of epidermal growth factor (EGF) upon hCG secretion in normal and malignant placental cells has not been examined. Because the human placenta is rich in EGF receptors, experiments were undertaken to determine whether EGF influences secretion of hCG in normal placenta in organ culture and to compare this secretion with that of choriocarcinoma cells. Despite the presence of EGF receptors on both the placental organ cultures and the JAr cells, under the conditions employed in these experiments, EGF did not modulate hCG production in normal first trimester placenta in organ culture. These conditions included time of exposure (start of culture, or 1 to 4 days later), length of exposure (24 or 48 hrs), media (CMRL 1066 or DMEM) or EGF concentration (2 to 20,000 ng/ml). In contrast, the JAr line of choriocarcinoma responded to EGF exposure with a 2-fold or greater increase in hCG secretion. The increases in hCG levels seen was not due to cell replication as protein content of the treated cultures relative to the controls was the same during the 1- to 3-day EGF exposure periods. In addition, there was no increase in the rate of DNA synthesis during the exposure to EGF as measured by incorporation of [methyl-³H]-thymidine.

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 precursor forms, 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 antigen or Goodpasture antigen, 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 trophoblast. 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 with term gestation or parity greater than 10. 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 retrovirus in four of ten cases. With all four retroviral isolates, p26 expression was detected in the cell monolayers between two and five weeks, and Mn^{2+} -dependent DNA polymerase activity was evident in the culture supernatants between five and nine weeks after initiation of cocultivation. Five of the remaining six sets of cocultivations grew Simian foamy virus and were discontinued. The two previous isolates from Macaca (MAC-1 and MMC-1) were both obtained in single long term experiments (over seven months) after multiple attempts had failed to yield virus. Consequently, primary trophoblast cells represent a useful differentiated cell source for isolation of infectious retrovirus from a "low producer" primate species.

While the buoyant density of all the purified new Macaca mulatta isolates is 1.14 cm^3 and all have a type C morphology by EM, and compete in parallel in a radioimmunoprecipitation assay with the Macaca arctoides MAC-1 p26, the host range of the initial two new Macaca mulatta isolates (MMC-2 and MMC-3) is different in that neither replicate in human A549 cells. Besides host range studies, an additional means to discriminate among these isolates involves nucleic acid hybridization. Accordingly, experiments were carried out to detect the degree of sequence homology between the various Macaca mulatta viral isolates and the single Macaca arctoides isolate. This was undertaken because previous work (Todaro, et al., *Virology*, 72, 278-282, 1976) demonstrated that type C viruses from 3 species of baboons (Papio cynocephalus, P. hamadryas, and P. papio) differ in their nucleic acid sequence thermal stability by 2-4°C, whereas several independent isolates from one species of baboon had nearly identical thermal stabilities (<0.5°C). Accordingly, cellular DNA was isolated from cell lines infected with the various isolates and hybridized to a ³H-DNA transcript prepared from Macaca arctoides MAC-1 virus. The cellular DNA from various Macaca mulatta isolates melted 1.1-2.7°C lower than the homologous hybrid. The lower melting temperatures obtained with the viral isolates from M. mulatta are consistent with base-pair mismatching of the genome. The data, therefore, suggest that the M. mulatta isolates can be distinguished readily from the previously described M. arctoides isolate and conversely, the similarity of melting temperatures between the initial M. mulatta isolate (MMC-1) and those viruses isolated from rhesus trophoblasts (MMC-2 through MMC-5) by cocultivation with feline embryo cells indicates rhesus origin. The fact that MMC-1 was never present within the laboratory, and the isolates of MMC-2 through MMC-5 were obtained separately and so rapidly and so consistently provides evidence against cross-contamination as the source of viral origin.

To determine possible intraspecies variation among MMC-1 through MMC-5 viral isolates, restriction enzyme analysis with BamH1, EcoR1, HindIII, and SacI (all which cleave within the colobus type C genome) is being carried out with all Macaca mulatta isolates as well as that from Macaca arctoides. The band pattern after enzyme digestion will provide further evidence of uniqueness of origin of the various isolates.

As an extension of the ease of retroviral isolation in the rhesus system, cocultivation of indicator lines from six species began with isolated trophoblast target cells from two Erythrocebus patas (red) and four Cercopithecus aethiops (African green) monkeys, as well as three Pan troglodytes (chimpanzee). Over a three-month period, supernatant DNA polymerase activity appeared in one chimpanzee embryo cell cocultivation and one African green-human A549 cocultivation. Although morphologically altered, in neither case was there obvious cytopathic effect with formation of syncytia. However, electron microscopy indicated foamy virus contamination and these particular cocultivations were discarded.

4. Growth factors in primate placenta and amniotic fluid. Growth factors of apparent molecular weights of 6,000, 10,000, 20,000 and one in excess of 30,000 can be isolated from acid/ethanol extracts of human term placentas. Each size class of growth factor resembles transforming growth factor (TGF) in that it stimulates anchorage-independent growth of normal rat kidney cells and competes with EGF for binding to EGF membrane receptors. The 6,000, 10,000 and 20,000 molecular weight polypeptides also resemble TGF in their acid and heat stability, and their requirement for intact disulfide bonds for growth-promoting activity. By homologous radioimmunoassay, neither the 6,000 nor 10,000 dalton polypeptide is related to human epidermal growth factor (hEGF). The presence of these TGFs in ample concentrations (approximately 100 ng of EGF equivalents per term placenta for the 10,000 dalton polypeptide) indicates the usefulness of this tissue source for study of human TGFs.

Using similar procedures, a pool of amniocentesis samples has indicated that human (at first trimester), as well as subhuman primates (using amniotic fluid samples obtained at term gestation), contain a transforming growth factor of approximately 8,000 molecular weight. Of interest is the additional presence in human amniotic fluid of factors that appear to suppress the colony growth of A375 cells, a malignant human melanoma line, in soft agar. An inhibitory growth factor of approximately 20,000 molecular weight is also found in the serum-free conditioned medium of short-term human placental organ cultures, which is the particular interest of Dr. Dennis Pigott. Another concern has been human urine TGFs and in an effort to obtain these TGFs in larger amounts, several human tumors have been established in nude rats; the urine is being processed for comparative TGF production.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of the mechanism of modulation of expression of biochemical markers of malignancy, such as hCG and growth factors is essential for the markers to be useful clinically. These markers can be easily studied in normal and malignant human placental tissue in cell and organ culture.

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 retrovirus from higher primates. Clearly, rhesus trophoblast is a preferential tissue source for isolation of endogenous retrovirus, 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.

Transforming growth factors (TGFs), particularly those present in urine, may prove useful markers of pre-clinical cancer development in man. The human placenta provides an in vitro way to study TGF synthesis and mechanism of action.

Proposed Course:

Because of limited technical help and resources, the approach to primate retroviral isolation using cocultivation with trophoblast cells is currently inactive with the relevant cocultivations simply frozen down for future use. The use of human placental culture system to study TGF action awaits further characterization of human urinary TGFs. For example, is the placental TGF of approximately 30,000 daltons equivalent to the urinary TGF of similar molecular weight? Initial results indicate that in normal nude rats there is no urinary growth factor over 10,000 M.W. Consequently, the major emphasis will be to evaluate the urine of nude rats bearing human tumors as a model system to obtain sufficient human TGFs for biochemical characterization.

Publications:

Huot, R.I., Foidart, J.M., Nardone, R.M., and Stromberg, K.: Differential modulation of human chorionic gonadotropin secretion by epidermal growth factor in normal and malignant placental cultures. J. Clin. Endocrinol. Metab. 53: 1059-1063, 1981.

Ruddon, R.W., Hartle, R.J., Peters, B.P., Anderson, C., Huot, R.I., and Stromberg, K.: Biosynthesis and secretion of chorionic gonadotropin subunits by organ cultures of first trimester human placenta. J. Biol. Chem. 256: 11389-11392, 1981.

Stromberg, K., Pigott, D.A., Ranchalis, J.E., and Twardzik, D.R.: Human term placenta contains transforming growth factors. Biochem. Biophys. Res. Comm. 106: 354-361, 1982.

Project Description

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 cell growth and tumorigenicity. Specifically: (1) to obtain from promotable cell lines, clonal cell lines which are resistant to promotion of transformation by the phorbol diester 12-O-tetradecanoylphorbol-13-acetate (TPA) 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; and (3) to continually characterize the clonal cell lines and monitor them for stability.

Methods Employed:

Characterization and cloning of mouse epidermal 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 dioctyl phthalate as well as to human and rodent transforming growth factors.
2. Retinoids inhibit promotion of anchorage-independent growth of JB6 cells by TPA. Promotion of tumor cell phenotype by the phorbol diester TPA is inhibited by retinoids which also act as antipromoters in mouse skin tumor promotion. The structure/activity relationship for antipromotion by retinoids in vitro paralleled that found in vivo with retinoic acid showing the highest activity. Antipromotion occurs when retinoic acid is added simultaneously with TPA or up to 24 hours subsequently but not with prior exposure. Retinoic acid enhances promotion of transformation by EGF. Enhancement of EGF receptor binding by retinoic acid may explain the promotion enhancement since it occurs in JB6 cells. Modulation of receptor binding does not explain the retinoid inhibition of phorbol ester promotion, however, since phorbol ester binding is unaffected by retinoic acid.
3. Analogy of JB6 model to second stage promotion in vivo. Slaga et al. (Proc. Natl. Acad. Sci. USA, 77, 3659, 1980) have identified two stages of promotion in mouse skin which must occur sequentially, are produced by different agents and are sensitive to different promotion inhibitors. Promotion of transformation in

JB6 cells occurs in response to second-stage promoters such as mezerein but not to first-stage promoters such as the calcium ionophore A23187. Inhibition of the promotion response occurs with second-stage inhibitors such as retinoids but not with first-stage inhibitors such as antiproteases.

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 vs. the correlative ones.

Proposed Course:

The following activities are planned: 1) to extend this work to study new non-phorbol 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 promoters of two different classes 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 to then 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., and Srinivas, L.: Responses of preneoplastic epidermal cells to tumor promoters and growth factors: Use of promoter resistant variants for mechanism studies. J. Cell. Biochem. 18: 261-270, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05143-03 LVC

PERIOD COVERED

October 1, 1981 to May 5, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Pathogenesis of Virus-Associated Human Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Laboratory of Viral Carcinogenesis

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PROFESSIONAL:

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OTHER:

0.05

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A serum factor which inhibits the stimulation of lymphocytes (LSI) by Epstein-Barr viral antigens was detected in patients with undifferentiated nasopharyngeal carcinoma (NPC). A positive correlation between favorable prognosis and decline of LSI titers was demonstrated. Information was collected relating to the clinical features, pathologic features and hormone receptor status of breast cancer patients in Tunisia. Evidence for the unusual progression of disease in Tunisian breast cancer patients was documented with the finding of a poorer nuclear grade in rapidly progressing breast cancer (RPBC) patients than non-RPBC patients. In contrast to American patients with inflammatory breast cancer, who are reported to have low levels of hormone receptors, patients with RPBC had levels comparable to non-RPBC patients. Information collected on 256 pathologically confirmed cases of Burkitt's lymphoma (BL) diagnosed in the Western Hemisphere demonstrated the curability of BL, particularly in patients with limited disease. Target organs appeared to be related to age, suggesting a predilection of the disease for rapidly dividing cells. Laboratory and epidemiologic studies provided evidence for environmental as well as genetically determined predisposing factors.

Project Description

Objectives:

The purpose of this project is to correlate clinical, epidemiologic and pathologic findings in patients with virus-associated tumors with the subsequent attempt to relate these parameters to virologic assays.

Methods Employed:

Sera from 25 patients with undifferentiated nasopharyngeal carcinoma (NPC), 20 patients with other head and neck tumors, and 20 healthy controls were tested for the presence of a serum factor which inhibits lymphocyte stimulation (LSI) by viral and soluble antigens of Epstein-Barr virus (EBV). Eight of the NPC patients were followed longitudinally for a minimum period of twenty months. The LSI titers as well as antibodies to viral capsid antigen (VCA), early antigen (EA) and nuclear antigen (EBNA) were compared with the response to therapy to see which assay was the best predictor of prognosis. Information was collected from more than 100 patients with breast cancer followed at the Institute Salah Azaiz in Tunis, Tunisia. Information from three separate studies, a chemotherapy study involving 112 patients, a pathology study involving 102 patients, and a hormone receptor study involving 94 patients, were integrated with an attempt to interrelate all of the clinical, pathologic and laboratory values. The American Burkitt Lymphoma Registry was maintained and cases continued to be collected from the Clinical Center at NIH, from the literature, and from personal contact with various physicians from different parts of the country. The patients' physicians were contacted for clinical information, diagnostic blocks, and sera. The diagnostic blocks were sent to Dr. Berard (NIH) for review and confirmation of the diagnosis. The sera obtained from these cases were tested for antibody titers to the EBV, VCA and EA. The data from 256 Burkitt's lymphoma (BL) patients were then analyzed for various racial, geographical and epidemiological patterns and compared to the African patterns.

Major Findings:

1. Identification of a serum factor (LSI) indicating disease activity in NPC. All 25 NPC patients showed the presence of EBV-related LSI which was associated with the IgA fraction of the serum, whereas it was found in none of the other head and neck tumor patients or healthy controls. Five of eight patients who were followed longitudinally and who showed good response to treatment showed a decline in titers of LSI. Three other patients who had early relapses and who did not respond to treatment showed consistently elevated titers. A preliminary comparison with other antibodies, including IgA antibody to VCA and IgG antibody to EA, which had previously been reported to be related to prognosis, revealed that LSI was a better and earlier predictor of response.
2. Characterization, pathogenesis and control of rapidly progressing breast cancer in Tunisia. Rapidly progressing breast cancer (RPBC) or pousse'e evolutive (PEV) proved to be an entity with certain pathologic features, such as poor nuclear grade and involvement of the dermal lymphatics with tumor microemboli. The evaluation of hormonal levels in Tunisian breast cancer cases showed increased estrogen receptor (ER) and progesterone receptor (PR) levels in comparison to American patients with inflammatory breast cancer. ER and PR levels were lower

in pre-menopausal and older patients than post-menopausal and younger patients. Age was more important than menopausal status in predicting hormone receptor levels. Cyclophosphamide methotrexate 5-fluorouracil (CMF) proved to be effective in controlling RPBC. Forty-two percent of patients showed a complete response to CMF, 54.7% had a partial response (more than 50% decrease), and an additional 17% showed objective improvement. Of all the factors relating to response to CMF, stage of disease proved to be most important. No difference was seen between surgery and radiotherapy as an adjunct to chemotherapy except in patients with advanced disease (PEV-3) who responded better to radiation therapy. Patients with higher ER and PR levels showed a longer disease-free interval than those with negative values. Regarding PEV level, lower receptor levels were found in PEV 1,2,3 as compared to PEV-0.

3. Burkitt's lymphoma in the United States. On evaluation of data from the American BL Registry, it was noted that the major characteristics of American BL patients tended to be male predominance, a young age group involvement (4 to 12 yrs.), and a higher incidence in Caucasians. More recently, older patients have been frequently reported, perhaps due to better awareness of the disease on the part of non-pediatricians. In contrast to the earlier report showing a paucity of patients in the high altitudes, cases were obtained from all parts of the country. A positive cancer history was obtained in first degree relatives of 18 of the 52 cases on whom detailed pedigrees could be obtained. Abdominal viscera were affected most often, the most outstanding being the ileum. Unlike African cases, cervical lymph nodes were also among the most common organs to be involved. Better median survival rates for Stage I and II cases were observed than for patients with Stage IV disease. No relapse was observed in American cases beyond 2 years, whereas 14 late relapses were observed in a series of 117 African patients. The survival seemed to be prolonged in patients with elevated VCA antibody titers. In contrast to African BL patients, Americans had more older patients, less EBV-positive patients, and fewer patients with jaw tumors.

Significance to Biomedical Research and the Program of the Institute:

In order to determine the significance of certain viral markers in human cancer, it is important to have well-characterized specimens from patients who have been appropriately studied. This project provides the characterization of patients with three diseases under close study by the National Cancer Institute. NPC is one of the tumors associated with EBV. Due to its relative inaccessibility, which makes it difficult to diagnose, and its close proximity to major blood vessels and nerves, early detection is imperative. Hence, there is a constant need for diagnostic and prognostic markers. LSI proves to be one such marker worth pursuing. The Tunisian breast cancer patients are of importance because they have an unusually aggressive disease and provide an opportunity to study factors which promote tumors and which limit their growth, the information also being applicable to breast cancer in the United States. The studies on Burkitt's lymphoma are of importance because BL is one of the tumors with the most prominent epidemiologic and laboratory evidence indicating a viral etiology. Two major cofactors implicated in the cause of BL are malaria and the Epstein-Barr virus. Studies on BL in an area where malaria is absent help to clarify the role of EBV in the pathogenesis of this disease.

Proposed Course:

These studies have been completed and the results prepared for publication. Some of the work will be continued as part of other projects.

Publications:

Costa, J., Webber, B.L., Levine, P.H., Muenz, L., O'Connor, G.T., Tabbane, F., Belhassen, S., Kamaraju, L.S., and Mourali, N.: Histopathological features of rapidly progressing breast cancer in Tunisia. Int. J. Cancer, in press.

Levine, P.H., Kamaraju, L.S., Connelly, R.R., Berard, C.W. Dorfman, R.F., Magrath, I., and Easton, J.M.: The American Burkitt's Lymphoma Registry: Eight year's experience. Cancer 49: 1016-1022, 1982.

Mourali, N., Tabbane, F., Muenz, L.R., Bahi, J., Belhassen, S., Kamaraju, L.S., and Levine, P.H.: Preliminary results of primary systemic chemotherapy in association with surgery or radiotherapy in rapidly progressing breast cancer. Br. J. Cancer 45: 367-369, 1982.

Sundar, S.K., Ablashi, D., Kamaraju, L.S., Levine, P.H., Faggioni, A., Armstrong, G.R., Pearson, G.R., Krueger, R.F., Hewetson, J.F., and Menezes, J.: Sera from patients with undifferentiated nasopharyngeal carcinoma contain a factor which abrogates specific Epstein-Barr virus antigen-induced lymphocyte response. Int. J. Cancer, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05149-03 LVC
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Mechanisms of Tumor Promotion		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Mohammed E. Shoyab Expert LVC NCI OTHER: George J. Todaro Medical Officer 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: 2.15	PROFESSIONAL: 1.05	OTHER: 1.10
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) (1) The mechanism of 12-O-tetradecanoylphorbol-13-acetate (TPA)-elicited inhibition of EGF binding (TIEB) was investigated. (2) Specific receptors for phorbol esters were obtained in soluble form from mouse brain and their purification and characterization are being pursued. (3) The putative endogenous ligands for phorbol receptors in milk, intestine, pancreas and stomach were found. (4) An enzyme (phorbol-12,13-diester 12-ester hydrolase, PDEH) which specifically cleaves phorbol diester at the 12 position was isolated and characterized. PDEH is not expressed in mouse skin but is present in rat, rabbit, guinea pig and hamster skin. (5) A protein from mouse serum which specifically binds biologically active phorbol diesters was purified and characterized. (6) Endogenous oncopromotone(s) and oncoretardine(s) have been detected in brain. Their purification and characterization are in progress. (7) Isolation and characterization of very potent oncostatin (antitumor promoter) from clams and other mollusks are being carried out.		

Project Description

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.

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, isochromato-focusing 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. This work was further extended. 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 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. Based on our work on this subject, we have reached the conclusion 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. We have purified a protein ($\sim M_r$ 71,000) from murine serum 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 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 phorbol-12,13-dibutyrate (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 liver. 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 $F1^{-}$ 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^{-6} 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, chlorpenthiolal, 2-chloroimpiramine and impiramine competitively decrease the binding of 3H -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 benzantracene (DMBA)-induced mammary tumorigenesis in Sprague Dawley rats by flufenazine. Sprague-Dawley rats were treated with a sub-optimal dose of DMBA (5 mg; one treatment) and after two weeks secondary treatment was started with either 0 μ g, 100 μ g or 200 μ g of flufenazine intragastrically or intraperitoneally three times a week. Flufenazine itself did not induce any mammary tumors. However, the rate and number of mammary tumors in DMBA-treated rats was significantly enhanced by flufenazine.

7. Purification and characterization of soluble specific receptors for biologically active phorbol esters from mouse brain. We have solubilized these receptors from crude particulate fractions of mouse brain with Triton X-100 and 3-[cholamidopropyl] dimethyl-ammonio]-1-propanesulfonate (CHAPS). However, the removal of detergent results in reaggregation of the solubilized receptors. During the course of our investigation we observed that about 1-2% of receptor activity is extracted in water in soluble form. Soluble brain receptors for PDBu resolve into three distinct peaks (\approx 440 K, 230 K, and 94 K) during gel filtration on Bio-Gel A-0.5 in PBS. All three fractions have the same affinity for TPA or PDBu as the crude particulate receptors. The kinetics of interactions of PDBu with all three soluble receptor fractions was similar to that of particulate receptors.

8. Isolation and characterization of putative endogenous ligands for PDBu receptors. We have purified from defatted cow milk a compound(s), lipid in nature, using chloroform:methanol extraction, acetone extraction, silicic acid chromatography and high pressure liquid chromatography (HPLC). This lipid(s) competitively inhibits the binding of PDBu to its receptor. It also inhibits the interaction of EGF to its receptor, as does TPA. We call it putative endogenous ligand (PEL). PEL is not a phorbol diester as it is not inactivated by PDEH. PEL itself does not inactivate or alter PDBu or TPA. PEL appears to be a nonpolar lipid. We are in the process of determining its chemical nature. This chloroform:methanol-soluble, PDBu binding-inhibiting activity is present in murine pancreas, intestine, stomach, testicles, skin, brain, kidney, liver, muscle, spleen, lung, and heart in decreasing order. No activity was detected in murine serum. Pancreas, intestine and stomach contain exceptionally high concentrations of this activity.

9. Endogenous transforming growth factor(s) (oncopromotine) and growth-inhibitory factor(s) (oncoretardine) from beef brain. We have partially purified a protein factor (\approx 30 K) from aqueous extracts of beef brain which induces anchorage-independent growth of rat kidney fibroblastic cells in soft agar. We have used ultrafiltration, Sephadex G-200 chromatography and DEAE-cellulose chromatography to purify this protein. 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 rat kidney fibroblastic and murine and human transformed cells in soft agar. We have purified the factor using ultrafiltration, Bio-Gel P-10 chromatography, ethanol fractionation and anion exchange chromatography. It is a low molecular weight compound(s) (\approx M_r 1,000), it 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.

10. Partial purification of anti-growth factor(s) (oncostatin) from clam digestive glands. We have found that 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. We have partially purified the factor from 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 suggests 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 facilitate the design of methods for the control and prevention of cancer.

Proposed Course:

This project will continue and extend the findings described to better define the mechanism(s) of tumor promotion. Further isolation and characterization of PDBu receptor from brain will be pursued in order to understand the molecular mechanisms of TPA and its interaction with membrane receptors. The characterization of putative endogenous ligand(s) will be given very high priority. In addition, we will continue our 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 and differentiation-modulating substances (endogenous tumor promoter[s]) should provide useful information in understanding the mechanism 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, in press.

Shoyab, M., Gunnell, M., and Lubiniecki, A.S.: Thymidine uptake and incorporation is defective in cultured fibroblasts from Fanconi anemia patients. Hum. Genet. 57: 296-299, 1981.

Shoyab, M., and Todaro, G.J.: Partial purification of a binding protein for biologically active phorbol and ingenol esters from murine serum. J. Biol. Chem. 257: 439-445, 1982.

Shoyab, M., Todaro, G.J., and Tallman, J.F.: Certain neuroleptic and antipsychotic tricyclic drugs competitively inhibit interaction between tumor-promoting phorbol esters and their specific receptors. Cancer Lett., in press.

Shoyab, M., Warren, T.C., and Todaro, G.J.: Isolation and characterization of an ester hydrolase active on phorbol diesters from murine liver. J. Biol. Chem. 256: 12529-12534, 1981.

Shoyab, M., Warren, T.C., and Todaro, G.J.: Phorbol-12,13-diester 12 ester hydrolase (PDEH) may prevent tumor promotion by phorbol diester in skin. Nature 295: 152-154, 1982.

Shoyab, M., Warren, T.C., and Todaro, G.J.: The tissue and species distribution and developmental variation of specific receptors for biologically active phorbol and ingenol esters. Carcinogenesis 2: 1273-1276, 1981.

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 Receptors for Phorbol and Ingenol Esters. In Hecker, E. (Ed.): Carcinogenesis, A Comprehensive Survey, New York, Raven Press, 1982, pp. 443-462.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05150-03 LVC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (60 characters or less)

Chemical Carcinogenesis and Cocarcinogenesis In Vitro

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ulf R. Rapp	Visiting Scientist	LVC	NCI
OTHER:	Cha-Mer Wei	Expert	LVC	NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

1.2

PROFESSIONAL:

0.6

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS

(b) HUMAN TISSUES

(c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An in vitro system was established for the quantitative chemical transformation of the mouse epithelial cells Mus musculus castaneus epithelial (MMCE). These cells can also be transformed by C3H/MuLV in conjunction with 12-O-tetradecanoyl-phorbol-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. MMCE cells have also appeared to provide the first system for two-stage carcinogenesis in culture with ethylnitrosourea (ENU) as carcinogen and TPA as promoting agent. Clones of transformed cells were examined for transformation-sensitive glycoproteins. MMCE cells were also transformed by a new transforming virus 3611-MSV and the pattern of glycoprotein changes that were induced was determined. These cells show a different profile of glycoprotein changes and two new transformation-induced cellular proteins were identified. The observation of cocarcinogenesis of MMCE cells with MuLV and TPA prompted a search for factors homologous to TPA in normal sera. Such a transforming factor was found and purified from normal mouse serum.

Project Description

Objectives:

Development of improved in vitro assays for chemical transformation of fibroblastic and epithelial cells. Use of cocarcinogenesis for the isolation of new cell-derived tumor genes after linkage with type C viral sequences. Specifically: (1) develop cell substrates for chemical transformation of a variety of differentiated cells in vitro; (2) improvement of assay systems to allow processing large numbers of carcinogen-treated cells and development of transformation experiments in a shorter period of time; (3) use of cocarcinogenesis for the isolation of efficiently transforming type C viruses or type C virus-linked cellular tumor genes in culture; and (4) isolation of growth-promoting and transforming factors from chemically transformed epithelial cells using nontransformed epithelial cells as test cells.

Methods Employed:

During the past ten years several lines of evidence have developed to indicate a possible interaction between chemical carcinogens and endogenous RNA viruses. Most of this work has been done in vivo where it is difficult to sort out the relative contributions of transformation frequency versus the host immune response to the overall tumor incidence. We have therefore concentrated on developing quantitative in vitro systems for chemical carcinogenesis. Since most human neoplasias arise in epithelial tissues, emphasis was placed on establishing epithelial cells. Drawbacks of chemical transformation assays in vitro are the required large size of the experiments (low transformation frequencies) and the long time (4-6 weeks) necessary for completion. We developed conditions which allowed assay of large numbers of treated cells within 1 to 2 weeks. To facilitate the generation of new transforming type C viruses, both in vivo and in vitro, we examined the effect of treatment with carcinogens and tumor promoters. The viral genomes in nonproductively transformed Mus musculus castaneus epithelial (MMCE) cells are now being determined using recombinant DNA techniques.

Major Findings:

1. Transformation of cells in culture by MuLV and TPA. MuLV does not carry a tumor gene but is capable of indirectly transforming cells in vivo. The mechanism by which this occurs occasionally leads to the formation of transmissible new transforming virus, and more commonly, to linkage of a potential cellular tumor gene with part of the MuLV genome (Payne, et al., 1981; Neel, et al., 1981; Hayward, W.S., 1981; Rapp and Todaro, 1980; and Stahl, et al., 1977). Such a sequence labeling of the cellular gene should then allow its identification by molecular cloning. To this end, an in vitro system was developed for the transformation of mouse epithelial cells by MuLV in conjunction with 12-O-tetradecanoylphorbol-13-acetate (TPA). The target cells were MMCE Cl 7 (Rapp et al., 1979), a cell line that can be reversibly transformed by TPA. MMCE Cl 7 can be transformed at very low frequency by infection with ecotropic C3H/MuLV. When infection of MMCE cells was followed by growth in TPA-substituted medium (100 ng/ml) and the cells were subsequently tested for growth in soft agar in the absence of TPA, a dramatic increase in the frequency of transformation was observed (from 0-4 colonies/10⁵ cells to 600-800 colonies/10⁵ cells). Cells transformed by MuLV and TPA retained

a high plating efficiency in soft agar and generally did not produce virus particles. This basic protocol was also applied to rat fibroblast cells and, while background growth of rat cells in agar with TPA was higher than that observed with the MMCE cells (50-70 colonies/10⁵ cells), a 2- to 5-fold increase in the number of agar colonies was observed after infection of the rat cells with ecotropic C3H/MuLV and subsequent treatment with TPA.

2. Analysis of genes involved in chemical transformation of C3H/10T1/2 and MMCE cells in vitro. Two cell systems were used for chemical transformation in vitro, the fibroblast cell line C3H/10T1/2 and the epithelial mouse cell line MMCE. Carcinogens used included methylcholanthrene (MCA), ethylnitrosourea (ENU) and ENU plus the tumor promoter TPA. Transformed cells were cloned in soft agar before further analysis. The DNAs from several of these clones have been tested by others for transforming activity (Shih et al., Proc. Natl. Acad. Sci. USA, 76, 5714, 1979), Wigler, unpublished data) and were found to be positive. From these experiments it appeared that, in these cells, transformation was due to the action of presumably a single gene. We have examined these transformed cells for expression of molecularly cloned retroviral oncogenes (Bister, Rapp, and Duesberg, unpublished data). None of the known viral oncogenes were found to be expressed. We also have prepared a cosmid gene library (pJB8) from C3H/MCA5 cells and are sorting out transforming genes by transfection and sib selection. This library is also being used for isolation of the cellular homologs of tumor genes from new transforming viruses that originated after IUDR induction of these 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. Considering the fact that carcinomas are especially prevalent in humans, we have recently put special emphasis on transformation of epithelial cells. A family of cell-derived transforming genes isolated as portions of type C viruses might provide the tool necessary for both an understanding of mechanisms of transformation, and the development of strategies to neutralize their action.

Proposed Course:

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. The genes may be altered as a consequence of carcinogen treatment or may represent derepressed normal genes. Those chemically transformed cells that were efficient donors of transforming DNA will be tested for: (1) their effectiveness as target cells for the generation of new transforming retroviruses; and (2) tumor gene expression in chemically transformed C3H/10T1/2 and epithelial MMCE cells will be determined by dot blot and Northern blot analyses. Transforming genes will also be isolated from C3H/MCA cellular DNA by transfection and sib selection with cosmid cloned DNA. C3H/MCA5 cells will also be used for the construction of cDNA libraries using expression vectors. It appears that both DNA transfection and type C virus-mediated transduction will lend themselves to the study of genetic changes related to chemical carcinogens.

Publications:

Rapp, U.R., and Keski-Oja, J.: Murine leukemia virus-mediated transformation of a mouse epithelial cell line MMC-E. Cancer Res., in press.

Rapp, U.R., and Keski-Oja, J.: Transformation of cultured mouse epithelial cells by ethylnitrosourea and effects on cellular glycoproteins. Cancer Res., in press.

PERIOD COVERED
 October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
 Purification and Characterization of Growth Factors Produced by Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hans Marquardt	Visiting Scientist	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI
	Joseph E. De Larco	Research Chemist	LVC	NCI
	Daniel R. Twardzik	Biochemist	LVC	NCI

COOPERATING UNITS (if any)
 M.B. Sporn, LC, NCI, Bethesda, MD; L.E. Hood, Cal. Tech., Pasadena, CA; E.G. Krebs, University of Washington, Seattle, WA

LAB/BRANCH
 Laboratory of Viral Carcinogenesis

SECTION
 Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
 NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	0.3

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) A low molecular weight human, mouse and rat transforming growth factor (TGF) was isolated from serum-free medium conditioned by a human metastatic melanoma tumor line, a Moloney MuSV-transformed mouse 3T3 cell line, and a Synder-Theilen FeSV-transformed Fisher rat embryo fibroblast cell line. The estimated molecular weight of TGF is 7,400. It is a single chain polypeptide of 67 residues with three intrachain disulfide bridges. A comparison of the amino-terminal region of the first 26 residues of TGF from different species and different cell types shows 91% homology. Alignment of the amino acid sequence of TGF with the sequence of epidermal growth factor (EGF) reveals statistically significant sequence homology. TGF competes with I-125-EGF for the same receptor completely and equivalently. I-125-TGF did not discriminate between TGF and EGF. TGF stimulates, in the presence of A431 cell membranes, the phosphorylation of a synthetic polypeptide which corresponds to the sequence of the reported site of tyrosine phosphorylation in pp60-src. In contrast, TGF enabled normal anchorage-dependent cells to grow in soft agar, whereas EGF did not stimulate growth of these cells in semi-solid medium. Based on the chemical and biological data, it is proposed that TGF and EGF are chemically distinct, but share a common receptor binding site.

Project Description

Objectives:

To purify and characterize the major classes of growth factors released by transformed 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 growth factors from serum-free supernatants of cultured cells. Various standard techniques were used to characterize and compare the purified growth factors on the level of the primary structure.

Major Findings:

1. Isolation and purification of transforming growth factors. A low molecular weight transforming growth factor (TGF) was isolated from serum-free medium conditioned by a human metastatic melanoma tumor line, A2058. The purification of TGF was achieved by gel permeation chromatography of the acid-soluble growth-promoting activity, followed by reverse phase high pressure liquid chromatography using sequentially acetonitrile and 1-propanol as the mobile phase and trifluoroacetic acid as the ionic modifier in the organic solvent. The purification of TGF was approximately 7,000-fold after gel permeation chromatography with an overall recovery of 75%. The purity of the final preparation was established by SDS-polyacrylamide gel electrophoresis. Only one major single band, with an apparent molecular weight of 7,400, was observed. Reduction with 2-mercaptoethanol did not change the mobility of TGF, which is evidence for a single chain polypeptide.

Sarcoma growth factor (SGF) isolated from Moloney MuSV-transformed mouse 3T3 cells, and rat TGFs from either Abelson MuLV-transformed or Snyder-Theilen FeSV-transformed Fisher rat embryo fibroblasts were purified to apparent homogeneity using the same methodology developed for the purification of human TGF.

2. Chemical characterization of transforming growth factors. The amino acid composition of human melanoma-derived TGF, murine SGF and rat TGFs were determined. The closest approximation to integral values for all residues was obtained by assuming that TGF/SGF has a molecular weight of 7,400. TGF/SGF is a single chain polypeptide of 67 residues with three intrachain disulfide bridges and no free sulfhydryl groups. The amino acid compositions of TGF/SGF are similar, but not identical. Human TGF lacks tyrosine, whereas both rat TGF and murine SGF have two tyrosine residues. The amino acid compositions of TGF/SGF are unique and show a number of important differences when compared with the amino acid compositions of either human or mouse EGF. Both human and mouse EGF have one methionine, whereas TGF/SGF lacks methionine. Human and mouse EGF have 5 tyrosine and no phenylalanine residues. In contrast, human TGF has no tyrosine, but 3 phenylalanine residues; murine SGF and rat TGF both have 2 tyrosine and 3 phenylalanine residues. TGF/SGF is a less hydrophobic polypeptide than EGF, in agreement with its lower retention time relative to EGF on reverse phase columns.

The amino terminal sequences of human TGF, murine SGF and rat TGF have been determined in collaboration with Dr. Leroy E. Hood. The sequence homology of the first 41 residues of rat TGF and murine SGF is 100%. The homology of the first 34 residues of human TGF compared with rat TGF and murine SGF is 91%. Thus, human, rat and murine TGF/SGF are structurally closely related and are more conserved than are mouse and human EGF (68% sequence homology for residues 1 through 34). Alignment of the amino acid sequences of TGF/SGF with the sequence of mouse or human EGF reveals statistically significant sequence homology. The alignment results in 12 common or conserved amino acid residues out of 42 possible comparisons. To obtain such an alignment, the introduction of only a single break was necessary in the TGF/SGF sequence. Thus, the conserved amino terminal sequences of TGF/SGF and EGF could represent the receptor binding site common to both TGF/SGF and EGF.

3. Biological characterization of transforming growth factors. TGF showed remarkable competing activity in a radioreceptor assay developed for EGF, and completely displaced ^{125}I -EGF binding to the EGF receptor of A431 human carcinoma cells. Approximately equimolar concentrations of TGF and EGF were required to inhibit EGF binding by 50%. Conversely, ^{125}I -TGF did not discriminate between TGF and EGF, indicating highly conserved receptor binding sites on the TGF and EGF polypeptides.

TGF, like EGF, stimulates, in the presence of A431 cell membranes, the phosphorylation of a synthetic polypeptide which corresponds to the sequence of the reported site of tyrosine phosphorylation in pp60^{SRC}. These studies were performed in collaboration with Dr. Krebs. The data suggest that peptides with sequences similar to the site of tyrosine phosphorylation in pp60^{SRC} are preferred substrates for the kinase in these membranes. Thus, the EGF- and TGF-stimulated protein kinase has the potential to interact with and phosphorylate pp60^{SRC}.

Experiments performed in collaboration with Dr. Sporn have clearly shown that TGF (or TGF-alpha), like EGF, potentiates the colony-forming activity of TGF-beta. This intracellular novel growth factor, isolated from neoplastic and non-neoplastic cells, does not compete with EGF for receptor binding nor does it induce by itself anchorage-dependent indicator cells to form progressively growing colonies in soft agar. The physiological significance of the interaction between EGF or TGF-alpha and TGF-beta is unknown, but it is possible that, in addition to a role in malignant transformation, these factors may function in the natural mechanism of wound repair and in embryogenesis, where both EGF (or TGF-alpha) and TGF-beta have been postulated to play a part.

In contrast to EGF, TGF enabled normal anchorage-dependent rat kidney cells to grow in soft agar. Picomolar concentrations of TGF were required for a measurable effect on specific target cells. In summary, the highly purified TGFs/SGF can stimulate cells, that possess an active EGF receptor system, as well as EGF.

Significance to Biomedical Research and the Program of the Institute:

A new and unique family of growth factors was successfully isolated and purified to homogeneity from different species (human, mouse, rat) and different cell types (melanoma, virally transformed fibroblasts). The production of TGF appears to be a common feature of transformed cells and may be induced either directly or indirectly by phosphorylation of one or more cellular tyrosine acceptor sites. The isolated growth factors are conserved within different species and within different

cell types. These results will provide the basis for synthesizing antigenic haptens to prepare antibodies as a diagnostic tool for screening human tumor tissues for the expression of TGF, or TGF-like materials, and to study the relationship of TGF to the activation of genetic information exhibited by malignant cells.

Proposed Course:

The continuation of this work includes the preparation of sufficient TGF to determine its complete amino acid sequence. Synthetic TGF peptides will be prepared and antibodies raised in rabbits against synthetic peptides after linkage to a protein carrier. Radioimmunoassays will be developed using these antibodies.

Publications:

Marquardt, H., and Todaro, G.J.: Human transforming growth factor: Production by a melanoma cell line, purification, and initial characterization. J. Biol. Chem. 257: 5220-5225, 1982.

Marquardt, H., Todaro, G.J., Henderson, L.E., and Oroszlan, S.: Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures: Homology with human insulin-like growth factor II. J. Biol. Chem. 256: 6859-6865, 1981.

Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., Frolik, C.A., Marquardt, H., Todaro, G.J., and Sporn, M.B.: Isolation from murine sarcoma cells of novel transforming growth factors potentiated by EGF. Nature 295: 417-419, 1982.

Todaro, G.J., Marquardt, H., De Larco, J.E., Fryling, C.M., Reynolds, F.H., Jr., and Stephenson, J.R.: Transforming Growth Factors Produced by Human Tumor Cells: Interaction with Epidermal Growth Factor (EGF) Membrane Receptors. In Mozes, L.W., Schultz, J., Scott, W.A., and Werner, R. (Eds.): Cellular Responses to Molecular Modulators. New York, Academic Press, 1981, Vol. 18, pp. 183-204.

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, Vol. 4, in press.

Twardzik, D.R., De Larco, J.E., Marquardt, H., Sherwin, S.A., and Todaro, G.J.: Transforming growth factors: Detection in the urine of pregnant and tumor-bearing humans. Med. Pediatr. Oncol., in press.

Twardzik, D.R., Todaro, G.J., Marquardt, H., Reynolds, F.H., Jr., and Stephenson, J.R.: Transformation induced by Abelson murine leukemia virus involves production of a polypeptide growth factor. Science 216: 894-897, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05172-02 LVC
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PERIOD COVERED
October 1, 1981 to June 11, 1982

TITLE OF PROJECT (80 characters or less)
Investigations on Transformation-Specific Glycolipids and Glycoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Mohinder S. Kang	Visiting Fellow	LVC	NCI
OTHER: George J. Todaro	Medical Officer	LVC	NCI

COOPERATING UNITS (if any)
I. Singh, Johns Hopkins University, Baltimore, MD; A.D. Elbein, University of Texas Health Science Center, San Antonio, TX; I.K. Vijay, University of Maryland, College Park, MD

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.8	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibiotics which specifically alter the synthesis of glycolipids and glycoproteins by either altering the synthesis of intermediates of protein glycosylation or changing the post-translational process were used to study the role of the carbohydrate portion of glycoproteins in virus synthesis and infectivity. It was found that the antibiotic streptovirudin which specifically inhibited glycosylation of viral glycoproteins also inhibited virion formation, however, the inhibitor swainsonine, which alters glycoproteins of vesicular stomatitis virus (VSV), had no effect either on virus formation or its infectivity. The effects of tumor promoters, such as the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced changes in cultured rat brain cells, were studied and the results suggested specific changes in ganglioside synthesis are associated with tumorigenicity.

Project Description

Objectives:

The objective of this project is to study the role of glycolipids and glycoproteins in neoplastic transformation. The studies are divided into two groups: (1) the effect of known chemical carcinogens on the alterations in glycolipids and glycoproteins is being investigated; and (2) specific inhibitors of glycoprotein biosynthesis were used to investigate the role of the carbohydrate portion of glycoproteins and glycolipids on viral assembly, infectivity and transformation. Transformation-specific changes in glycolipid and glycoprotein syntheses and changes in the activities of transformation-associated glycosyl transferases as tumor markers will be investigated. Binding sites for the binding of tumor promoters (e.g., phorbol esters, sarcoma growth factors and epidermal growth factor) will be investigated.

Methods Employed:

Analytical and preparative ultracentrifugation; affinity, column, thin layer and paper chromatography; density gradient centrifugation; paper, agarose and polyacrylamide gel electrophoresis; isotopic labeling with ^3H , ^{14}C and ^{35}S radionuclides, gas liquid chromatography and high pressure liquid chromatography (HPLC). Standard cell culture techniques for initiation, growth, maintenance, passage and synchronization of mammalian and human cell cultures for virus production, infectivity assays and for in vivo and in vitro testing of various drugs were employed. Primary mixed and isolated cell culture techniques were also used.

Major Findings:

1. Effect of tumor promoters on glycolipid synthesis. Since brain is a relatively rich source of glycolipids, the effect of promotable concentrations of 12-O-tetradecanoylphorbol-13-acetate (TPA) was studied on primary cultures of fetal rat brain. The results indicate that incubation of primary cultured brain cells with as low as 10 ng/ml of TPA induces specific changes in the biosynthesis of gangliosides. These results indicate that specific changes in ganglioside biosynthesis are associated with tumorigenicity.

2. Effect of glycoprotein biosynthesis on virus assembly, infection and transformation. Studies with tunicamycin and streptovirudin, both inhibitors of lipid-linked saccharides which are intermediates of glycoprotein biosynthesis, indicate that the carbohydrate part of the glycoproteins of vesicular stomatitis virus (VSV) is necessary for virus replication. However, alterations produced in VSV glycoproteins by swainsonine, an inhibitor of α -mannosidase, one of the enzymes responsible for the processing of high mannose glycoproteins to the complex type of glycoproteins, had no effect on VSV assembly, replication, infectivity and transformation in baby hamster kidney cells. These studies suggest that the carbohydrate portion of VSV is necessary for VSV synthesis and infectivity, but alterations which lead to the synthesis of the hybrid structure of the carbohydrate part of the glycoprotein were not required for VSV formation and infectivity.

Significance to Biomedical Research and the Program of the Institute:

The changes in the carbohydrate part of glycolipids, glycoproteins and certain glycosyl transferases associated with tumorigenicity in cell culture should help to better understand the complicated process of carcinogenesis in humans, aid in early detection of tumors, and possibly give opportunities to intervene in the carcinogenesis process or selectively kill the cancer cells.

Proposed Course:

Project terminated.

Publications:

Phillips, L.A., Kang, M.S., and Narayanan, R.: Life Cycle Leukemia and Sarcoma Viruses with Particular Reference to the Viral Nucleic Acids. In Phillips, L.A. (Ed.): Viruses Associated with Human Cancer. New York, Marcel Dekker, in press.

Phillips, L.A., Kang, M.S., and Narayanan, R.: 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, in press.

Phillips, L.A., Kang, M.S., Narayanan, R., and Hollis, V.W., Jr.: Complementary DNA Copies of Leukemia and Sarcoma Virus RNA Contain Sequences of Deoxycytidylate and Deoxyguanylate. In Nieburges, H.E. (Ed.): Cancer Detection and Prevention. New York, Alan R. Liss, Vol 4, 1981, pp. 67-77.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Hormone and Growth Factor Requirements of Transformed Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Angie Rizzino	Expert	LVC	NCI
OTHER:	Nancy H. Colburn	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:

0.4

PROFESSIONAL:

0.3

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cell culture models developed to study the action of tumor promoters are often limited by the requirement for serum in the culture medium. This problem can be overcome by the use of defined media selectively supplemented with highly purified and well-characterized attachment factors, growth factors and hormones. In this project a defined medium has been developed for the mouse epithelial cell line JB6 Clone 41, which has previously been shown to respond to tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) in serum-containing media. In place of serum, the defined medium (referred to as 4F) contains fibronectin, insulin, transferrin and epidermal growth factor. JB6 Clone 41 was grown in 4F for over 35 generations without any noticeable change in cell behavior. In contrast, JB6 Clone 41 cells that had been induced to grow in soft agar by treatment with TPA were unable to grow in 4F, even though their growth in serum-containing media was unaffected. These results demonstrate that the use of defined media offers the advantage of exposing differences in the growth requirements of cells before and after treatment with tumor promoters.

Project Description

Objectives:

It is an established fact that phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), can function as promoters of transformation in vivo. However, the mechanisms involved are poorly understood and are likely to be quite complex. To simplify the task of studying this phenomenon, several cell culture models have been developed. One such system is a series of epithelial cell lines (e.g., JB6 Clone 41) that respond to TPA by irreversibly acquiring the ability to grow in soft agar. However, the study of transformation using in vitro models of transformation such as this, is limited by the requirement for serum in the culture medium. Therefore, the objectives of this project were to develop a totally defined culture medium for the long-term growth of the mouse cell line JB6 Clone 41 and to determine whether transformation of these cells by TPA alters their requirements for growth.

Methods Employed:

The major role of serum in cell culture is to provide factors that fall into several major categories: attachment factors, growth factors and hormones. Today, the majority of required factors are available in highly purified form. Using these factors, and drawing upon what is now known about the growth requirements of mammalian cells (Rizzino et al., Nutr. Rev., 37, 369-378, 1979), it is possible to design a defined medium selectively supplemented with the factors required by a particular cell line. The basic methodology involved: 1) determining which attachment factors permit adequate cell attachment (in most cases this is the major hurdle in the development of a defined medium); and 2) determining which hormones and growth factors are required to promote continuous cell proliferation.

Major Findings:

1. Development of a hormone-supplemented defined medium for JB6 Clone 41. JB6 Clone 41 cells are normally grown in medium containing 8% fetal calf serum. When they are transferred from serum-containing to unsupplemented serum-free medium, the cells rapidly attach to the culture dish. However, during the next 48 hrs nearly all of the cells detach and lyse. This problem was eliminated by coating the culture dishes with the attachment factor fibronectin (FN). Under these conditions the cells exhibit little proliferation and eventually detach unless the required hormones and growth factors are also added. The required supplements were found to be insulin, transferrin (an iron transport protein) and epidermal growth factor (EGF). In a defined medium containing the four factors (to be referred to as 4F), JB6 Clone 41 can be grown and subcultured for numerous generations (>35) without any noticeable changes in cell growth or response to TPA. Although each of the four factors is essential for prolonged growth in defined medium, EGF has the most effect on cell growth. Thus, net cell growth does not occur in the presence of FN, insulin and transferrin, unless EGF is also present. These data demonstrate that EGF is a potent mitogen for JB6 Clone 41 and is sufficient in the presence of FN, insulin, and transferrin to maintain the long-term growth of these cells in the absence of serum.

2. Growth requirements of JB6 Clone 41 cells after exposure to TPA. The availability of the defined medium 4F makes it possible to compare the growth requirements of JB6 Clone 41 before and after TPA treatment. For this purpose 5 TPA-induced colonies that had formed in soft agar were selected. Cells from each colony were initially grown in serum-containing medium to expand their population. Each of these colonies was then tested for growth in various formulations of defined media in order to determine their response to the factors in 4F. In short-term experiments (3 days) cells from 3 of the 5 colonies exhibited little or no growth in 4F. The remaining 2 colonies exhibited a small increase (approximately 80%) in cell number which is much less than that observed with the parent JB6 Clone 41 cells (600%) during the same period of time. In addition, the response to EGF by these 2 colonies is greatly reduced (>80%) with respect to that observed with untreated JB6 Clone 41 cells. In studies over a longer period (9 days), none of the cells from the 5 colonies were able to proliferate in 4F beyond 1 or 2 generations. During the same time period JB6 Clone 41 in 4F typically reaches 6-8 generations and will continue to grow. Thus, these results indicate that cells treated with TPA undergo a major change in their requirements for growth. It should be noted, that as expected, these differences were not apparent in serum-containing culture medium.

Significance to Biomedical Research and the Program of the Institute:

Hormones, growth factors and attachment factors are known to play a central role in the control of cell proliferation. It is likely that alterations in the response to these factors can partially explain the lack of normal controls exhibited by malignant cells. This possibility is supported by the finding that viral transformation can lead to alterations in cellular growth requirements. The project described here focuses on the serum factor requirements of epithelial cells before and after exposure to tumor promoters. The use of defined media makes it possible to carefully examine these requirements and thus brings a new and powerful tool to the study of tumor promotion. In particular it provides a new means of: 1) characterizing the differences between the treated and the parent cells; and 2) studying the mechanisms of tumor promotion. The importance of this work is further enhanced by the use of epithelial cells, which should help increase our understanding of the most prevalent form of human cancers, i.e., carcinomas.

Proposed Course:

This project has been terminated.

Publications:

None

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Control of Cellular Differentiation by Extracellular Signals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Angie 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:

0.5

PROFESSIONAL:

0.4

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Hormone-supplemented defined media able to support the growth and differentiation of several embryonal carcinoma (EC) cell lines are being developed in order to identify and study the extra-cellular signals that induce differentiation. Previous studies have demonstrated that the "nullipotent" EC cell line F9, can be grown in a defined medium (EM-3) containing fibronectin, insulin and transferrin. The F9 cells do not differentiate in EM-3 unless exposed to an inducer such as retinoic acid. Current studies indicate that multipotent EC cells do not proliferate in EM-3. In direct contrast to F9, 3 different multipotent EC cell lines rapidly differentiate when cultured in EM-3 and the differentiated cells that form continue to proliferate for several generations provided EM-3 is supplemented with high density lipoprotein. In each case the entire EC cell population differentiates in the defined medium and one major cell type forms - greater than 85% of the cells exhibit the properties of parietal extraembryonic endoderm. Attempts to understand these results led to the finding that fibronectin and laminin induce multipotent EC cells to differentiate and provide the first evidence that these molecules may directly influence the appearance of extraembryonic endoderm during early mammalian development.

Project Description

Objectives:

Embryonal carcinoma (EC) cells represent an excellent model system for the study of cellular differentiation in general and early mammalian development in particular. EC cell lines are established from germ line tumors known as teratocarcinomas, and can differentiate into a wide variety of embryonic and adult cell types (muscle, nerve, etc.). In many cases, it is possible to direct the differentiation of EC cells to specific cell types by manipulation of their culture conditions. Furthermore, under appropriate culture conditions, certain EC cell lines can differentiate in vitro and mimic, very closely, important stages of early mammalian development. These and other studies demonstrate that the extracellular environment provides important signals able to direct the differentiation of EC cells. In culture, these signals can originate: 1) from the culture medium, in particular from the hormones and attachment factors present in serum; 2) from the cells themselves in the form of secreted factors; and/or 3) by cell-cell contact. The primary objective of this project is to identify the extracellular signals that affect the differentiation of EC cells and to examine their molecular modes of action.

Methods Employed:

Until recently the study of EC cells could only be conducted in serum-containing media (SCM). Due to the complexity and undefined nature of serum, it is very difficult to pinpoint the factors that direct differentiation. Clearly, it is preferable to study EC cell differentiation in completely defined culture media that are selectively supplemented with required factors. The use of defined media not only makes it possible to systematically determine which molecules are involved but also permits a careful examination of their modes of action without interference from extraneous serum factors. The first major step toward the study of EC cell differentiation under defined conditions was the development of a hormone-supplemented defined medium for the long-term 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 SCM, little or no differentiation occurs unless an inducer, such as retinoic acid (a natural metabolite of vitamin A) 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 during the 5th 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 SCM is that F9 will clone in SCM 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 be grown and induced to differentiate in defined media raises two important questions: 1) do all EC cell lines proliferate in EM-3; and 2) do all EC cell lines differentiate when EM-3 is supplemented with inducers (e.g., retinoic acid) that are known to affect EC cell differentiation? Therefore, the first aim of the current study was to examine the behavior of various EC cell lines in the defined medium EM-3.

Major Findings:

1. Growth of multipotent embryonal carcinoma cell lines in defined media. F9 is a type of EC cell line that exhibits only a limited potential to differentiate. Even in the presence of an inducer, the F9 cells appear to only differentiate into extraembryonic endoderm (parietal and visceral endoderm). Therefore, it was important to examine the growth and differentiation of other EC cell lines in EM-3, especially those EC cell lines that are able to differentiate into cells derived from each of the three embryonic germ layers. For this study, two multipotent EC cell lines were selected: OC-15S1 and 1003. In striking contrast to F9 cells in EM-3, the multipotent EC cell lines OC-15S1 and 1003 rapidly and irreversibly differentiate. However, the differentiated cells that form fail to survive and to proliferate after 48 hrs, unless EM-3 is supplemented with HDL. Under these conditions, greater than 90% of the EC cells differentiate within 12 hr (greater than 99% by 24 hr) and none of the EC cells remain undifferentiated after 48 hr. The differentiated cells that form exhibit a uniform morphology throughout the culture, and their morphology is characteristic of parietal endoderm. In addition, at least 85% of the differentiated cells secrete the type of plasminogen activator that is secreted by only one embryonic cell type - parietal endoderm.

2. Fibronectin and other serum factors influence the differentiation of multipotent embryonal carcinoma cells. One of the major questions raised by the above findings is why do these multipotent EC cells differentiate in this defined medium (EM-3 plus HDL) yet exhibit very little differentiation in SCM? There are two major possibilities to consider: 1) one or more of the components of the defined medium induces differentiation; and/or 2) one or more serum factors directly or indirectly supports growth but not differentiation. The evidence indicates that both possibilities are involved. Fibronectin, which is the attachment factor used in EM-3, can induce both OC-15S1 and 1003 to differentiate in SCM. In these experiments, fibronectin will only induce differentiation if it is bound to the surface of the culture dish. However, under these conditions, some of the EC cells continue to proliferate and do not undergo differentiation, indicating that one or more factors in serum partially interferes with the ability of fibronectin to induce differentiation. 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 induce the differentiation of the multipotent EC cells in SCM.

3. The growth of other embryonal carcinoma cell lines in defined media. The contrasting results obtained with F9 EC cells and the multipotent EC cell lines OC-15S1 and 1003, suggested that additional EC cell lines should be examined in defined media. Thus far, two other EC cell lines have been tested, PC-13 and P-19. The EC cell line PC-13, which only differentiates into a few cell types in culture and, like F9, requires an inducer to differentiate, was found to proliferate in EM-3. In contrast, P-19, which is a multipotent EC cell line, rapidly differentiates when transferred to EM-3 plus HDL. Furthermore, fibronectin also induces P-19

to differentiate in SCM. Thus, EC cell lines that require an inducer to differentiate in culture and that only differentiate into a few cell types will apparently only proliferate in EM-3 plus HDL, whereas multipotent EC cell lines will differentiate when transferred to this defined medium.

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 3 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 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. Third, the use of defined media has revealed important differences between a number of EC cell lines. This is particularly important in our attempt to understand the mechanisms that induce the formation of early embryonic cell types (e.g., parietal endoderm). Previous work had led to the argument that retinoic acid is necessary for parietal endoderm formation. However, the results described above demonstrate that, in the case of the multipotent EC cells examined, retinoic acid is not necessary for the formation of parietal endoderm. In contrast, the results of this investigator strongly implicate fibronectin and laminin as inducers of parietal endoderm formation. 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 this 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 Teratomas and Differentiation, Sherman, M. and Solter, D. (Eds.), Academic Press, pp. 3-12, 1975] 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:

Future studies will continue the work discussed above and focus on several major areas. First, identification of factors required for the long-term growth of multipotent EC cells. This is necessary in order to fully understand why the multipotent EC cells differentiate when cultured in defined media. Second, examination of the mechanism(s) by which fibronectin and laminin promote differentiation. The

data thus far suggests that fibronectin and laminin induce differentiation by affecting cell shape and/or cell-cell interactions. Third, since greater than 90% of the multipotent EC cells commit to differentiate within 12 hr after being placed in defined media, attempts will be made to determine if they undergo commitment at a particular point in the cell cycle. Lastly, the findings of this project suggest that fibronectin and laminin affect extraembryonic endoderm formation during early mammalian development. This will be tested directly by culturing mouse embryos at various stages (in particular, during the stage of extraembryonic endoderm formation) in defined media (Rizzino, A. and Sherman, M., *Exp. Cell Res.*, 121, 221-233, 1979) with and without fibronectin and/or laminin.

Publications:

Rizzino, A: The Growth and Differentiation of Embryonal Carcinoma Cells in Defined Media: The Role of Fibronectin. In Sirbasku, D., Sato, G., and Pardee, A. (Eds.): Growth of Cells in Hormonally Defined Media. New York, Cold Spring Harbor Press, in press.

Rizzino, A.: Two multipotent embryonal carcinoma cell lines irreversibly differentiate in defined media. Dev. Biol., in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Transforming Growth Factors in Urine of Patients with Disseminated Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Daniel R. Twardzik	Biochemist	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI
	Stephen A. Sherwin	Clinical Associate	BRMP	NCI
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COOPERATING UNITS (if any)

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Laboratory of Viral Carcinogenesis

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INSTITUTE AND LOCATION

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TOTAL MANYEARS:

0.5

PROFESSIONAL:

0.4

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

High molecular weight transforming growth factor (TGF) activities can be detected in the urine of patients with a variety of disseminated cancers. These acid- and heat-stable polypeptides compete for binding to epidermal growth factor (EGF) membrane receptors and promote the anchorage-independent growth of non-transformed cells. This high molecular weight TGF activity (30,000-35,000 Mr), which coelutes with EGF-competing activity, was found in the urine of 18 of 22 cancer patients with diagnoses including carcinoma of the lung, breast and colon, as well as sarcomas and melanomas. Low, but detectable, amounts of this TGF activity were found in only 5 of 30 nonmalignant controls which included both normal individuals and patients with a variety of inflammatory disorders. This high molecular weight TGF differs from EGF in apparent molecular weight, soft agar growth-stimulating activity and high pressure liquid chromatography elution profile. These findings suggest that urine may provide a convenient source for the biochemical characterization of these TGF-like activities which may be clinically useful biological markers of certain types of cancer.

Project DescriptionObjectives:

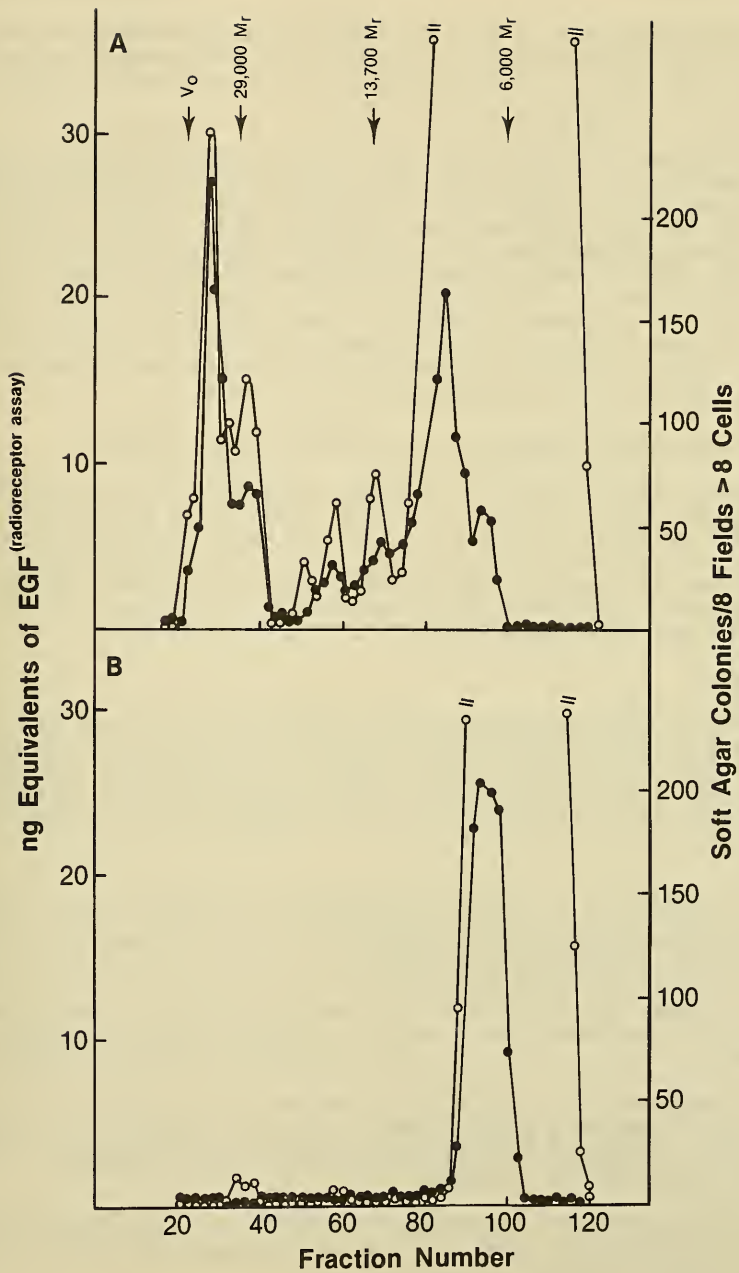
High molecular weight transforming growth factors (TGFs) which compete for epidermal growth factor (EGF) receptors and stimulate anchorage-independent cell growth will be purified from the urine of cancer patients. Their biochemical and biological properties will be compared to human EGF and TGFs released by tumor cells in culture. In addition, a clinical study has been designed to examine the usefulness of the urine-derived 30,000-35,000 M_r TGF as a marker of the transformed phenotype in pediatric cancer patients prior to and after therapy.

Methods Employed:

Urine was collected from previously untreated cancer patients and peptides solubilized utilizing an acid/ethanol extraction procedure. The high molecular weight TGF (30,000-35,000 M_r) was initially resolved from 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 pressure liquid chromatography 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 factors in cancer patient urine. In initial experiments, liter volumes of urine from selected cancer patients and normal control pools were extracted with acidified ethanol and chromatographed on Bio-Gel P-100 columns. Aliquots of each fraction were tested for EGF-competing and soft agar growth-stimulating activity. A typical experiment is presented in Figure 1, in which urine from an 18-year old female with metastatic alveolar cell soft tissue sarcoma and a normal control pool were compared for these two activities. The urine (panel A) from the sarcoma patient, in addition to containing EGF which elutes in the region of the 6,000 M_r insulin marker (fractions 95-105), also contained a peak of EGF-competing activity (fractions 25-35), eluting slightly larger than the 29,000 M_r carbonic anhydrase marker. This 30,000-35,000 M_r EGF-competing activity coeluted with NRK soft agar growth-stimulating activity. All urine samples examined also contained an 8,000 M_r soft agar growth-stimulating activity (fractions 75-85) which is chromatographically separable from EGF. In contrast to the sarcoma patients' urine, urine from a normal control pool (panel B) contained only one major peak of soft agar growth-stimulating activity which eluted with an apparent molecular weight of 8,000 M_r . The high molecular weight TGF activity was not present in urine derived from healthy individuals or mid- to late-term pregnant donors. In addition to differing from EGF in its apparent molecular weight and transforming activity, the 30,000-35,000 M_r TGF activity from this patient also demonstrated a differential elution profile on high pressure liquid chromatographic analysis. The EGF-competing and soft agar growth-stimulating activities coeluted as a major doublet from C_{18} μ Bondapak columns at an acetonitrile concentration of approximately 40%; a minor TGF activity eluting at 33% acetonitrile was also found. Human EGF eluted under similar conditions from these columns at 28% acetonitrile. The 30,000-35,000 M_r TGF from the



urine of cancer patients also exhibited a more basic charge relative to EGF when examined by carboxymethyl cellulose chromatography. EGF does not adsorb to this resin at pH 5.0, whereas the urine-derived TGF activity demonstrated high affinity, thus eluting at a high salt concentration.

In order to determine if the high molecular weight TGF activity observed in the urine of the metastatic sarcoma patient could also be detected in other patients with disseminated cancer a rapid screening assay using smaller urine volumes was designed. Urine specimens were acidified, chromatographed on small Bio-Gel P-30 columns and fractions tested for soft agar growth-stimulating activity. The 30,000-35,000 M_r TGF activity was found in the urine of 18 of 22 cancer patients (S. Sherwin) and in only 5 of 22 nonmalignant control specimens. Cancer patients included diagnoses of carcinoma of lung, breast and colon in addition to several sarcomas and melanomas. Control urine specimens were collected from a population in which half of the individuals had nonmalignant infections and inflammatory conditions including bronchitis, pneumonia, colitis, and diverticulitis.

Significance to Biomedical Research and the Program of the Institute:

The high molecular weight urinary TGF activity may be useful as an indicator of the extent of tumor burden and thus be of value in monitoring patients with cancer and their response to treatment. Unlike the oncofetal antigens alpha fetal protein and carcinoembryonic antigen to which no functional role in neoplasia has been assigned, the high molecular weight urinary TGF promotes anchorage-independent cell growth in soft agar, an *in vitro* property of the transformed phenotype which correlates with tumorigenicity *in vivo*.

Proposed Course:

The high molecular weight transforming growth factor will be purified from urine of patients with pathologically different tumor types and compared as to tumor specificity. These *in vivo* growth factors will then be compared to transforming growth factors produced by tumor cells *in vitro*. Urine from cancer patients will be monitored prior to and after therapy for the 30,000-35,000 M_r TGF activity.

Publications:

Sherwin, S.A., Twardzik, D.R., Bohn, W., Cockley, K., and Todaro, G.J.: Tumor-associated transforming growth factor activity in the urine of patients with disseminated cancer. Cancer Res., in press.

Stromberg, K., Pigott, D.A., Ranchalis, J.E., and Twardzik, D.R.: Human term placenta contains transforming growth factors. Biochem. Biophys. Res. Comm. 106: 354-361, 1982.

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, Vol. 4, in press.

Twardzik, D.R., De Larco, J.E., Marquardt, H., Sherwin, S.A., and Todaro, G.J.: Transforming growth factors: Detection in urine of pregnant and tumor bearing humans. Annals Pediatr. Oncol., in press.

Twardzik, D.R., Ranchalis, J.E., and Todaro, G.J.: Mouse embryos contain transforming growth factors related to those isolated from tumor cells. Cancer Res. 42: 590-593, 1982.

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., in press.

Twardzik, D.R., Todaro, G.J., Marquardt, H., Reynolds, F.H., Jr., and Stephenson, J.R.: Transformation induced by Abelson murine leukemia virus involves production of a polypeptide growth factor. Science 216: 894-897, 1982.

PERIOD COVERED

October 1, 1981 to January 15, 1982

TITLE OF PROJECT (80 characters or less)

Transfer of Gene(s) into Animal Cells Using Retroviruses as Eukaryotic Vectors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Expert

LVC

NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:

1.1

PROFESSIONAL:

0.8

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Retroviruses can be used as true vectors for genes other than genes that lead to oncogenesis. A recombinant retrovirus containing the src gene of Harvey murine sarcoma virus (Ha-MuSV) and the thymidine kinase gene (TK) of Herpes simplex virus type 1 (HSV-1) were constructed and isolated. The new viruses can induce focus formation on NIH 3T3 cells and convert NIH 3T3 (TK-) cells into the TK+ phenotype by carrying into the TK- cells the HSV-1-tk gene. In the TK+ transformants, HSV-1-specific thymidine kinase can be identified by immunoassays. Hybridization analysis indicates that the recombinant virus contains both the Ha-MuSV src sequence and the tk gene sequence in a single RNA species of approximately 4.9 kilobases. The HSV-1 tk gene linked to the deleted Moloney murine leukemia virus (Mo-MuLV) genome in double recombinants. The mouse TK-cells can be transformed into the TK+ phenotype with much higher efficiency by transfection using recombinant DNA containing both the tk and the defective Mo-MuLV than using the tk DNA alone.

Project Description

Objectives:

In order to gain insight into the genomic organization and gene expression of murine RNA tumor viruses in mammalian cells, one approach is to use recombinant DNA technology to obtain DNA copies of the RNA tumor viruses. The availability of such cloned DNA molecules enables one to study the viral genes at the nucleotide level and to manipulate the genomes at strategic regions which are involved in tumorigenesis and leukemogenesis. Experiments include construction of point and deletion mutants at these regions *in vitro*. Similar approaches could be employed to examine other important viral functions, including sequence recognition for integration and gene expression at the transcriptional level. To study the expression of a particular gene in animal cells, one has to transfer the cloned gene back into a homologous or heterologous host cell. One of the approaches to this gene transfer is to introduce the naked DNA into an appropriate cell; another is to use an animal virus as the eukaryotic vector providing that this vector is not lytic or lethal to the host cell. Retroviruses will provide an additional animal virus vector system. The knowledge and techniques acquired from gene transfer research could be applied to correct genetic disorders in animals or humans.

Methods Employed:

Southern's gel blotting technique to identify viral DNAs in a mixture of cellular DNAs; RPC-5 column chromatography to fractionate DNA fragments according to their AT-GC contents and sizes; analytical agarose gel electrophoresis to analyze DNA fragments; preparative agarose gel electrophoresis to separate DNA fragments based on molecular size; molecular cloning system using lambda phage as vector to obtain viral DNA sequences; calcium phosphate-dependent transfection assay to test for transforming and lytic functions of the DNA molecules; and Northern blotting technique to identify viral RNAs in viruses or animal cells.

Major Findings:

1. Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of Herpes simplex virus type I. Recombinants have been constructed which contain both the Harvey murine sarcoma virus (Ha-MuSV) genome and the thymidine kinase gene (TK) of Herpes simplex virus type 1 (HSV-1) linked to each other. The TK gene was placed in a position downstream from the long terminal repeat (LTR) and the src gene of Ha-MuSV. The DNAs from the recombinants were used to transfect NIH 3T3 mouse fibroblasts in order to obtain Ha-MuSV DNA-induced foci of transformed cells. The transformed foci were superinfected with a helper-independent retrovirus, and from the superinfected foci new individual retroviruses were isolated. The new viruses can induce focus formation on NIH 3T3 cells and convert NIH 3T3 (TK) cells into the TK phenotype by carrying the HSV-1 TK gene into the TK cells. Nonproducer foci on NIH 3T3 and TK transformants on NIH 3T3 (TK) cells containing one such new viral genome coding for the dual properties have been isolated from virus-infected cells. The new retroviral sequence in the nonproducer cells can be rescued into virus particles at high titers by superinfection with a helper-independent retrovirus. Hybridization analysis indicates that the recombinant virus contains both the Ha-MuSV src sequence and the TK gene sequence in a

single RNA species of approximately 4.9 kilobases. It is concluded that retroviruses can be used as true vectors for genes other than genes that lead to oncogenesis.

2. Long terminal repeats of Moloney murine leukemia virus may be responsible for increased efficiency of transformation by the thymidine kinase gene of Herpes simplex virus type 1. Double recombinants were constructed containing both the deleted Moloney murine leukemia virus (Mo-MuLV) genome and the thymidine kinase gene of HSV-1. The TK gene was placed in a position upstream from the LTR. One λ recombinant was isolated which contained a Mo-MuLV promoter and a TK promoter in tandem position, another recombinant with two promoters opposed. During DNA transfection experiments, the DNAs isolated from either recombinant have higher efficiency for transforming NIH 3T3 (TK⁻) into the TK⁺ phenotype than transformation by the TK DNA alone. Since the TK gene will not be transcribed by the linked Mo-MuLV promoter without gene rearrangement in vivo, it is suggested that the high efficiency of transformation may be due to more specific and more efficient integration of the transfected DNAs.

Significance to Biomedical Research and the Program of the Institute:

Application of the recombinant DNA technology to RNA tumor viruses would add a new dimension to our understanding of the organization of viral genomes and their biological functions. The methodology developed and biological functions revealed from studies with DNA viruses would be applied to RNA tumor viruses by analyzing their DNA copies from molecular cloning. It is now possible to construct deletion mutants in vitro at defined positions. When these mutants become available, it should be possible to analyze in detail the DNA sequences which are involved in integration, replication and further expression of viral genes. A transmissible retrovirus has been isolated which contains both the *src* gene and the TK gene. The recombinant Ha-MuSV containing an extra genetic marker may be used to study the regulation of expression of either the transforming gene of Ha-MuSV or the TK gene. Since neither gene is essential for viral replication, one can potentially analyze for unusual mutants from one gene while monitoring their existence through the functional activity of the other gene.

Proposed Course:

This project terminated January 15, 1982.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05180-02 LVC
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Evolution and Sequence Organization of Mammalian Retroviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Raoul E. Benveniste	Medical Officer	LVC	NCI
OTHER:	Kurt J. Stromberg	Senior Surgeon	LVC	NCI
	Stephen O'Brien	Geneticist	LVC	NCI
	Tom Bonner	Expert	LVC	NCI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Primate Virus Working Group

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 3.2	PROFESSIONAL: 1.2	OTHER: 2.0
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nucleic acid hybridization studies using cloned retroviral DNA are being used to examine the sites of integration and 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. A wide variety of these tissues have been examined for nucleic acid sequences related to various subclones of the endogenous baboon type C virus; the DNA of all normal and malignant tissues examined contain the same class of related sequences as detected by Southern blot analysis. The 12 kb and 5.5 kb fragments of normal human DNA that hybridize to the cloned baboon type C viral probe are being cloned in lambda vectors. Various Macaca mulatta (rhesus monkey) type C isolates have been compared by restriction enzyme analysis to the previously isolated type C virus from Macaca arctoides (stumptail macaque, MAC-1). Preliminary results indicate that the rhesus isolates can be distinguished from MAC-1. The organization of the two endogenous feline viruses (RD and FeLV) in various backcrossed animals which are offspring of a genetic cross between an F1 hybrid and a viro-gene-negative parent (leopard cat) is also being examined.

Project Description

Objectives:

To study the evolution and organization of endogenous primate and feline retroviruses within the mammalian genome. Recombinant DNA techniques will be employed to develop probes appropriate for the detection of virus-related sequences in human cells and tissues. In addition, the factors involved in the expression of primate viral sequences will be investigated.

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 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. The cloned proviral DNA can then be used as probes for cloning viral sequences integrated in the genomes of mammalian species. The expression of primate retroviral sequences in cells was being 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 rhesus trophoblast tissue. Several isolates have been obtained by cocultivation of rhesus (Macaca mulatta) trophoblast tissue with various heterologous cell lines. Nucleic acid hybridization experiments can be used to detect the degree of sequence homology between various isolates. For example, type C viruses isolated from three species of baboons (Papio cynocephalus, P. hamadryas, P. papio) have been shown to differ in their nucleic acid sequence homology by an extent that results in a 2-4°C reduction in the thermal stability of their nucleic acid hybrids, whereas several independent isolates from one species of baboon have nearly identical thermal stabilities (<0.5°C difference) (Todaro, G.J., Sherr, C.J., and Benveniste, R.E., Virology, 72, 278-282, 1976).

The nucleic acid homology of the new rhesus isolates was compared to that of a previously isolated virus from a stump-tail macaque (MAC-1) and from a rhesus monkey (MMC-1). Cellular DNA was isolated from cell lines infected with the various isolates and hybridized to a ³H-DNA transcript prepared from the MAC-1 isolate. As can be seen from Table 1, the cellular DNA from the various rhesus isolates melt 1.1-2.7°C lower than the homologous hybrid. The lower melting temperatures obtained with the rhesus viral isolates are consistent with base-pair

mismatching of the genome. The data therefore suggest that the M. mulatta isolates can be distinguished readily from the previously described M. arctoides isolate.

Table 1:

Species of origin	Viral isolate ^a	Thermal stability of hybrids ^b	
		T _m ^o C	T _m ^o C
<u>Macaca arctoides</u>	MAC-1	84.4	0.0
<u>Macaca mulatta</u>	MMC-1	82.0	2.4
<u>Macaca mulatta</u>	80R8A	82.4	2.0
	80R15A	81.7	2.7
	80R17	82.3	2.1
	80R5A	83.3	1.1

^aThe MAC-1 virus was grown in a human lung carcinoma cell line (A549), MMC-1 in a canine thymus line (FCf2Th), and the other viruses in a feline line (FEC).
^bH-DNA transcript prepared from MAC-1 virus was hybridized to the cellular DNA of cultures releasing the viruses listed. Hybridization was carried out to a C_t of 10⁴, the actual final extent of hybridization varied from 65-75%. After hybridization, aliquots were heated for 5 min. in 0.75 M NaCl at various temperatures. The amount of hybrid remaining was determined with the single-strand specific nuclease S₁. The T_m is the temperature at which 50% of the hybrids had dissociated. ΔT_m is the difference in T_m between the other DNA-DNA hybrids and the T_m of the homologous hybrid. The T_m^s varied by + 0.5^oC in various experiments.

To further characterize the intra- and interspecies variations among the six macaque isolates, restriction enzyme analyses with EcoRI, BamHI, SACI and HindIII are being performed. Preliminary results suggest that the various M. mulatta isolates can be distinguished from MAC-1, the M. arctoides isolate.

2. Characterization of endogenous primate retrovirus sequences in monkey, ape and human tissues. The three classes of Old World monkey virus isolates include the baboon type C virus, the colobus-macaque group of type C viruses, and the langur type D virus. It has previously been shown that endogenous viral DNA sequences related to these three classes of viruses are present in most primate cellular DNA. The divergence of single copy cellular DNA between the primate species can be used as the framework for the evaluation of the evolution of virus-related sequences. Sequences related to both type C families appear to diverge more rapidly in the Asian primates than in African primates; for example, related sequences can be readily detected by liquid hybridization techniques in the African apes (chimpanzee and gorilla) but not in the Asian apes (orangutan and gibbon) or in human tissues. The type D sequences diverge so rapidly that we cannot detect related sequences in any of the apes or in man. The data suggest that viral nucleic acid sequences related to all three classes of Old World primate viruses are present in gibbon, orangutan and human tissues, but possess too little nucleic acid homology to be detected by these methods.

Recombinant DNA technology coupled with restriction enzyme analysis has provided more selective and specific probes to detect subviral gene sequences which could

be related to Old World monkey viruses. By using various cloned restriction fragments of the baboon type C genome we have detected various related sequences in all ape and human tissues examined. Thus, in higher primates and in man, it appears that viral sequences exist which are never, or perhaps only rarely, expressed as a virus particle. Specifically, a 12 kb and a 5.5 kb fragment of normal human DNA are being cloned in lambda in order to generate more specific probes to characterize these human viral sequences.

3. Detection of retroviral sequences in normal and malignant human tissues. With specific probes generated from cloned subgenomic fragments of the baboon type C virus we can detect multiple, related DNA sequences in all human tissues digested with various restriction enzymes. A wide variety of normal and malignant human tissues, including various sarcomas, leukemias (both myelogenous and lymphoblastic forms), lymphomas and melanomas have been examined. All samples tested yielded the same restriction band pattern on Southern blot analysis. There is, therefore, no major gene rearrangement or amplification of the baboon type C-related sequences in any human tumor tissue cellular DNA tested.

4. Segregation of endogenous type C and feline leukemia viral genes in crosses between domestic cat and leopard cat. The domestic cat *Felis catus* contains two classes of RNA tumor viruses, feline leukemia virus (FeLV) and RD-114 type C viruses. The former class causes lymphoma and myeloproliferative disease while the latter class has not yet been shown to cause disease in cats. Both sets of viruses are found in multiple copies in the cellular DNA and are inherited as stable Mendelian units from one generation to the next. The leopard cat from Southeast Asia, *Felis bengalensis*, does not contain either virus in its DNA. The offspring of matings between the leopard and domestic cats thus afford a unique opportunity to study the segregation of both sets of virogenes in F_1 hybrids and in the progeny of a backcross to the virogene-negative parent. Dr. Willard Centerwall has provided 51 backcrossed animals and 8 F_1 hybrid cats. Blot hybridization data with cloned RD-114 and FeLV viruses are being analyzed to examine the frequency and distribution of both viral sequences in the various backcrossed cats. These results will be correlated with virologic data on the ability of various cell lines established from these animals to restrict the growth of RD-114 and FeLV.

5. Evolutionary relationships among the family Felidae as determined by liquid DNA hybridization. In collaboration with Dr. S. O'Brien, we have completed an analysis of the evolutionary relationships among 25 species of cats as determined by the nucleic acid sequence homology of their unique sequence cellular DNA. ^3H -single copy cellular DNA from 8 feline species maintained in culture and characterized by their karyotype was hybridized to 25 feline species. The data reveal that the Felidae are a family of closely related animals, with the largest difference in thermal stability being approximately 2.5-3.0°C. The details of the evolutionary tree derived by this method are being compared to various published classification schemes based on anatomical features and habitat.

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.

Proposed Course:

The detection of type C viral sequences in human cellular DNA derived from normal and malignant tissues that are related to endogenous primate viruses suggests that humans also contain these retroviruses. We will attempt to clone these human viral sequences in order to devise a restriction map of the endogenous human virus and to use these probes to study the role of this class of viruses in neoplasia.

Hybrid cats that are offspring of matings between leopard cats and domestic cats containing a variable number of virogene copies will allow the study of the effects of gene dose on susceptibility and resistance to diseases mediated by this group of viruses. The knowledge gained from these tumor virus genome genetic transmission studies might also help us to better understand human neoplasia.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05202-02 LVC
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Structure, Function, and Utilization of Eukaryotic Promoter Sequences

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	George E. Mark	Expert	LVC	NCI
OTHER:	Tom I. Bonner	Expert	LVC	NCI
	Ulf R. Rapp	Visiting Scientist	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: 1.3	PROFESSIONAL: 1.2	OTHER: 0.1
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CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
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 provides a unique probe to dissect 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; (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 show 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.

Project Description

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 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.T 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: (1) to 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 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 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 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 C₁t 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 promoter 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 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 fractionality will subsequently be determined at the nucleotide sequence level.

2. Structure and evolution of endogenous retrovirus. 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 is in doubt. The difficulty of CPC-1 isolation suggested that its repression was the result of multiple factors. This is supported by the following observations: (1) both cloned endogenous proviral promoters are transcriptionally inactive; and (2) recent 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 inconsistent with the high proviral copy number

being the result of super-infections (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. Steven Oroszlan as its amino acid sequence. Comparison of the genes at the extremities of a retrovirus of fowl (REV), colobus and baboon families of retroviruses shows 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.

3. Retrovirus LTRs hold a significant promise of being 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 stimulate 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 undoubtedly 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., in press.

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.

Lovinger, G.G., Mark, G.E., Todaro, G.J., and Schochetman, G.: 5' terminal nucleotide noncoding sequences of retroviruses: Relatedness of two Old World primate type C viruses and avian spleen necrosis virus. J. Virol. 39: 238-245, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05211-02 LVC
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Studies on the Etiology and Control of Human Breast Cancer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul H. Levine	Medical Director	LVC	NCI
OTHER:	Jose Costa	Pathologist	LP	NCI
	Larry R. Muenz	Mathematical Statistician	BB	NCI
	Kamaraju Sreemahalakshmi	Visiting Fellow	LVC	NCI

COOPERATING UNITS (if any)
N. Murali, Inst. Salah Azaiz, Tunis, Tunisia; J.G. Bekesi, Mt. Sinai Hosp., New York, NY; S. Spiegelman, Columbia Univ., New York, NY

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Clinical Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.6	OTHER: 0.1
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(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is a multidisciplinary study of breast cancer in which epidemiologic, virologic, immunologic, and pathologic techniques are applied to the comparison of breast cancer in the United States and Tunisia. Pathologic studies supported the concept of rapidly progressing breast cancer (RPBC) or pousee evolutive (PEV) as a separate biologic entity with the demonstration that even PEV-1, which has no clinical evidence of inflammation but which is based primarily on the patient's subjective report of rapid tumor growth, has a significantly higher frequency of nuclear grade 3 than the non-PEV (PEV-0) cases. The similarity between the more advanced RPBC cases and the so-called inflammatory breast carcinoma in the United States was demonstrated by the frequent finding of lymphatic permeation involving the skin. Hormone receptor studies demonstrated lower levels in PEV cases vs. PEV-0 and American cases, again supporting the designation of PEV-1 as a biologic entity. A correlation between estrogen receptor levels and a favorable response to treatment was observed.

Project Description

Objectives:

To investigate the pathogenesis and treatment of rapidly progressing breast carcinoma (RPBC) in Tunisia as a model for aggressive breast cancer in the United States.

Methods Employed:

More than 100 Tunisian breast tumors from patients seen at the Institute Salah Azaiz (ISA) were studied by pathologic, biochemical and immunologic techniques to determine differences between RPBC and non-RPBC cases. Immunologic tests evaluated differences between Americans and Tunisians with and without breast cancer. Clinical and laboratory information on 112 patients entered into a chemotherapy study utilizing cyclophosphamide, methotrexate, and 5-fluorouracil were also analyzed. Hormone receptor assays were performed on 94 biopsies from Tunisian breast cancer patients and the results were compared with a series of biopsies from American women with breast cancer.

Major Findings:

1. Characterization of rapidly progressing breast cancer in Tunisia. Pathologic studies supported the concept of rapidly progressing breast cancer (RPBC) or pouseev evolutive (PEV) as a separate biologic entity with the demonstration that even PEV-1, which has no clinical evidence of inflammation but which is based primarily on the patient's subjective report of rapid tumor growth, has a significantly higher frequency of nuclear grade 3 than the non-PEV (PEV-0) cases. The similarity between the more advanced RPBC cases and the so-called inflammatory breast carcinoma in the United States was demonstrated by the frequent finding of lymphatic permeation involving the skin. Hormone receptor studies demonstrated lower levels in PEV cases vs. PEV-0 and American cases, again supporting the designation of PEV-1 as a biologic entity. A correlation between estrogen receptor levels and a favorable response to treatment was observed.

Significance to Biomedical Research and the Program of the Institute:

The development of a strong collaborative effort between the National Cancer Institute and the Institute Salah Azaiz in Tunisia is of great value in that it provides access to a group of patients with rapidly progressing breast cancer. Information gained as to the etiology and means of controlling this form of breast cancer in Tunisia can be expected to help in the control of fulminating breast cancer in the United States. The high frequency of RPBC in Tunisia allows information on this entity to be accumulated more rapidly than in the United States. Clinical and pathological data thus far indicate that RPBC in Tunisia is no different than fulminating breast cancer in the U.S. and, therefore, information obtained from the Tunisian patients would be directly applicable to breast cancer patients in the United States. The finding of the high content of antigen-reacting with murine mammary tumor virus (MMTV) indicates that human material will be available that will accelerate studies on the involvement of viruses in the cause of breast cancer. The chemotherapy studies have already been of value to breast cancer patients in the United States and the results of these studies

are encouraging American chemotherapists to treat patients with rapidly progressing breast cancer who in the past had not been treated with chemotherapy. The immunologic studies demonstrate the integrity of the immune system in patients with RPBC, providing guidelines to management of such patients.

Proposed Course:

This project will be completed this year with the final report on the following: (1) determination of the specificity of an antigen detected in Tunisian breast cancer biopsies; and (2) analysis of the long-term survival of Tunisian breast cancer patients with RPBC.

Publications:

Costa, J., Webber, B.L., Levine, P.H., Muenz, L., O'Connor, G.T., Tabbane, F., Belhassen, S., Kamaraju, L.S., and Murali, N.: Histopathological features of rapidly progressing breast cancer in Tunisia. Int. J. Cancer, in press.

Levine, P.H., Murali, N., Tabbane, F., Loon, J., Terasaki, P., Tsang, P., and Bekesi, J.G.: Studies on the role of cellular immunity and genetics in the etiology of rapidly progressing breast cancer in Tunisia. Int. J. Cancer 27: 611-615, 1981.

Murali, N., Tabbane, F., Muenz, L.R., Bahi, J., Belhassen, S., Kamaraju, L.S., and Levine, P.H.: Preliminary results of primary systemic chemotherapy in association with surgery or radiotherapy in rapidly progressing breast cancer. Br. J. Cancer 45: 367-374, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05223-02 LVC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Role of Mitogenesis in Tumor Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Nancy H. Colburn Expert LVC NCI

COOPERATING UNITS (if any)
R. Tucker, Johns Hopkins University Medical School, Baltimore, MD

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 0.9	PROFESSIONAL: 0.1	OTHER: 0.8
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CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
One hypothesis for tumor promotion proposes that promoters function to stimulate proliferation of initiated cell populations which produce either: (1) clonal selection of progressing cells; or (2) increased probability of gene rearrangements or fixation of DNA damage. Recent work in our laboratory suggests that the promoting activity of phorbol esters in JB6 mouse epidermal cells is not due to a release from the quiescence type of mitogenic stimulation by the tumor promoter. This conclusion derives from two lines of evidence. First, when JB6 cells are exposed to a promoter under conditions in which they cannot undergo promoter-dependent mitogenesis, promotion of tumor cell phenotype is not inhibited. Second, variants of promotable JB6 cells which have been selected for resistance to the mitogenic activity of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) have been found to retain promotability, thus ruling out mitogenic stimulation as a required event in promotion of transformation in JB6 cells. These 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 EGF (or other growth factor) binding to EGF receptors and stimulated hexose transport.

Project Description

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 ^3H -2-deoxyglucose uptake.

Major Findings:

- Promotion of transformation without promoter-induced mitogenesis. Promotion of anchorage-independent growth of JB6 cells occurs when cells are exposed to TPA only during the logarithmic growth phase. Since TPA-dependent mitogenesis in JB6 cells (and a variety of other cells) only occurs at plateau density as a release from quiescence, TPA mitogenesis appears not to be a requirement for promotion of anchorage-independent growth of JB6 cells by TPA.
- Selection of mitogen-resistant JB6 cell lines. A promotable clone of JB6 cells has been subjected to selection for resistance to TPA mitogenesis by exposure to TPA plus colchicine at plateau density (procedure of Pruss and Herschman, Proc. Natl. Acad. Sci. USA, 74, 3918, 1977). Under these conditions the sensitive cells are trapped in mitosis, washed off, and the resistant cells remaining are then collected. After cloning, a number of cell lines which are completely resistant to TPA mitogenesis (at 1-100 ng/ml) have been obtained, including some which are transformed (anchorage-independent) and some which are not (anchorage-dependent).
- TPA mitogen-resistant variants are promotable. The question was next asked whether selected mitogen-resistant variants had been consistently coselected for nonpromotability, a result to be expected if TPA mitogenesis were required. It turns out that several TPA mitogen-resistant cell lines have been isolated which are promotable to anchorage-independent growth by TPA. This clearly rules out the TPA-dependent release from the quiescence type of mitogenesis, which occurs at plateau density and presumably in various other growth-inhibited states, as a requirement for promotion of transformation in JB6 cells. Thus, while the mitogenic activity of tumor promoters may be required for early stages of promotion, it does not appear to be required for later stages, at least in vitro in JB6 cells.

As indicated above, the selection for TPA mitogen-resistant cells yielded some anchorage-independent transformants. This result constitutes independent evidence dissociating mitogenesis from promotion of transformation by TPA since the transformants had to arise as a result of TPA exposure without cell division.

4. TPA mitogen-resistant variants show low to zero levels of 125 I-EGF binding.
 All of the TPA mitogenesis-resistant variants have shown low to zero levels of 125 I-EGF binding (See Project No. Z01 CP 05226-02 LVC), while all of the mitogenesis-sensitive JB6 lines show moderate to high levels of 125 I-EGF binding. This leads us to postulate that the binding of EGF or another ligand to EGF receptors may mediate the mitogenic activity of TPA.

5. Hexose transport may be involved in mediating the mitogenic response to TPA
 The TPA mitogenesis-resistant variants of JB6 show high basal hexose uptake and no stimulation of uptake in response to TPA, whereas their sensitive counterparts 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. This promoter-stimulated hexose transport may be a required event in mitogenesis by TPA.

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 mitogen-resistant clones are promotable. We can now deal separately with the question of which responses to TPA are on the mitogenesis pathway and which are on the promotion pathway.

Proposed Course:

Further use will be made of the TPA-resistant cells of various phenotypes to determine the basis for resistance. In particular, TPA mitogen-resistant cells of both promotable and nonpromotable phenotypes will be compared with the TPA mitogen-sensitive counterparts to ascertain further the biochemical and cellular basis for mitogen-resistance. The observation that the TPA mitogen-resistant variants assayed so far show from low to zero levels of available EGF receptors suggest the possibility that EGF is an obligatory mediator of TPA mitogenesis. This suggestion will be followed up by carrying out two experimental approaches. The first will repeat the selection for TPA mitogen resistance using a new JB6 parent clonal line to achieve an independent selection and thus avoid the problem of possible reisolation of the same variant. For this selection a phorbol ester derivative that is a strong mitogen but weak promoter will be used in order to minimize the production of transformants. If the independently selected TPA mitogen-resistant variants again turn out to lack EGF receptors, this makes more probable the role of EGF or EGF receptors as mediators of TPA mitogenesis. This second approach will involve 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). If both EGF binding and TPA mitogenic response are restored, this would constitute independent evidence in support of a role for EGF receptors in TPA mitogenic response. If hexose uptake response is also restored, this would be consistent with its role in TPA mitogenesis as well as a role for EGF receptors in the hexose uptake response.

Publications:

Colburn, N.H., Dion, L.D., and Wendel, E.J.: The Role of Mitogenic Stimulation and Specific Glycoprotein Changes in the Mechanism of Late-Stage Promotion in JB6 Epidermal Cell lines. In Hecker, E. (Ed.): Cocarcinogenesis and Biological Effects of Tumor Promoters. New York, Raven Press, 1982, pp. 231-235.

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., in press.

Colburn, N.H., Wendel, E., and Abruzzo, G.: Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phorbol esters: Mitogen resistant variants are sensitive to promotion. Proc. Natl. Acad. Sci. USA 78: 6912-6916, 1981.

Colburn, N.H., Wendel, E., and Srinivas, L.: Responses of preneoplastic epidermal cells to tumor promoters and growth factors: Use of promoter resistant variants for mechanism studies. J. Cell Biochem. 18: 261-270, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05224-02 LVC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Role of Cellular Glycoprotein Shifts in Promotion of Transformation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Louis D. Dion	Staff Fellow	LVC	NCI
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LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.6	PROFESSIONAL: 0.85	OTHER: 0.75
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CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Since tumor promoters are nonmutagenic and active on the cell membrane, a hypothesis proposes that the biological response is mediated by specific promoter-induced changes in cell surface glycoconjugate targets. Recent investigations have led to the discovery of three promoter-sensitive glycoproteins in JB6 mouse epidermal cells, gp150, gp180, and gp220, having molecular weights of 150,000, 180,000 and 220,000, respectively. The gp180 and gp150 have been identified as the pro-alpha-1 and pro-alpha-2 subunits of procollagen, respectively. Both phorbol and non-phorbol promoters such as epidermal growth factor and mezerein are active in decreasing these three glycoproteins. 12-O-tetradecanoylphorbol-13-acetate (TPA) exposure switches off procollagen synthesis pretranslationally (probably transcriptionally) as indicated by a lack of translatable collagen mRNA. Antipromoting concentrations of retinoic acid prevent decreases in procollagen levels, but the retinoid action appears to be post-translational. We propose that the transcriptional switch affecting collagen synthesis indicates a required event in promotion coordinately regulated with procollagen.

Project Description

Objectives:

To identify tumor promoter-sensitive cellular glycoproteins that might be implicated in the mechanism of tumor promotion. To determine whether they are also sensitive to antipromoting retinoids. To study the mechanism of action by which 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibits collagen synthesis in JB6-derived cells. Specific questions to be addressed: (1) What is the mechanism by which procollagen is decreased by tumor promoters; (2) Do retinoids inhibit promotion by antagonizing the TPA-dependent inhibition of collagen synthesis; and (3) Are other extracellular matrix components altered in a manner parallel with the procollagen decrease?

Methods Employed:

Collagen production is determined by metabolic labeling of the cells with ^3H -proline and SDS-PAGE analysis of the cell-associated proteins. Collagen synthesis is expressed as the percent optical density in the 180K and 150K collagenase-sensitive bands relative to total optical density on the gel. Collagen synthesis is also measured by labeling the cells with ^{14}C -proline and determining the percent of solubilized counts after exhaustive collagenase digestion.

In vitro translation of mRNA was performed by isolating total cellular RNA using 8 M guanidine hydrochloride and translating the isolated messenger with a rabbit reticulocyte lysate. Translated products were analyzed using SDS-PAGE.

Major Findings:

1. TPA inhibition of procollagen synthesis. The predominant effect of TPA on glycoprotein synthesis is the decrease in a 180,000 molecular weight glycoprotein which has been identified as pro-alpha-1 procollagen. Also inhibited are the 150,000 M.W. pro-alpha-2 protein and a 220,000 M.W. glycoprotein which is fibronectin. Initial studies utilized incorporation of ^3H -mannose into cellular glycoproteins to demonstrate these effects. The glycoprotein decreases occurred in parallel with morphological and cell adhesion changes (less flat and less adhesive) thus suggesting cell surface localization. The TPA-dependent inhibition of collagen synthesis is most easily demonstrated when ^3H -proline is used to label newly synthesized proteins. The half-maximal concentration of TPA needed to produce this effect was between 0.1 and 1.0 ng/ml which is consistent with the half-maximal dose for promotion of anchorage-independent cell growth in agar. The identification of the 180K ^3H -mannose-labeled glycoprotein as procollagen was demonstrated by its collagenase sensitivity. The inhibition of procollagen by TPA shows that the phorbol analog structure/activity response correlates with tumor-promoting activity in vivo. Mezerein and EGF, but not the calcium ionophore A23187, also inhibit procollagen synthesis in JB6 cells. This demonstrates that the procollagen response is not limited to phorbol promoters alone but occurs also with other complete and late-stage promoters. The data also suggest that the collagen response may not be involved in first-stage promotion since A23187 is a first-stage promoter.

2. The antipromoting retinoids enhance the amount of cell-associated procollagen. When retinoic acid (RA) is administered in conjunction with TPA, there is an antagonism of the TPA-dependent reduction in cell-associated procollagen as measured by long term (8-hour) labeling periods with ^3H -mannose or ^3H -proline. The time course of the RA effect is slower than that of the TPA. The effect of TPA on procollagen levels can be detected within several hours and is maximal by 24. The RA effect on procollagen is seen by 24 hours but is further enhanced by 72 hours of treatment.

3. TPA acts by regulating the amount of translatable collagen mRNA. Experiments in collaboration with Dr. Mark Sobel have shown that TPA acts to reduce the available collagen mRNA. In vitro translation of isolated messenger RNA confirms that by 24 hours essentially no new collagen is being synthesized. This indicates either a "switched off" collagen mRNA transcription, collagen mRNA degradation or a failure of collagen in RNA processing. Pulse labeling with ^3H -proline after TPA exposure showed that the reduced rate of collagen translation and, therefore, the onset of this pretranslational switch, occurred as soon as 2 hours. Using short (25 minute) pulse labeling, in which high specific activity ^3H -proline was used to measure translation rates, TPA produced a consistent inhibition of procollagen synthesis at 24, 48 and 72 hours. RA produced significant antagonism (enhanced collagen translation) only at 72 hours. Thus, TPA acts rapidly at a pretranslational level to inhibit procollagen synthesis while RA enhances procollagen translation rates only at later time points.

4. Retinoic acid acts initially to enhance the cell pools of presecretory molecules. The observation that retinoic acid enhances cell-associated procollagen levels seems to be determined by two experimental parameters: 1) The longer the duration of metabolic labeling, the sooner the RA effect can be demonstrated and the larger the effect appears. An 8-hour labeling period shows greater effect than a 20-minute labeling period. 2) The greater the elapsed time after initiating RA treatment, the larger the RA effect will be. RA treatment for 72 hours shows a greater effect than exposure for 24 hours. Taken together, these results indicate that the initial effect of retinoic acid is to enlarge the intracellular pools of presecretory molecules perhaps by enhancing the number of golgi bodies or secretory vesicles.

5. The procollagen loss is not sufficient for promotion. An extensive survey of JB6-derived cloned cell lines which differ with respect to sensitivity to promotion of transformation by TPA indicate that procollagen synthesis alone does not lead to promotion since the nonpromotable C1 25 and C1 30 show similar levels of procollagen loss. Whether the procollagen loss is necessary for promotion in JB6 cells is not clear since, to date, no cell lines have been obtained that are resistant to the collagen decrease by TPA.

Significance to Biomedical Research and the Program of the Institute:

The biochemical mechanism by which a nonmutagenic promoter irreversibly induces the transformed phenotype in preneoplastic cells is one of the important unanswered questions in carcinogenesis. Evidence such as we have developed for TPA-mediated regulatory events at the level of transcription of a major structural gene product like collagen should provide a new tool for studying the TPA mechanism(s) of action.

Proposed Course:

To characterize the major secreted macromolecules of JB6 cells and the effects of TPA and RA on their secretion; to determine whether colonies in agar have produced an extracellular matrix around themselves composed in part of collagen fibers and if this is the case, to determine whether there are distinctive differences between the promotable and nonpromotable clones; to investigate the mechanism whereby promoters inhibit procollagen transcription; and to investigate the target(s) of the antipromoting action of retinoids, be they secretional or transcriptional.

Publications:

Colburn, N.H., Dion, L.D., and Wendel, E.J.: The Role of Mitogenic Stimulation and Specific Glycoprotein Changes in the Mechanism of Late-stage Promotion in JB6 Epidermal Cell Lines. In Hecker, E. (Ed.): Cocarcinogenesis and Biological Effects of Tumor Promoters. New York, Raven Press, 1982, Vol. 7, pp. 231-235.

Dion, L.D., Bear, J., Bateman, J., DeLuca, L., and Colburn, N.H.: Tumor promoting phorbol ester inhibits procollagen synthesis in promotable JB6 mouse epidermal cells, J. Natl. Cancer Inst., in press

Dion, L.D., DeLuca, L., and Colburn, N.H.: Phorbol ester-induced anchorage independence and its antagonism by retinoic acid correlates with altered expression of specific glycoproteins. Carcinogenesis 2: 951-958, 1981.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Gangliosides and Other Membrane Lipids: Possible Regulatory Role During Promotion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leela Srinivas	Visiting Fellow	LVC	NCI
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Cell Biology Section

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TOTAL MANYEARS:

1.36

PROFESSIONAL:

1.1

OTHER:

0.125

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 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role in promotion of transformation of cell surface glycolipids, particularly gangliosides is being studied. Phorbol esters produce specific ganglioside changes under conditions in which they regulate differentiation; binding to specific surface gangliosides is required for the function of certain hormones and toxins which regulate growth and differentiation. Tumor-promoting but, not nonpromoting, phorbol esters produce specific changes in ganglioside synthesis, principally a 10-fold decrease in the synthesis of a trisialoganglioside (GT). This change is blocked by antipromoting concentrations of retinoic acid. Variants of JB6 mouse epidermal cells, which are promotable to tumor cell phenotype by phorbol esters, show the GT decrease while their nonpromotable counterparts do not, suggesting that a ganglioside shift may play a causal role in promotion of transformation. This suggestion has been further substantiated by the observation that reconstitution of JB6 cells with GT, but not with other sialic acid-containing glycoconjugates, blocks promotion of transformation by 12-O-tetradecanoylphorbol-13-acetate (TPA). Current efforts are directed to selecting JB6 cells for resistance to the GT response to TPA and asking whether they have been coselected for resistance to promotion of transformation.

Project Description

Objectives:

To demonstrate whether specific changes in cell membrane gangliosides and other lipids are involved in the process of promotion of transformation of JB6 mouse epidermal cells. Specifically, to study the ganglioside profile of promotable JB6 cells on exposure to phorbol esters and other tumor promoters; to study the correlation between activity for producing these changes and tumor-promoting activity; to determine whether a target for the antipromoting action of retinoids is at the level of ganglioside synthesis; to study the mechanisms whereby phorbol esters and retinoids regulate ganglioside biosynthesis; to ascertain whether specific ganglioside changes may play a causal role in promotion of transformation by studying: (1) the correlation of ganglioside response with promotability of variant cell lines obtained by independent methods of cloning; and (2) the possible inhibitory effects of certain gangliosides on phorbol ester promotion of transformation.

Methods Employed:

$1-^{14}\text{C}$ -glucosamine-labeled, newly synthesized gangliosides were extracted with chloroform:methanol, followed by water partitioning separation by thin layer chromatography and quantitated. Trisialoganglioside (G_T) in liposome form was inserted into the membranes of cells that were simultaneously treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) in experiments designed to test inhibitory activity. Since tetanus toxin (or toxoid) binds specifically to G_T , experiments are underway to sort cells on the basis of their G_T content by utilizing the binding of fluoresceinated tetanus toxoid and sorting on the FACS IV cell sorter for cells having high or low fluorescence.

Major Findings:

1. Tumor-promoting but not nonpromoting phorbol esters produce specific changes in ganglioside biosynthesis. The *de novo* biosynthesis of gangliosides was studied by labeling the cells with D-[$1-^{14}\text{C}$]-glucosamine for the terminal 4-hour period during a 24-hour TPA-treatment. It was observed that TPA produces a 10- to 20-fold decrease in the biosynthesis of a trisialoganglioside (G_T). This was accompanied by a two-fold increase in an unknown ganglioside and a two-fold increase in G_{D1b} . These three changes are observed at 6-48 hours of TPA exposure at concentrations of TPA reported by us to induce promotion of transformation as monitored by acquisition of anchorage-independent growth of JB6 cells. Activity for eliciting these ganglioside changes correlated with promoting activity for a series of phorbol esters. Simultaneous exposure to the antipromoter retinoic acid completely antagonized the changes brought about by TPA, suggesting that these ganglioside switches may be required in promotion of transformation.

2. Promotion-resistant variants lack the G_T response to TPA. We next asked whether clonal variants of JB6 which are promotion-sensitive could be distinguished from their promotion-resistant counterparts on the basis of ganglioside responses to TPA. TPA consistently produces a 10-20-fold G_T decrease in the promotable but not the nonpromotable variants of JB6. This is the first biochemical

response to TPA we have discovered which distinguishes promotion-sensitive from promotion-resistant JB6 cells. A G_{α} increase does not occur consistently in promotable cells and a G_{D1b} decrease occurs only in nonpromotable cells.

In order to further test the proposition that the G_T decrease may be a required event in promotion of anchorage-independent cell growth, promotable cells were reconstituted with G_T at 10^{-10} to 10^{-8} M and exposed to TPA (1.8×10^{-8} M) simultaneously. It was observed that G_T , but no other ganglioside or sialoglycoconjugate tested, blocked promotion of transformation by TPA.

3. GT reconstitution blocks promotion of transformation by TPA. To determine whether G_T inhibits at the level of expression of transformation as opposed to induction of transformation, the effect of G_T on soft agar colony formation by tumor cell lines was assayed. G_T at 10^{-8} M did not block colony formation by transformed cells, showing thereby that G_T inhibition of promotion of transformation is at the level of the induction of the anchorage-independent phenotype by TPA and not at the level of expression of transformed phenotype. To understand the mechanism by which G_T inhibits TPA action it was considered that G_T could inhibit TPA binding to its specific receptor. It was found that G_T does not inhibit binding or down modulation of TPA.

4. The mechanism of TPA-induced GT loss may involve oxidation. The possibility was tested that oxidation of sialic acid in cell surface glycolipids or glycoproteins by reactive oxygen produced by TPA would lead to transformation. Hence, a mild periodate oxidation of terminal sialic acids in surface glycoconjugates of JB6 mouse epidermal cells was carried out, with the result that anchorage-independent transformation was produced. Nonpromotable JB6 cells did not respond to periodate oxidation with induction of colonies. This may implicate oxidation of sialic acids, possibly G_T sialic acid, as a significant event in the promotion pathway.

5. The mechanism of GT loss may involve a neuraminidase activity. The mechanism by which lowered synthesis of G_T occurs could be due to reduced synthesis or increased breakdown of this particular ganglioside. Preliminary evidence has been obtained which shows that JB6 cells contain a neuraminidase with a substrate preference for G_T . Neuraminidase, a readily inducible enzyme, could be regulating the ganglioside concentrations in JB6 cells which in turn could be activated or induced by TPA.

6. Selection for resistance to the GT response to TPA coselects for promotion resistance. Another major approach towards understanding the importance of G_T involvement in the promotion process was to obtain cells deficient in the G_T response to TPA. Tetanus toxoid binds specifically to G_T . Fluoresceinated tetanus toxoid was allowed to bind to TPA-treated cells and the cells were sorted according to their fluorescent intensity. Cells which were resistant to TPA for the G_T response showed high fluorescence because they have more G_T and the sensitive ones showed low fluorescence as they have lost a major portion of their G_T . Isolated resistant and sensitive cell populations have been shown to be stable for their G_T response or lack of it.

Significance to Biomedical Research and the Program of the Institute:

If G_T is actually involved in preneoplastic progression and is proved to be effective in inhibiting tumor promotion in in vivo experiments it may be effective in cancer prophylaxis.

Proposed Course:

Variants that are resistant or sensitive to TPA-induced G_T loss will be obtained based on their ability to bind fluoresceinated tetanus toxoid. Studies on the mechanism of G_T loss will be continued. Biochemical events consequent to the G_T drop will be investigated. Attempts will be made to find a membrane or transmembrane protein that binds specifically to G_T which may participate in signal transduction. The ganglioside profile of nonpromotable cells transfected with promotable cellular DNA and showing promotable phenotype will be investigated to independently associate or dissociate the involvement of G_T in the promotion of transformation by TPA.

Publications:

Colburn, N.H., Wendel, E.J., and Srinivas, L.: Responses of preneoplastic epidermal cells to tumor promoters and growth factors: Use of promoter resistant variants for mechanism studies. J. Cell. Biochem. 18: 261-270, 1982.

Srinivas, L., and Colburn, N.H.: Tumor promoter induced ganglioside changes in promotable mouse epidermal cells: Antagonism by an antipromoter. J. Natl. Cancer Inst. 68: 469-473, 1982.

Srinivas, L., Gindhart, T.D., and Colburn, N.H.: Tumor promoter resistant cells lack trisialoganglioside response. Proc. Natl. Acad. Sci. USA, in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cell Surface Receptors and Signal Transduction in Responses to Tumor Promoters

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nancy H. Colburn	Expert	LVC	NCI
OTHER:	Thomas Gindhart	Expert	LEP	NCI

COOPERATING UNITS (if any)

None

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Laboratory of Viral Carcinogenesis

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INSTITUTE AND LOCATION

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TOTAL MANYEARS:

1.1

PROFESSIONAL:

0.3

OTHER:

0.8

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Phorbol diester binding sites appear stable biologically, showing no significant variation 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. 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. A partially purified preparation of transforming growth factor (TGF) from a human tumor line can promote anchorage-independent growth of a TPA-promotable mouse epidermal cell line lacking EGF receptors and responsiveness to EGF. I-125-labeled TGF shows specific binding to these EGF receptorless cells, suggesting that this TGF exerts its biological activity through receptors that are distinct from EGF receptors. The roles of membrane lipid methylation, reactive oxygen production and monovalent cation transport have been probed as possible mediators of signal transduction following receptor binding, and each has been found to be promotion relevant.

Project Description

Objectives:

To determine the role of phorbol diester (PD) receptor number, binding affinity and down modulation in promotion of transformation in JB6 mouse epidermal cell lines by 12-O-tetradecanoylphorbol-13-acetate (TPA); the relationship between phorbol diester receptors and receptors for growth factors; the relationship between phorbol diester receptors and transformation; the role of EGF receptors in TPA-induced mitogenesis and promotion; and the binding and promoting activity of transforming growth factors (TGFs) in preneoplastic JB6 cells. A second major question to be addressed is - what is the physiologic system most immediately perturbed by PDs? 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) phospholipid methyltransferases (3-deazadenosine and S-adenosyl-homocysteine); (2) reactive oxygen (catalase) (H_2O_2 has previously been shown to be a promotor in this system in our laboratory); and (3) sodium ion transport (amiloride).

Methods Employed:

Selection for mitogen resistance using colchicine plus TPA at plateau density. Assay of anchorage-independent cell 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 3H -2-deoxyglucose uptake.

Major Findings:

1. Phorbol diester resistance is not determined at the level of PD receptor number or affinity but may be determined at the level of PD-induced receptor down modulation. Mouse epidermal cell lines differing from each other in responsiveness to TPA with respect to mitogenic response in monolayer culture, promotability in soft agar and transformation were found to possess approximately the same number of PD binding sites of similar affinity. Both sensitive and resistant variants showed down modulation of 3H -PDBu binding following incubation with PDBu. However, when ligand-induced down modulation was analyzed separately from density-dependent down modulation (see below) it turned out that the nonpromotable cells were specifically lacking ligand-induced down modulation. This suggests that phorbol diester-induced down modulation may be involved in transducing the signal for promotion of transformation. Variant lines lacking receptors for epidermal growth factor (EGF) did not differ from cell lines possessing EGF receptors with respect to any of the above parameters of PD binding.

2. EGF may mediate the mitogenic but not the promotion response to TPA. All of the TPA mitogenesis-resistant variants obtained by colchicine plus TPA selection showed little or no binding of ^{125}I -EGF, indicating that EGF or EGF receptors may mediate the mitogenic effect of TPA on JB6 mouse epidermal cell derivatives.

Several of these EGF receptorless variants underwent promotion of anchorage-independent growth in response to TPA, thus indicating that EGF receptors are not required for promotion by TPA.

3. A TGF can bind and function as a promoter independently of EGF receptors. JB6 variants show cross-sensitivity and cross-resistance to the promoting activity of TPA and TGF derived from a human rhabdomyosarcoma. One line lacking EGF receptors which undergoes promotion in response to TPA is promotable by TGF but not EGF suggesting that TGF and other growth factors may act through receptors different from the EGF receptor. Recent studies have demonstrated specific TGF binding sites on JB6 cells lacking EGF receptors in support of this alternative mechanism.

4. Cell density regulates phorbol diester binding sites. A physiologic means of regulating phorbol diester number has been found which offers a novel approach to identifying this binding site and the physiologic system which it regulates. All of the JB6 epidermal cell derivatives show approximately a four-fold decrease in number of binding sites when they pass from the log phase of growth to the plateau phase. In contrast, the number of EGF receptors increases significantly at plateau density, indicating divergent mechanisms regulating these two different receptors.

5. Mechanism of phorbol diester receptor down modulation. The possible direct or indirect involvement of phospholipid hydrolysis in the process of PD receptor down modulation was investigated by asking whether stimulation of phospholipase A₂ (PLA₂) produces down modulation. Cells were incubated with either subtoxic concentrations of PLA₂ or with the nonpromoting activator of PLA₂ melitin with the result that there was no effect on PDBu binding.

6. Mechanism of retinoic acid enhancement of EGF promotion. In contrast to its antipromoting action when given with TPA to JB6 cells, retinoic acid enhances promotion of transformation induced by EGF. Retinoic acid has been shown to enhance EGF binding in various fibroblasts (Jetten et al., Nature, 284, 626-629, 1980). Retinoic acid (RA) alone or administered simultaneously with EGF produced an approximate 2-fold increase in ¹²⁵I-EGF binding relative to the respective non-RA treated controls in promotable JB6 cell lines. This is consistent with the possibility that the number of available EGF receptors may be limiting for promotion of transformation by EGF and contrasts with observations by Herschman et al. (J. Biol. Chem., 253, 3970-3977, 1978) that only a fraction of EGF receptors are necessary for maximal mitogenic response to EGF in 3T3 cells.

7. 3-Deazadenosine (3-DZA)-inhibited TPA-induced promotion. S-adenosylhomocysteine (5×10^{-7} M) and 3DZA (4×10^{-6} M), which inhibit phospholipid methyltransferases in the RBL-1 histamine release system, inhibited TPA-induction of anchorage-independent cell growth by 50%. Complete inhibition occurs at higher concentrations. Thus, methylation of membrane phospholipids may be involved in signal transduction following phorbol diester binding.

8. Catalase effect on TPA promotion. Various lots of impure catalase (50-100 U/ml) completely blocked TPA-induced promotion as well as the morphologic change usually induced by TPA in epidermal cells in monolayer culture. No effect on

expression of anchorage-independence by established tumors has been found. Highly purified catalase, however, showed no activity for inhibition of promotion of transformation, thus indicating that: 1) H_2O_2 is not a mediator of promotion by TPA in JB6 cells; and 2) there is a non-catalase inhibitory activity in the impure preparations. Benzoyl peroxide, a promoter of skin carcinogenesis also shows activity for promotion of transformation in JB6 cells. Mild oxidation with $NaIO_4$ also had promoting activity. 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 .

9. Vasopressin-induction of anchorage-independence. Arginine vasopressin (AVP) induces a sodium ion influx and synergistically enhances responses to growth factors in many cells. In initial experiments, AVP has induced anchorage-independent cell growth alone and in an additive fashion with TPA.

Significance to Biomedical Research and the Program of the Institute:

The molecules which represent the specific phorbol diester binding sites appear to be very stable in response to deliberate efforts to select for variant cells unresponsive to the mitogenic or promoting effects of TPA. Despite the multitude of biochemical systems which change with transformation, the PD binding site remains unaffected. This suggests that it is part of a basic physiologic mechanism whose functioning is essential for cell survival. Attempts to control pre-neoplastic 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 PDs at low concentrations. However, many less toxic agents, such as EGF and benzoyl peroxide, also activate irreversible transformation of JB6 cells, which suggests less clinically hazardous strategies for pharmacologic inhibition of this physiologic system involved in malignant transformation.

Phospholipid methylation, reactive oxygen generation and sodium ion influxes may all 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 PDs. Several 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 secondary events following PD 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) Characterization of changes in cell membrane phospholipid composition induced by tumor promoters and search for correlation of observed changes with promotion. Specific lipids known to modulate the functioning of membrane bound enzymes and receptors will be studied; (2) Identification of the target molecules attacked by reactive oxygen; (3) Determination of whether arginine vasopressin promotion is attributable only to induction of sodium influx by attempting inhibition with the sodium ion channel blocker amiloride and induction of sodium influx by monesin, a sodium-preferring monovalent ionophore.

Publications:

Colburn, N.H. and Gindhart, T.D.: Specific binding of transforming growth factor correlates with promotion of anchorage independence in EGF receptorless mouse JB6 cells. Biochem. Biophys. Res. Comm. 102: 799-807, 1981.

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. (Ed.): Organ and Species Specificity in Chemical Carcinogenesis. New York, Plenum Press, in press.

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., in press.

Colburn, N.H., Srinivas, L., and Wendel, E.J.: Responses of preneoplastic epidermal cells to promoters and growth factors: Use of promoter resistant variants for mechanism studies. J. Cell. Biochem. 18: 261-270, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05228-02 LVC
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Genes for Promotability

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nancy H. Colburn	Expert	LVC	NCI
OTHER:	Thomas Gindhart	Expert	LEP	NCI
	Donald G. Blair	Expert	LID	NCI

COOPERATING UNITS (if any)

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TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.1	OTHER: 0.9
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(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This research aims to identify the changes in the genetic material itself or its regulation which must occur for mammalian cells to undergo neoplastic progression in response to tumor promoters. Our results suggest that promotability behaves as a dominant trait. Complementation analysis is being carried out to estimate the number of different genes (and gene products) involved in promotability. We found that 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. In addition to P+, DNA retroviral long terminal repeat (LTR) sequences also appear to confer promotion sensitivity on transfection. Whether the P+ sequences specify controlling elements or gene products that are missing from the P- cells is an important question under investigation.

Project Description

Objectives:

To determine the number and nature of genetically distinct steps required for tumor-promoting phorbol diesters to induce anchorage-independent growth of the JB6 family of mouse epidermal cell lines. Characterization of the genetic loci in terms of their function(s), regulation and structure will be conducted. Identification of regulatory mechanisms at the DNA level through cloning and sequencing of the relevant genes are the ultimate objectives.

Methods Employed:

Cell hybridization using polyethylene glycol fusion of cells differentially labeled with fluorescent beads, followed by cell sorting and assay of promotion of anchorage-independent cell growth by TPA. The use of fluorescent bead labeling has the advantage of avoiding selection for genetic markers which might perturb the genes being studied. DNA transfection using calcium phosphate DNA precipitates to transfer DNA from promotable cells into nonpromotable cells. Restriction enzyme analysis to characterize and fractionate DNA sequences showing activity for promotability.

Major Findings:

1. Promotability appears to be a dominant trait. If promotability is dominant and is determined by one locus, fusion of promotable (P-) and nonpromotable (pp) lines should yield a promotable (P-pp) fusion product, whereas if promotability is recessive and is determined by one locus, fusion of promotable (pp) and nonpromotable (P-) lines should yield nonpromotable (ppP-) fusion products. A JB6 P- clonal line was fused with a JB6 P+ 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. In addition, the P+/P+ and the P-/P- fusion products were obtained. The anchorage-independence response to TPA of the P+/- fusion product in comparison with control P+/P+ and P-/P- fusion products was assayed. Of three sorted P+/- fusion products produced, all three were promoted to anchorage-independence in response to TPA, thus indicating dominance of the P+ trait.
2. No evidence for more than one genetic locus specifying promotability has so far been obtained. Of several P-/P- fusion products assayed, none have shown complementation to yield promotability. More products will need to be assayed before we can consider that promotability is determined by a single genetic locus.
3. Promotability can be transferred by transfection of whole cell DNA from P+ into P- cells. When DNA isolated from one of two P+ lines, JB6 C1 41 or JB6 C1 22, was precipitated with calcium phosphate and used to treat the P- cell line JB6 C1 30, the transfected cells yielded 200-400 agar colonies per 10^5 cells in response to TPA. Control C1 30 cells transfected with C1 30 DNA yielded only 40 colonies per 10^5 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) promotability is determined by genetic components in the P+ DNA but NIH 3T3 cells synthesize a suppressor of expression of the P+ donor sequences.

4. 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. Lines established from Eco gpt positive colonies were P+ with a frequency greater than 1% after co-transfection with P+ DNA. These P+ lines 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- C1 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.

5. Moloney virus oncogene DNA confers promotability on P- C1 30 cells when transfected. Initial experiments indicate that transfection of *v-mos* with one long terminal repeat (LTR) sequence (supplied by Dr. D. Blair) or one LTR alone with carrier C1 30 DNA yields cells that respond to TPA with induction of anchorage-independent growth. *V-mos* alone is inactive. Hence, insertion of a transcriptional promoter may be sufficient to convert the P- C1 30 cells to P+ phenotype.

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:

Continue experiments confirming the dominant nature of TPA promotability indicated by preliminary experiments using the FACS sorted products of polyethylene glycol fusions; continue complementation analysis of nonpromotable mouse epidermal cell lines using FACS sorted fusion products; determine the restriction enzyme sensitivity of P+ DNA; determine whether transforming sequences and promotability sequences are the same or different on the basis of restriction enzyme sensitivity; 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; confirm and extend the observation that retroviral LTRs can transfer promotability; determine the frequency and distribution of LTRs on secondary transfections yielding promotability by TPA; aim to clone P+ DNA sequences after restriction enzyme digestion and choice of appropriate fraction(s).

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, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05229-02 LVC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Production of Monoclonal Antibodies to Viral Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Fulvia Veronese	Visiting Fellow	LVC	NCI
OTHER:	John R. Stephenson	Visiting Scientist	LVC	NCI
	Gary J. Kelloff	Senior Surgeon	LVC	NCI

COOPERATING UNITS (if any)

F.H. Reynolds, Jr., Litton Bionetics, Inc., (FCRF), Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Carcinogenesis Mechanisms and Control Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

1.1

PROFESSIONAL:

0.9

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER

(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nonproducer transformed rat cells have been derived which contain each of the three strains of defective feline sarcoma viruses. The transforming proteins encoded by these feline sarcoma viruses are expressed in these rat cells and the latter have been used to hyperimmunize their syngeneic hosts. The immune cells of these highly immunized hosts have been fused with myeloma cell lines and cloned yielding growing hybridomas which produce high-titered monoclonal antibodies to the transforming proteins. Analysis of the monoclonal antibodies by polyacrylamide gel electrophoresis revealed a battery of monospecific reactivities to unique determinants of the transforming and structural proteins encoded by the different sarcoma viruses. Monoclonal antibody to the transforming proteins block their associated tyrosine-specific protein kinase activity indicating that this activity represents an intrinsic property of these onc gene products.

Project Description

Objectives:

Production of hybridomas from immune lymphocytes obtained from rats immune to syngeneic tumor cell lines containing the polyprotein of the feline sarcoma virus(es). The hybridomas are to provide a library of high-titered monospecific antibodies to the different antigenic determinants of the transforming proteins.

Methods Employed:

Cell culture techniques include maintenance of myeloma lines, cell fusions of myeloma cells with immune lymphocytes, cell cloning, and production of monoclonal hybridomas. Immunologic procedures include immunoprecipitation and gel electrophoresis.

Major Findings:

A series of hybridomas have been derived from spleen cells of Fisher rats immunized with syngeneic Gardner, Snyder-Theilen and McDonough feline sarcoma virus (FeSV)-transformed cells. These include two hybridomas producing IgG₁ antibody against feline leukemia virus (FeLV) gag gene structural components, one of which is directed against FeLV p15 and the second, specific for FeLV p30. The FeLV p15 determinants recognized by the first of these clones are shared by polyprotein gene products of all three strains of FeSV while the p30 determinants recognized by the second are unique to P170^{gag-fms}. The three remaining hybridomas, one initially isolated from spleen cells of Gardner FeSV-immunized rats and two from spleen cells of rats immunized with Snyder-Theilen FeSV, lack detectable reactivity with FeLV structural proteins. Of these latter monoclonal antibody reagents, one is an IgG_{2a}, one an IgG_{2b} and the remaining one, an IgM. The Gardner and Snyder-Theilen FeSV-encoded polyproteins exhibit tyrosine-specific protein kinase activity following immunoprecipitation by F72 hybridoma antibody, supporting the possibility that the observed reactivities represent intrinsic properties of the polyproteins themselves rather than associated cellular reactivities. In contrast, P170^{gag-fms}, when assayed under similar conditions, lacked detectable protein kinase activity, but was phosphorylated in tyrosine when coimmunoprecipitated with either the Gardner or Snyder-Theilen FeSV-encoded polyproteins. These findings are consistent with our previous results indicating P170^{gag-fms} to lack intrinsic protein kinase enzymatic activity and may account for a reported low level of P170^{gag-fms} phosphorylation in immunoprecipitates obtained with conventional hyperimmune antisera. Monoclonal antibodies produced by hybridomas F113, F115 and F123 are specific for the acquired sequence (v-fes) components of the Gardner- and Snyder-Theilen-encoded polyproteins. None of these three reagents exhibit detectable cross-reactivity with FeLV Pr65^{gag}, or with any of the FeLV gag and env gene-encoded structural proteins. In contrast to polyproteins immunoprecipitated by F72 antibody, the F113, F115 and F123 precipitated polyproteins lack detectable enzymatic activity, and in appropriate mixing experiments direct inhibition of the protein kinase by these latter monoclonal reagents can be demonstrated. Immunoprecipitation of Snyder-Theilen (ST) P85^{gag-fes} by antibody secreted by F123, the one hybridoma originally isolated from spleen cells of a Gardner FeSV-transformed cell immunized rat argues that it is specific for immunologic determinants mapping within the region of Gardner (GA) P110^{gag-fes} shared with ST P85^{gag-fes}. Finally, the

lack of detectable immunoprecipitation of P170^{gag-fms} by any of these three v-fes specific reagents is consistent with previous studies indicating the translational products of the v-fes and v-fms genes to be structurally and immunologically unrelated.

Significance to Biomedical Research and the Program of the Institute:

The production of a library of monoclonal antibodies reactive with single antigenic determinants of these viral- and cellular-encoded transforming proteins will make possible the search for these proteins in, and a determination of, their role in spontaneous and chemically-induced tumors.

Proposed Course:

Completion of the above outlined studies.

Publications:

Veronese, F., Kelloff, G.J., Reynolds, F.H. Jr., Hill, R.W., and Stephenson, J.R.: Monoclonal antibodies specific to transforming polyproteins encoded by independent isolates of feline sarcoma virus. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 CP 05245-01 LVC

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effectors of Anchorage-Independent Growth in the Primate Feto-Placental Unit

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dennis A. Pigott	Visiting Fellow	LVC	NCI
OTHER:	Kurt J. Stromberg	Senior Surgeon	LVC	NCI
	Daniel R. Twardzik	Research Chemist	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:

1.3

PROFESSIONAL:

1.1

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A study was undertaken to determine whether effectors of anchorage-independent growth (AIG) were present in the amniotic fluid and/or the placenta of human and subhuman primates. Assays depended on the ability of positive effectors to induce soft agar colony formation in a clone of normal rat kidney cells (NRK-49F), while negative effectors inhibited the spontaneous soft agar colony formation of a clone of human melanoma cells (A375 Ag5). It was determined that while acid/alcohol extraction of term placenta yielded a large quantity of several size classes of both positive and negative effectors of AIG, in organ cultures of first trimester human placenta, only a single 20,000 M.W. negative effector was secreted to the serum-free conditioned medium. Also, in both human (first trimester) and monkey (term) amniotic fluid, a low activity, 8,000 M.W. positive effector was detected, while inhibitors of AIG exhibited considerable variability, perhaps related to gestational age. The discovery of these effectors in the feto-placental unit encourages speculation that they play a role in embryology.

Project DescriptionObjectives:

To determine whether effectors of anchorage-independent growth (AIG) can be identified in: (1) amniotic fluid; (2) placental extracts; or (3) conditioned medium of placental organ cultures obtained from human and sub-human primates - Erythrocebus patus (red) and Ceropithecus aethiops (African green monkeys).

Methods Employed:

Positive effectors of AIG (transforming growth factors, TGFs) were assayed by their ability to induce colony formation in ^{125}I -clone of normal rat kidney cells (NRK-49F) in soft agar and to compete with ^{125}I -labeled epidermal growth factor (EGF) for binding to specific receptors of A431 cells. Negative effectors of AIG were assayed by their ability to inhibit the spontaneous colony formation in a clone of human melanoma cells (A375-Ag5) in soft agar. Negative effectors which interfered with TGF stimulation could be detected by reduction of NRK colony formation induced by either added TGF or a feeder layer of TGF-producing cells.

Major Findings:

1. Abundance of modulators in extracted placenta. Acid/alcohol extraction of term human placentas yield a large quantity (up to 100 ng equivalents of EGF) of several size classes of TGFs as well as negative effectors of AIG. The ready availability of this human source, coupled with its high yield, make the placenta an important source of human cellular AIG modulators. Their relevance to embryology and usefulness in the study of malignancy is at yet unclear.
2. Inhibitor secreted by placental organ cultures. Organ culture of first trimester human placenta revealed a single 20,000 M.W. inhibitory factor secreted to the serum-free conditioned medium. This inhibitor showed no evidence of interference with the TGF system. The relatively high activity, chromatographic integrity and secretory nature of this factor makes it amenable to further investigation.
3. Modulators present in amniotic fluid. Both human (first trimester) monkey and (term) amniotic fluid contained an 8,000 M.W. TGF. The low activity of TGFs implies that excess fetal TGFs are not excreted and accumulated in the amniotic fluid in a biologically active form. Inhibitors of AIG in the amniotic fluid show no clearly reproducible pattern, but may reflect gestational age. Of considerable interest, however, was the discovery in Erythrocebus patus of an activity chromatographically similar to the inhibitor secreted by first trimester human placental organ cultures.

Significance to Biomedical Research and the Program of the Institute:

The capacity for anchorage-independent growth (AIG) in vitro, shows a strong correlation with malignant potential in vivo. Thus, factors which act as effectors of AIG - especially negative ones - are of considerable interest in the area of cancer control. The discovery of these effectors in the primate fetoplacental unit allows speculation on their importance in embryology.

Proposed Course:

Of particular interest is the finding of a stable inhibitor of AIG in the conditioned medium of human placental organ cultures. This implies a naturally occurring, secreted factor which could exert a negative growth regulatory influence on the fetus. Biochemical characterization of this factor, its mechanism of action on sensitive cells, and the regulation of its production are all areas for further research.

Publications:

Stromberg, K., Pigott, D.A., Ranchalis, J.E., and Twardzik, D.R.: Human term placenta contains transforming growth factors. Biochem. Biophys. Res. Comm. 106: 354-361, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 CP 05246-01 LVC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Isolation and Characterization of Growth Regulatory Factors from Human Tumor Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth K. Iwata	Staff Fellow	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI
	Daniel R. Twardzik	Biochemist	LVC	NCI

COOPERATING UNITS (if any)
J. Massague and M.P. Czech, Department of Biochemistry, University of Massachusetts, Worcester, 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:
1.1	0.8	0.3

CHECK APPROPRIATE BOX(ES)
 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER
 (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Serum-free conditioned media from several human tumor cell lines (e.g. rhabdomyosarcoma, melanomas, and carcinomas) were observed to produce several growth regulatory factors. One class of factors competes for EGF binding and allows growth of normal rat fibroblasts in soft agar. These factors are designated transforming growth factors (TGFs). Certain of these human tumor cells also produce another 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 and rat fibroblasts, however, are stimulated to proliferate by these same factors. Currently, extensive efforts are being directed towards the purification and characterization of this new class of factors produced by human tumor cells. Using commercially available crosslinking reagents, a cell surface receptor for a transforming growth factor was identified on normal rat fibroblasts. This putative TGF receptor was specifically competed for by unlabeled TGFs but not EGF.

Project Description

Objectives:

To isolate and characterize a new class of growth regulatory factors produced by human tumor cells which inhibit tumor cell growth yet stimulate the proliferation of normal human and rat fibroblasts. When a sufficient quantity of the inhibiting factors are purified, studies are planned to determine the role of these factors in the control of cellular proliferation and tumor cell growth.

Methods Employed:

Biochemical and biological techniques previously developed in this laboratory were used to obtain factors from serum-free supernatants of cultured cells. New techniques were developed to assay tumor cell growth inhibitors. Published procedures and commercially available crosslinking reagents were used in the affinity-labeling of TGF receptors.

Major Findings:

1. Tumor cell inhibiting factors. Supernatant fluids from human tumor cell lines (e.g., rhabdomyosarcoma, melanoma, and carcinoma) were observed to produce several growth regulatory factors. As has been previously reported, human tumor cells release proteins (transforming growth factors, TGFs) which confer the transformed phenotype on untransformed fibroblasts. Another class of factors released by some of these tumor cells, however, inhibits growth of certain tumor cells (melanomas and carcinomas) in soft agar and in monolayer cultures. The isolation and characterization of this class of factors is the main focus of this project. A rapid and sensitive assay was developed to follow the tumor inhibiting activity through the various purification steps. The activity of these tumor cell growth inhibitors is measured by comparing the incorporation of radiolabeled IUDR by indicator tumor cells (melanomas and carcinomas) treated with aliquots containing the test factor with the amount of incorporation in the untreated control. The major tumor inhibiting factor (TIF) activities fractionate by gel permeation chromatography into three molecular weight classes (5,000, 10,000-12,000, and 20,000). These TIFs were found to inhibit several human tumor cell lines (e.g., rhabdomyosarcomas, melanomas, and carcinomas) and mink lung epithelial cells. TIFs stimulate normal human and rat fibroblasts to proliferate in monolayer cultures. Both inhibitory and stimulatory activities were concentration dependent. The concentration of the 5,000 molecular weight species of TIF required for inhibition of tumor cells or stimulation of normal human fibroblasts was 10-20 ng/ml. This is the concentration at which potent mitogens (e.g., epidermal growth factor) stimulate mitogenesis. TIFs were found to possess none of the antiviral activity found in interferon.

2. Receptor for transforming growth factor. ^{125}I -labeled transforming growth factor was observed to crosslink a 53 kilodalton membrane component that exhibited properties expected for a high affinity receptor for this ligand. Unlabeled sarcoma growth factor (SGF) and TGFs were able to displace the ^{125}I -labeled TGF from the 53 kilodalton membrane component and from the EGF receptor. Unlabeled EGF was unable to displace the ^{125}I -labeled TGF from the 53 kilodalton membrane component. Crosslinking of the ^{125}I -labeled TGF to the 53 kilodalton membrane component and the EGF receptor was obtained with either hydroxysuccinimidyl p-azidobenzoate or disuccinimidyl suberate crosslinking reagent.

Significance to Biomedical Research and the Program of the Institute:

The isolation and preliminary characterization of factors derived from human tumor cells which inhibit tumor cell growth provides new tools to study the control of tumor cell growth as well as a diagnostic marker for the presence of the tumor. Understanding the role and biochemical action of these factors would provide insight into the mechanism and control of tumor cell growth.

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 of action of these factors on tumor cells. Eventually, recombinant DNA technology will be used to produce quantities of TIFs for in vivo and further in vitro studies.

Publications:

None

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Transforming Growth Factors Produced by Independent Isolates of FeSV

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Daniel R. Twardzik	Biochemist	LVC	NCI
OTHER:	George J. Todaro	Medical Officer	LVC	NCI
	John R. Stephenson	Visiting Scientist	LVC	NCI
	Kenneth K. Iwata	Staff Fellow	LVC	NCI
	Hans Marquardt	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)

F.H. Reynolds, Jr., Litton Bionetics, Inc. (FCRF), Frederick, MD

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS:

1.1

PROFESSIONAL:

1.0

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

 (a) HUMAN SUBJECTS (b) HUMAN TISSUES (c) NEITHER (a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cells transformed by acute transforming viruses of feline origin which differ in cellular acquired sequences were used to examine the effect the of v-fes and v-fms gene on both the production and biological activity of transforming growth factors (TGFs). Cell cultures nonproductively transformed by the Snyder-Theilen and Gardner strains of feline sarcoma virus (FeSV), both of which contain the v-fms gene product release a small (10,000 Mr) and large (20,000 Mr) form of TGF. Both size classes of TGF compete with EGF for EGF membrane receptors and stimulate anchorage-independent cell growth and thus resemble sarcoma growth factor (SGF) and TGFs released by human tumor cells. Snyder-Theilen FeSV-transformed cultures which contain a polyprotein associated tyrosine specific protein kinase consistently release the highest liters of TGF (approximately 200 ng/l of cell culture) relative to all transformants examined. In contrast, cells transformed by McDonough FeSV (v-fms gene) which lack detectable kinase activity produce low levels of TGF (less than 10 ng/l). Large scale production of Snyder-Theilen transformed cell culture supernatants currently provide a rich source of transforming growth factor for use in primary sequence studies.

Project DescriptionObjectives:

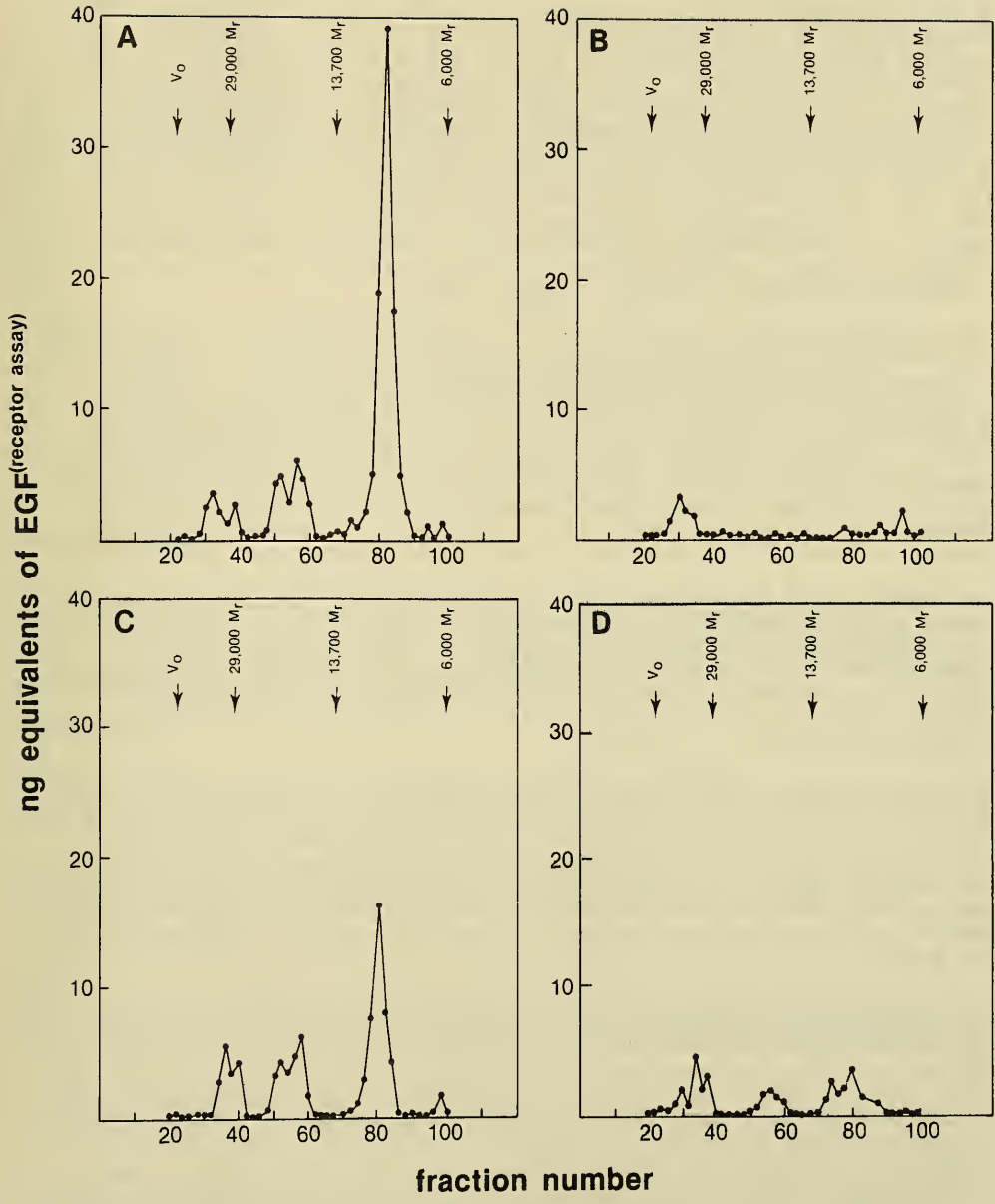
Transformation of tissue culture cells by independent isolates of feline sarcoma virus (FeSV) including Snyder-Theilen, Gardner and McDonough strains has been shown to result in a reduction in available epidermal growth factor (EGF) membrane receptors. This reduction in EGF binding has been shown in other viral transformants to correlate with the production of transforming growth factors (TGFs) which bind to and block EGF receptor sites. The object of this study is to determine if these FeSV transformants, which differ in their acquired cellular sequences, produce and release TGFs. One such acquired cellular sequence, identified as the *v-fes* gene found in Snyder-Theilen and Gardner FeSV, encodes a polyprotein containing a protein kinase activity with specificity for tyrosine residues, whereas in contrast, the *v-fms* gene is restricted to McDonough FeSV and lacks detectable enzymatic activity. Experiments will be designed to determine the effect of *v-fes* and *v-fms* gene expression on both the production and biological activity of TGFs from these transformants.

Methods Employed:

Cell cultures nonproductively transformed by isolates of FeSV containing the *v-fes* gene (Snyder-Theilen and Gardner) and the *v-fms* gene (McDonough) were grown to confluency. Serum-free conditioned media was harvested at 12-hour intervals for a period of three days, clarified by centrifugation and immediately lyophilized. Acid solubilized, nondialyzable peptides were resolved into different size classes using gel filtration columns. Fractions were assayed for competition with radiolabeled EGF for binding to EGF membrane receptors on receptor-rich human carcinoma cells and also for stimulating normal rat kidney cells to form colonies in soft agar. Dilution assays were constructed to quantitate: 1) the ng of both size classes of TGF produced per equivalent volume of cell culture supernatant; and 2) transforming activity per ng equivalent of the TGFs isolated from representative *v-fes* and *v-fms* transformants. A comparison of the net hydrophobicity of the major TGFs produced by these transformants based on their differential solvent elution from reverse phase high pressure liquid chromatographic systems was made.

Major Findings:

1. Comparison of TGFs produced by cell cultures transformed by FeSV isolates containing *v-fes* and *v-fms* acquired cellular sequences. Fisher rat embryo (FRE) cell nonproductively transformed by independent isolates of FeSV release TGFs into culture supernatants. As shown in Figure 1, Panel A, Bio-Gel P-100 column chromatography of processed conditioned media from Snyder-Theilen-transformed FRE cells contains a major peak of EGF-competing activity eluting in the region of 10,000 M_r . Additional minor peaks of EGF-competing activity eluting in the 20,000 and 35,000 M_r range are also seen. Both the 10,000 and 20,000 M_r activity stimulate the growth of nontransformed rat kidney cells in soft agar. The smaller TGF promotes the growth of large, compact, round colonies in agar containing hundreds of cells and concomitant acid production in these cultures. The larger (20,000 M_r) TGF promotes smaller and slower colony growth, whereas no anchorage-independent cell growth is observed in agar plates to which the 35,000 M_r EGF-competing activity was added. As shown in Figure 1, Panel B, this 35,000



M_r minor component is the only EGF-competing activity found in processed conditioned media from highly contact-inhibited normal Fisher rat embryo cell cultures and again, no transforming activity was found in any column fraction tested. A chromatographic profile similar to Snyder-Theilen transformants was also seen in conditioned media processed from Gardner FeSV-transformed cells (Fig. 1C). Again, a major EGF-competing and soft agar-promoting activity was found eluting in the 10,000 M_r range as is an additional minor 20,000 M_r TGF activity. Conditioned media harvested from equivalent cultures of McDonough FeSV also contain, albeit low, two peaks of EGF-competing activity eluting in the same relative positions as Snyder-Theilen and Gardner FeSV TGFs with apparent molecular weights of 10,000 and 20,000 (Fig. 1D). When tested on an equivalent ng basis TGFs released by all FeSV transformants exhibit similar transforming activity in soft agar growth assays.

Nontransformed Fisher rat embryo cells have available EGF membrane receptors and bind 125 I-EGF, whereas these same cells transformed by various FeSV isolates or by Abelson murine leukemia virus score negative for EGF binding. Concordant with unoccupied EGF receptors, highly contact-inhibited FRE cells release no detectable TGFs into culture supernatants as determined by either EGF competition or by the more sensitive NRK soft agar assays. In contrast, the highest amounts of 10,000 M_r TGF (200 ng equivalents of EGF/liter of conditioned media) are released by Snyder-Theilen FeSV transformants, whereas Gardner FeSV-transformed cells release 3-fold less of the smaller TGF. In contrast, McDonough FeSV-transformed cells produce very low amounts of 10,000 M_r TGF (<10 ng/l).

The 10,000 M_r TGFs derived from Snyder-Theilen FeSV- and McDonough FeSV-transformed FRE cells were further compared by reverse phase high pressure liquid chromatography (HPLC). The small 10,000 M_r TGF from Snyder-Theilen and McDonough FeSV-transformed cell culture supernatants elute from C_{18} Bondapak columns at approximately 20.0% acetonitrile. Mouse submaxillary gland EGF elutes from these columns at 28% acetonitrile and does not stimulate the growth of nontransformed cells in soft agar.

Both the soft agar growth-promoting and EGF-competing activities of the 10,000 M_r TGF from Snyder-Theilen-transformed FRE conditioned media were susceptible to treatment with trypsin and dithiothreitol as previously reported for sarcoma growth factor and TGFs from human tumor cells. In addition, the small TGF activity was also stable in 1 M acetic acid at various temperatures. As previously reported for other TGFs, the rat small TGF does not show any detectable immunological cross-reactivity when tested in a sensitive homologous radioimmunoassay for mouse EGF.

2. Production and partial purification of the low molecular weight TGF from conditioned media from Snyder-Theilen transformed cell culture. Conditioned media harvested at 12-hour intervals for four days from monolayer (T150 flasks) cultures of Snyder-Theilen-transformed rodent cells contained predominantly the small molecular weight form of TGF. Concentration by lyophilization was found to be a more efficient method of concentration than filtration. Yields ranged from approximately 120-200 ng of TGF per liter of culture supernatant following acid/ethanol extraction and chromatography on acidified Bio-Gel P-10 columns. Although yields were high, the presence of extraneous protein in large volumes of supernatants (>100 l) facilitated the acid/ethanol extraction of lyophilized conditioned media prior to gel filtration chromatography.

Large scale production of conditioned media harvested at 24-hour intervals from confluent roller bottle cultures of Snyder-Theilen FeSV transformants has resulted in the production of μg quantities of the small 10,000 M_r rodent TGF for subsequent purification and primary sequence analysis. Early harvests of these cultures also gave high TGF yields, however, unlike monolayers, more of the high molecular weight (20,000 M_r) TGF was produced relative to the smaller form. Unlike supernatants harvested from monolayers, however, less contaminating protein is found in conditioned media from roller bottle cultures.

Significance to Biomedical Research and the Program of the Institute:

The availability of acute transforming viruses of feline origin which differ in cellular acquired sequences allows us to examine the effect of the v-fes and v-fms gene and their gene product(s) on both the production and transforming activity of TGFs. Since the v-fes-encoded polyprotein contains an associated tyrosine-specific protein kinase, whereas the v-fms-encoded polyprotein does not, experiments can be designed to examine the role of tyrosine phosphorylation in the expression and modulation of TGF activity. The high level of TGF released by Snyder-Theilen-transformed cells relative to other transformed cell systems also provides an invaluable source of growth factor for use in primary sequence determination.

Proposed Course:

The role of preferred tyrosine phosphorylation in the modulation of TGF expression and function will be examined using cell cultures transformed by FeSV isolates differing in the v-fes and v-fms genetic complements. The low molecular weight TGF will be produced in μg amounts from Snyder-Theilen-transformed rodent cell cultures for use in primary sequence studies.

Publications:

- Sherwin, S.A., Twardzik, D.R., Bohn, W., Cockley, K., and Todaro, G.J.: Tumor-associated transforming growth factor activity in the urine of patients with disseminated cancer. Cancer Res., in press.
- Stromberg, K., Pigott, D.A., Ranchalis, J.E., and Twardzik, D.R.: Human term placenta contains transforming growth factors. Biochem. Biophys. Res. Comm. 106: 354-361, 1982.
- 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, Vol. 4, in press.
- Twardzik, D.R., De Larco, J.E., Marquardt, H., Sherwin, S., and Todaro, G.J.: Transforming growth factors: Detection in urine of pregnant and tumor bearing humans. Pediatr. Oncol., in press.
- Twardzik, D.R., Ranchalis, J.E., and Todaro, G.J.: Mouse embryos contain transforming growth factors related to those isolated from tumor cells. Cancer Res. 42: 590-593, 1982.

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., in press.

Twardzik, D.R., Todaro, G.J., Marquardt, H., Reynolds, F.H., Jr., and Stephenson, J.R.: Transformation induced by Abelson murine leukemia virus involves production of a polypeptide growth factor. Science 216: 894-897, 1982.

Project Description

Objectives:

Transforming growth factors (TGFs) are known to induce anchorage-independent growth of nontransformed cells, and are released by a variety of cells, including fibroblasts transformed by Moloney murine sarcoma virus (MSV) (De Larco and Todaro, Proc. Natl. Acad. Sci. USA, 75, 4001-4005, 1978). One of the major questions concerning TGFs relates to their function(s). In regard to malignant cells, it has been proposed that the ectopic production of TGFs by transformed cells contributes to the malignant phenotype that they express. However, TGFs may also have normal physiological functions and it has been suggested that they may play a role during embryogenesis (Todaro et al., Nature, 276, 272-274, 1978). The objective of this project was to examine the possible role of transforming growth factors during early mammalian development. This possibility has been examined by determining whether TGFs influence the growth and differentiation of mouse embryonal carcinoma (EC) cell lines. EC cells, which are the stem cells of the germ line tumors known as teratocarcinomas, are recognized as a good model system for the study of early mammalian development. These cell lines are unique in several ways: 1) they are very similar to the cells of the inner cell mass at the 4th day of gestation (the inner cell mass forms the embryo proper); 2) they can differentiate into a wide range of cell types, including those that are formed during early mammalian development; 3) their differentiation *in vitro* can be directed to specific cell types; and 4) EC cells can colonize mouse embryos and give rise to normal differentiated cells that are fully functional.

Methods Employed:

Two embryonal carcinoma cell lines, F9 and PC-13, were specifically selected for this study. 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 during the 5th day of gestation and, thus, are two of the first cell types to appear during early mammalian development. PC-13 cells were selected for a different reason. Their differentiated cells, which also form after RA treatment, exhibit a large increase in the ability to bind EGF and can 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. PC-13 and F9 EC cells were selected for an additional reason. Both can be grown and induced to differentiate in defined media (Rizzino, A. and Crowley, C., Proc. Natl. Acad. Sci. USA, 77, 457-461, 1980), which makes it possible to examine the influence of TGFs in the absence of extraneous serum factors. This is doubly important since serum itself (e.g., fetal calf serum) has been reported to contain TGFs. Therefore, the growth and differentiation of F9 and PC-13 EC cells, as well as the growth of their differentiated cells, was examined in defined medium with and without TGFs. A final reason for selecting F9 and PC-13 EC cells is that both EC cell lines condition their culture medium, and this raised the possibility that EC cells produce factors with TGF activity. Thus, EC cells were examined for the production of TGFs by determining whether they release factors that are able to promote the growth of nontransformed cells (normal rat kidney, NRK) in soft agar.

Major Findings:

1. Response of embryonal carcinoma cells and their differentiated cells to factors released by MSV-transformed cells. The effect of TGFs on the growth and differentiation of EC cells was examined using TGFs prepared from MSV-transformed 3T3 cells. Two preparations were used in the experiments described below: 1) acid-extracted serum-free conditioned medium, referred to as crude TGFs (cTGFs); and 2) sarcoma growth factor (SGF), which is prepared from cTGFs. The first question examined was: Do TGFs 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 cTGFs or SGF. No effect on the differentiation of either cell line was observed. Next, the question of whether TGFs affect the growth of the EC cells and/or their differentiated cells was examined. Neither cTGFs nor SGF significantly influenced the growth of F9 or PC-13 EC cells. In direct contrast, growth of the differentiated cells derived from F9 and PC-13 was stimulated by cTGFs and SGF in serum-free media (SFM) and, to a lesser degree, in serum-containing media (SCM). In SFM, the growth rate of the differentiated cells increased significantly in the presence of cTGFs. In the case of the F9 differentiated cells, the generation time in SFM was approximately 48 hrs versus 24 hrs in SFM containing cTGFs. It was also observed that growth of the differentiated cells was stimulated by epidermal growth factor (EGF), and the degree of stimulation was approximately equivalent to that observed with SGF. The findings that the differentiated cells responded to cTGFs, SGF and EGF, all of which have the ability to bind to EGF receptors, 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 resulted in increases in EGF binding of 24.5- and 3.8-fold, respectively, relative to the undifferentiated parental cells. Thus, these findings are consistent with the argument that the ability of PC-13 and F9 to bind EGF increases as they differentiate, and, as a result, they become responsive to EGF and other factors able to bind to the EGF receptors, such as SGF.

2. Characterization of the growth-promoting activity of cTGFs and SGF. The fact that EGF stimulated the growth of the differentiated cells raised the possibility that the preparation of cTGFs might contain significant levels of EGF. This possibility was tested by determining if the growth stimulation of cTGFs or SGF could be blocked by antibodies directed against EGF. It was found that antibodies to EGF completely block the growth stimulation by EGF, but have little or no effect on the growth stimulation by cTGFs or SGF. The growth-promoting activity of cTGFs and SGF was characterized further by examining its stability under conditions that have been used to characterize the TGFs produced by MSV-transformed cells. Incubation at 50°C for 2 hrs or at 100°C for 3 mins did not affect the growth-promoting activity, but treatment with trypsin or dithiothreitol destroyed nearly all of the growth-promoting activity. These chemical properties closely resemble the properties of the TGFs (heat- and acid-stable disulfide-requiring peptides) and argue that the same, or similar, molecules are responsible for both activities.

3. Production of TGFs by embryonal carcinoma cells. The failure of F9 and PC-13 EC cells to respond to cTGFs or SGF, together with their low level of EGF binding and their ability to condition their culture medium, raised the possibility that EC cells produce TGFs. This possibility was examined by preparing conditioned media from F9 and PC-13 EC cells grown in the absence of serum. The conditioned

media were dialyzed, concentrated and tested for the ability to induce nontransformed indicator cells (normal rat kidney cells) to grow in soft agar. Both F9 and PC-13 EC cells were found to release factors that promote the growth of nontransformed cells in soft agar.

Significance to Biomedical Research and the Program of the Institute:

Attempts to understand the possible roles of TGFs have led to the suggestion that these factors may have normal physiological functions during embryogenesis. The results of this project support this suggestion and provide two findings that argue strongly for TGFs playing a significant role during the early stages of mammalian development. First, the differentiated cells derived from F9 and PC-13 EC cells, but not the parental cells themselves, respond by increased growth to several factors released by MSV-transformed cells, including sarcoma growth factor. This finding is consistent with the possibility that certain TGFs represent embryonic forms of growth factors (e.g., sarcoma growth factor may represent an embryonic form of epidermal growth factor). Second, and perhaps more importantly, F9 and PC-13 EC cell lines, which do not respond to the TGFs released by MSV-transformed cells, were found to release factors with transforming growth factor activity. Given the close relationship between EC cells and the cells of early embryos (in particular, the cells of the inner cell mass), the findings of this project indicate that a role for TGFs during early development must be seriously entertained.

Proposed Course:

Future studies will continue the work discussed above and will focus on several questions. 1) Do all EC cells produce TGFs? 2) Does the differentiation of EC cells alter their production of TGFs? 3) Do the TGFs released by F9, PC-13 and other EC cell lines resemble the TGFs released by other cells (e.g., MSV-transformed cells)? In particular, do the TGFs released by EC cells bind to EGF receptors and are they chemically related to the TGFs released by transformed cells? 4) Do TGFs, in particular those released by EC cells, influence the growth and differentiation of early mouse embryos cultured in vitro? 5) Do early mouse embryos (blastocyst stage) produce transforming growth factors?

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, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CP 05249-01 LVC
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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Structural Analysis of the Mammalian c-fes/c-fps Genetic Locus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nora Heisterkamp	Visiting Fellow	LVC	NCI
OTHER:	John Groffen	Visiting Fellow	LVC	NCI
	John R. Stephenson	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)
F.H. Reynolds, Jr., Litton Bionetics, Inc. (FCRF), Frederick MD

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Carcinogenesis Mechanisms and Control

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MANYEARS: 1.2	PROFESSIONAL: 0.9	OTHER: 0.3
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(a1) MINORS (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Molecular cloning and restriction endonuclease mapping techniques have been utilized in an effort to define the relatedness of the avian type C viral v-fps and mammalian v-fes genes. The results establish that these independently derived transforming sequences correspond to a common cellular genetic locus which has remained highly conserved in evolution. The chromosomal localization of this sequence has been determined by analysis of a series of mouse and human cell hybrids.

Project Description

Objectives:

To define, molecularly clone and fine structure map human c-fes/c-fps homologous sequences as part of an overall determination of their potential involvement in human cancer.

Methods Employed:

Molecular cloning of viral and cellular genes in plasmid, phage and cosmid vector systems; use of expression plasmids for identification of gene products and application of nucleic acid sequencing techniques for analysis of cloned genes.

Major Findings:

The Gardner and Snyder-Theilen isolates of feline sarcoma virus (FeSV) represent genetic recombinants between feline leukemia virus (FeLV) and transformation-specific sequences of cat cellular origin (v-fes gene). A related transforming gene (v-fps) common to the Fujinami (FSV) and PRC II strains of avian sarcoma virus has also been described. Polyprotein gene products of each of these virus isolates exhibit tyrosine-specific protein kinase activity. By restriction endonuclease and molecular hybridization analysis, the v-fes and v-fps genes have been found to contain highly related sequences. Moreover, molecular probes corresponding to each hybridize to a single well-resolved 12 kb EcoRI restriction fragment of human cellular DNA.

To accurately define regions of sequence homology of the v-fps gene with the human cellular homolog of v-fes, a series of three cosmid clones containing v-fes homologous sequences within a total of 56 kb of contiguous human DNA sequences, were examined. Hybridization with FSV v-fps was restricted to a single 12.0 EcoRI restriction fragment containing v-fes homologous sequences. Following subcloning, the 12.0 EcoRI fragment was digested with a series of restriction endonucleases and analyzed for hybridization to v-fes and v-fps specific probes. FSV v-fps homology to human DNA most closely resembled that of Gardner (GA) FeSV v-fes. However, the 3' terminal v-fes homologous region of human DNA common to Snyder-Theilen (ST) and GA v-fes did not hybridize to a detectable extent with the FSV v-fps probe. ST v-fes homologous sequences differ from those of both GA v-fes and FSV v-fps in that they are not represented within the 5' terminal region hybridizing to the other two. Finally, two regions of human DNA homology unique to FSV v-fps were identified in the 5' half of the 12.0 kbp human EcoRI DNA fragment. Thus, although all three viral "oncogenes" appear to be entirely represented by homologous sequences within a single c-fes/c-fps human genetic locus, the exact positions at which regions of homology map differ among the individual viral transforming genes. The lack of detectable hybridization of v-fps with the 3' region of the c-fes/c-fps locus could reflect evolutionary divergence. Alternatively, a comparison of the 5' terminal position of ST v-fes, and 3' position of FSV v-fps homologous sequences within the human c-fes/c-fps locus may provide an estimate of human DNA sequences required for transformation.

Significance to Biomedical Research and the Program of the Institute:

The molecular cloning and characterization of the human c-fes/c-fps sequences should be of value in a determination of the possible involvement of this locus in tumors of man.

Proposed Course:

Future studies will involve fine structure analysis of the human c-fes and c-abl genes, identification of their transcriptional products and studies of regulatory controls influencing their expression.

Publications:

Groffen, J., Hesiterkamp, N., Grosveld, F., Van de Ven, W.J.M., and Stephenson, J.R.: Isolation of human oncogene sequences (v-fes homolog) from a cosmid library. Science 216: 1136-1138, 1982.

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.): Gene Transfer and Cancer. New York, Raven Press, in press.

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Application of Cosmid Vectors to Molecular Cloning of Mammalian Oncogenes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John Groffen	Visiting Fellow	LVC	NCI
OTHER:	Nora Heisterkamp	Visiting Fellow	LVC	NCI
	John R. Stephenson	Visiting Scientist	LVC	NCI

COOPERATING UNITS (if any)

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SUMMARY OF WORK (200 words or less - underline keywords)

A highly representative cosmid library of Mbol partially digested human lung carcinoma DNA has been constructed. Important features of this library include the size of the cellular inserts (30-40 kb) and the presence of thymidine kinase and SV40 promoter sequences in the vector arms. A series of clones containing the complete c-fes and c-abl genes have been isolated, subcloned and subjected to detailed restriction endonuclease mapping.

Project DescriptionObjectives:

To molecularly clone and determine the structural organization of the human c-fes and c-abl genes.

Methods Employed:

Molecular cloning of viral and cellular genes in plasmid, phage and cosmid vector systems and application of nucleic acid sequencing techniques for analysis of cloned genes.

Major Findings:

To define the human homolog(s) of transforming sequences (v-fes gene) common to the Gardner (GA) and Snyder-Theilen (ST) isolates of feline sarcoma virus (FeSV). independent virus isolates, a representative library of human lung carcinoma DNA in a cosmid vector system was constructed. Two cosmids containing the entire cellular homolog of the GA/ST FeSV-acquired cellular sequences, and a third cosmid containing a major portion of these sequences were isolated. Cellular inserts within these three cosmids range from between 32 to 42 kb in length and represent overlapping regions corresponding to a total of 56 kb. Sequences, both homologous to and colinear with, GA/ST FeSV v-fes are distributed discontinuously over a region of up to 9.5 kb and contain a minimum of 3 regions of nonhomology representing probable introns. Upon transfection to RAT-2 cells, using a thymidine kinase selection system, the human c-fes sequence lacked detectable transforming activity. Using a similar approach the human lung cosmid library has also been utilized for the molecular cloning of human cellular sequences homologous to v-abl, an oncogenic sequence with specificity for lymphoid transformation. The human c-abl gene is distributed over a much more extensive region of the human genome than c-fes, thus requiring several overlapping cosmid clones for its complete representation.

Significance to Biomedical Research and the Program of the Institute:

The isolation of human DNA sequences homologous to viral genes encoding transforming proteins with tyrosine-specific protein kinase activity should be of value in determining the functional significance of this enzymatic activity and its possible involvement in naturally occurring tumors of man.

Proposed Course:

Future studies will involve fine structure analysis of the human c-fes and c-abl genes, identification of their transcription and translational products and studies of regulatory controls influencing their expression.

Publications:

Groffen, J., Heisterkamp, N., Grosveld, F., Van de Ven, W.J.M., and Stephenson, J.R.: Isolation of human oncogene sequences (v-fes homolog) from a cosmid library. Science 216: 1136-1138, 1982.

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.): Gene Transfer and Cancer. New York, Raven Press, in press.

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