Division of

Cancer Etiology

1988 Annual Report
Intramural Activities
Volume II

October 1, 1987
September 30, 1988

U.S. DEPARTMENT
OF HEALTH
AND HUMAN SERVICES

National
Institutes of
Health

National
Cancer
Institute

Bethesda,
Maryland 20892

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ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

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ANNUAL REPORT OF

THE LABORATORY OF BIOLOGY
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The Laboratory of Biology plans, develops, and conducts in vitro and in vivo investigations to elucidate the role of chemical, physical and biological agents in the modulation of carcinogenesis. Coordinated biochemical and biological studies utilizing human and animal cell models are used to characterize the cellular alterations associated with the transition to the neoplastic state. These include: assessment of the effect of physiologic host mediating factors; determination of cell surface changes; and evaluation of the relationships between DNA metabolism, chromosome alterations, and carcinogenesis.

The major objective of the Somatic Cell Genetics Section is to understand changes in chromosomes and DNA metabolism which regulate gene expression responsible for neoplastic transformation. The superimposing of molecular events on biological observations leads to conclusions concerning gene expression relevant to control of differentiation and cancer. The Tumor Biology Section emphasizes host interactions during carcinogenesis. Development of immunological intervention capable of preventing development of the transition to neoplasia receives particular emphasis.

A primary objective of the Laboratory of Biology is elucidation of signals associated with the development and expression of the malignant state, particularly on developing reproducible in vitro models of human carcinogenesis. Human cells are notable in being more resistant than animal cells to neoplastic transformation by any of the well-known carcinogens. As a result, recombinant human papillomavirus (HPVs) DNA has been selected for transformation studies because of the evidence associating HPVs with human cancer, particularly with cervical carcinoma. Furthermore, epidemiologic reports suggest that environmental agents enhance the frequency of these cancers. The laboratory's approach to determining the role of human papillomaviruses in human carcinogenesis has been to develop model systems utilizing mammalian cells in conjunction with human papillomaviruses, particularly HPV-16 and HPV-18 which belong to the subgroup of papillomaviruses associated with cancer of the cervix. Although there is substantial evidence for their oncogenic potential, the question remains whether the virus causes cancer or is a necessary factor for carcinogenesis.

The Laboratory of Biology has been responsible for developing in vitro models that use human epithelial cells derived from the foreskin and from the cervix. Immortalization of human epithelial cells by human papillomavirus type 16 is reproducible at a high frequency, is due to the presence of viral sequences in the cells, and occurs independently from the genetic characteristics of the cells. Because the virus cannot be grown in vitro as yet, the epithelial cell lines were obtained by transfection with a plasmid containing the HPV sequence and selected either by G418 or growth in the absence of antibiotic. The

latter was possible because the normal cells have a finite period of approximately 50 doublings. Thus far, approximately 12 immortalized lines have been obtained from foreskin epithelial cells transfected with either HPV-16 or HPV-18. Each line has a constant number of copies of the viral genome integrated into the cellular DNA as well as unique patterns of integration of HPV sequences into the cellular genome; however, all lines express similar patterns of viral messages. Foreskin epithelial cell lines which would grow in the presence serum were obtained by selection from serum-free medium, demonstrating that immortalization and differentiation can be separated. Thus, the establishment of continuous cell lines is the direct consequence of the presence of viral sequences. Although all cell lines had viral sequences, none of the lines formed tumors in nude mice, suggesting that additional events are necessary for progression to malignancy.

The foreskin epithelial-transfected HPV lines have also been analyzed for chromosome alterations. The immortal lines exhibited clonal chromosomal abnormalities with complex translocations, deletions, telomeric associations, achromatic lesions, partial chromosome duplications, and variable number of cells with minute chromosomes. Such alterations are never present in normal cells and reflect gene amplification seen in tumors. In three lines the integration sites of the viral sequences were localized by _in situ_ hybridization on normal chromosomes, at the junction of chromosome translocations, at the site of achromatic lesions, and within duplicated chromosome segments. Partial duplication of the long arm of chromosome 1 was observed in three lines; these regions had integrated HPV-16 near _arg_ and _trk_ proto-oncogenes. Duplication and amplification of cellular sequences may have originated at nearby fragile sites. HPV-16 integration in the cellular genome is directly responsible for the induction of chromosome alterations. Integration of HPV-18 viral sequences associated with cervical adeno- and squamous carcinomas, such as HeLa and SW756, is a non-random event because of its association with constitutive or heritable fragile sites and proto-oncogenes. Thus, the formation of abnormal chromosomes and HPV16 integration near proto-oncogenes may be essential steps for neoplastic development of human keratinocytes.

Because transfected foreskin epithelial cells failed to form tumors in mice and because HPV-16 and, to a lesser extent, HPV-18 are associated primarily with advanced cervical neoplasia, an in vitro system was developed to study the interaction between human papillomavirus (HPV) DNA and normal exocervical epithelial cells (HCX). Most cervical cancer occurs at the squamocolumnar junction of the cervix. HCX were isolated from the cervical transformation zone, grown in serum-free medium, and transfected with recombinant HPV-16 or -18 DNA. Although normal HCX senesced after 30 to 40 population doublings, HPV-transfected HCX continue to proliferate (>200 population doublings to date). The HPV-immortalized HCX lines contained between 2 to 20 integrated copies of HPV DNA per cell and expressed 1.8 and 4.2kbp HPV mRNAs and various other HPV transcripts. Expression of keratins, major differentiation products of HCX cells, by HPV-immortalized HCX was consistent with their origin from exocervical epithelium. Sublines resistant to serum-induced terminal differentiation were selected by culture in the presence of serum. These differed in keratin expression from the parent lines. These sublines may represent another stage in carcinogenesis because they have acquired additional cervical characteristics of transformed cells. None of the HCX immortalized lines, regardless of abnormal karyotype, formed tumors in nude

mice. However, when a serum-selected HPC-16 HCX line was cotransfected with H-ras and the multidrug resistance gene vector, which confers resistance to colchicine, a tumorigenic line was developed. When this line was injected subcutaneously into nude mice, it formed cystic tumors that were well-differentiated squamous cell carcinomas.

HPV-immortalized cervical epithelial cells and HPV-positive cervical carcinomas are also being evaluated as a model of lymphokine modulation of epithelial cell sensitivity to natural immunologic cytotoxicity. Epithelial cells, immortalized by transfection with HPV-16 DNA, are treated with 2.5 U/ml leukoregulin for 1 hr and mixed with natural killer (NK) cells or interleukin-2 (IL-2)-induced lymphokine-activated killer (LAK) cells at effector to target cell ratios up to 50:1 in a 4 hr 51Cr release assay. Several HPV-16 DNA immortalized HCX lines have now been examined with regards to their sensitivity to NK and LAK cell cytotoxicity and modulation of NK and LAK sensitivity by leukoregulin. HPV-16 DNA HCX lines are resistant to NK and sensitive to LAK cell cytotoxicity. Leukoregulin treatment of these target cells induces and/or up-regulates their sensitivity to NK and LAK cell cytotoxicity, with LAK cell cytotoxicity being up-regulated more than NK cell cytotoxicity. When the HPV-16 DNA-immortalized cell lines are evaluated according to their length of time in culture, early passage cells are less sensitive to the enhancing effect of leukoregulin than are late passage cells. The early passage cells react similarly to the control nontransfected cervical cells. These observations are the first demonstration that leukoregulin up-regulates the sensitivity of papilloma viral DNA immortalized human epithelial cells to different forms of natural lymphocyte cytotoxicity.

As part of the study of understanding the molecular mechanisms of chromosome alteration, a number of proto-oncogenes were localized by an *in situ* hybridization procedure developed in the Laboratory of Biology. A high proportion of proto-oncogenes exists at breakpoints of chromosomes. The *Fyn* gene, a novel human gene structurally related to the *Src* gene, was localized to 6q21 within the same region as *ros* and *myb* proto-oncogenes, suggesting that they may be closely linked at the molecular level. Furthermore, the existence of a fragile site at 6q21 suggests that these alterations may be important to specific hematologic malignancies and solid tumors. On the X chromosome *dbl* was localized to q27, a site associated with mental retardation and anti-hemophilic factor IX. From a thyroid-binding protein DNA library the key enzyme, P55, responsible for post-translational modification of collagen synthesis, has been localized. The gene for P55 is at q25 of chromosome 17; other nuclear thyroid hormones are not nearby. Because HPV-18 of HeLa cells is found on several chromosomes, the flanking sequences associated with HPV-18 and chromosome 8 were isolated. This flanking sequence was found only on chromosome 8, indicating that integration on the various chromosomes was independent of chromosome 8. The integration was also accompanied by amplification of viral sequences and induced *myc* RNA expression.

In the guinea pig multistage carcinogenesis model, there is coordinate N-*ras* up-regulation, mutational activation, and acquisition of tumorigenicity. The N-*ras* promoter region contains a regulatory element similar to that found in growth control genes and inducible genes, and is identical to that in the adenovirus major late promoter which can be transactivated by cellular transcription factor AP-1. This suggests that AP-1 regulates N-*ras* expression.

Characterization of the molecular and biological pathways of leukoregulin action this past year focused upon further elucidation of the mechanism of action and potential usefulness of this direct-acting anti-cancer lymphokine. Examination of the physiologic role of leukoregulin demonstrates that its ability to up-regulate target cell sensitivity to natural killer cell immunocytotoxicity extends to LAK lymphocyte cytotoxicity and to T lymphocyte killing of allogeneic human tumor cells. Continued evaluation of leukoregulin's ability to facilitate the target cell uptake of pharmacologically active molecules indicates the leukoregulin-induced membrane channel freely admits molecules as large as 20,000 daltons. In the third area concentrating upon the biotechnological production of leukoregulin, clones of the RPMI 1788 leukoregulin-producing human lymphocyte cell line have been developed that produce large amounts of the lymphokine required for cloning of the leukoregulin gene and expression of recombinant human leukoregulin.

Leukoregulin interaction with tumor cells up-regulates their sensitivity to LAK cytotoxicity similar to the previously established ability of the lymphokine to increase the sensitivity of carcinoma, leukemia, and sarcoma cells to natural killer lymphocyte cytotoxicity. One to two units of leukoregulin/ml increases target cell sensitivity two- to tenfold at effector to target cell ratios as small as 1:1. Leukoregulin up-regulation of LAK cytotoxicity as observed in up-regulation of NK cytotoxicity, is opposite the action of gamma interferon which down-regulates target cell sensitivity to lymphocytotoxicity. The up-regulation of target cell sensitivity by leukoregulin is unique; it does not occur in target cells exposed alone or in combination to tumor necrosis factor, interferons, or colony stimulating factors. The up-regulation of target cell sensitivity to natural cytotoxicity also occurs in T lymphocyte-directed cytotoxicity as studied in collaboration with C. Slingluff and H. L. Seigler in the Department of Surgery at Duke University. Up-regulation of tumor cell sensitivity by T cytotoxic lymphocytes, however, is directed against allogeneic, not autologous cells, in investigations with melanoma target cells and effector lymphocytes derived from the individual with the melanoma. These observations suggest that cytotoxic T-cells have the capacity to react with more than one target cell receptor configuration or that the configuration of the target cell recognition site is rapidly regulated by leukoregulin. The molecular events involved in this aspect of leukoregulin target cell regulation are under investigation as is the question as to whether they extend to antibody-dependent cellular cytotoxicity (ADCC), another major physiologic mechanism for the elimination of abnormal target cells.

Leukoregulin is produced by NK lymphocytes and destabilizes the target cell membrane. A major question is what is the relationship of leukoregulin to natural killer cytotoxic factor (NKCF), another lymphokine secreted by NK lymphocytes which is a critical molecular mediator for target cell lysis. Molecular sizing and calcium dependence biochemical studies indicate that the two lymphokines are distinct. In collaboration with J. Ortaldo, Laboratory of Experimental Immunology, NCI, antibody neutralization studies employing polyclonal and monoclonal anti-NKCF antibodies demonstrate no inhibition of leukoregulin activity providing further evidence that leukoregulin and NKCF are distinct molecules.

The up-regulation of target cell sensitivity to natural lymphocyte cytotoxicity occurs concurrently with an increase in target cell plasma

membrane permeability and increased uptake of pharmacologically active macromolecules. The uptake of metabolic inhibitory antibiotics as large as 4000 daltons is augmented as much as 30-fold within 60 minutes after leukoregulin target cell interaction. Various sized preparations of fluorescein-labelled dextran demonstrate that molecules as large as 20,000 daltons are taken up more rapidly by leukoregulin-treated cells. Leukoregulin also facilitates tumor cell uptake of insulin and rapidly modulates growth factor receptors as indicated flow cytometrically by down-regulation of transferrin uptake in K562 leukemia cells. Modulation of target cell drug uptake and growth factor binding in combination with lymphocyte cytotoxicity provides new and potentially powerful methods for controlling target cell function and, in particular, for more specific and potent tumor cell destruction.

New sources for improved biotechnological production of leukoregulin necessary for isolation and molecular cloning of the leukoregulin gene have been developed this past year. Leukoregulin is not constitutively synthesized; it must be induced following antigen or other ligand membrane interaction or acute perturbation as occurs with phorbol diester treatment. Stimulated lymphocytes secrete only small quantities of labile leukoregulin along with large amounts of many other lymphokines. Molecularly homogeneous leukoregulin, with a pI of 5.1 and a molecular weight of 50,000 daltons at pH 7.4 and conditions approximating physiological ionic strength, has been isolated from both phytohemagglutinin-stimulated human peripheral blood lymphocytes and from 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated RPMI 1788 lymphocytes using sequential tangential ultrafiltration, anion exchange chromatography, preparative immobiline polyacrylamide flat bed gel electrophoresis and preparative spherical matrix molecular sizing high performance liquid chromatography (HPLC). Quantitative protein and activity measurements indicate that leukoregulin has a very high specific activity, estimated at greater than one million cytostatic K562 units/picogram. This approximates 5 x 10E19 units/mole, indicating that one molecule of leukoregulin/target cell is sufficient to modulate target cell function.

As a result of the high specific activity of leukoregulin, lymphokine preparations produced by either fresh human peripheral blood lymphocytes or by lymphocyte cell lines contain extremely small quantities of the lymphokine. This necessitates pilot scale biotechnological production of leukoregulin in hundreds of liters of unfractionated lymphokine to generate sufficient leukoregulin for molecular cloning of the gene. Cloned RPMI 1788 cell lines have been developed which proliferate rapidly in chemically defined serum-free medium and high producer clones have been isolated. The availability of larger quantities of native biologically active leukoregulin from cloned cell lines will accelerate production of antibodies to leukoregulin and cloning of the leukoregulin gene necessary for expression and production of recombinant human leukoregulin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04629-23 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. A. DiPaolo	Chief	LB	NCI
Others:	P. E. Bowden	Visiting Associate	LB	NCI
	J. Doniger	Senior Staff Fellow	LB	NCI
	N. C. Popescu	Research Microbiologist	LB	NCI
	M. Ruthsatz	Visiting Fellow	LB	NCI
	C. D. Woodworth	Senior Staff Fellow	LB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biology

SECTION

Somatic Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	5.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A number of the human papillomaviruses (HPVs) associated with human genital tract lesions were examined for their ability to immortalize cells. Those associated with benign genital warts, such as HPV-6 and HPV-11, did not immortalize foreskin epithelial cells or become integrated after transfection in a suitable vector. Others such as human papillomavirus (HPV)-16, HPV-18, HPV-33, and HPV-35, which are found associated with a high percentage of cervical carcinomas, did immortalize epithelial cells from either the foreskin or exocervical area. The immortalizing ability of HPV-16 is tentatively associated with E6/E7 genes. That immortalization is not due to a rather unusual genetic background of the cells used was demonstrated by utilization of epithelial cells from a number of different individuals, none of whom had any HPV genes in their cells. Furthermore, immortal cervical cells express keratin synthesis identical to that associated with exocervical epithelial cells, proving that the lines immortalized were of exocervical origin. An in-depth chromosome analysis of foreskin-derived epithelial cells transfected and immortalized by HPV-16 DNA demonstrated that there were complex translocations, deletions, telomeric associations, achromatic lesions and partial chromosome duplications. Furthermore, integration sites of the viral sequences were localized at the junction of chromosome translocations, at site of achromatic lesions, and within duplicated chromosome segments. In some lines these regions of duplication had integrated HPV-16 near proto-oncogenes such as _arg_ and _trk_. Furthermore, duplication and amplification of cellular sequences may have originated at nearby fragile sites. HPV-16 integration in the cellular genome is directly responsible for the induction of chromosome alterations and may be an essential step for neoplastic development.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. DiPaolo	Chief	LB NCI
P. E. Bowden	Visiting Associate	LB NCI
J. Doniger	Senior Staff Fellow	LB NCI
M. Rathsatz	Visiting Fellow	LB NCI
N. C. Popescu	Research Microbiologist	LB NCI
C. D. Woodworth	Senior Staff Fellow	LB NCI

Objectives:

This study investigates factors and mechanisms responsible for modulating neoplastic transformation of human and other cells and vital aspects in the etiology and prevention of cancer. The specific objectives are: (1) to define the role of chemical, physical, and biological agents pertinent to the process of carcinogenesis; (2) to characterize the cellular and chromosomal alterations associated with carcinogenesis; (3) to evaluate the relationships between DNA metabolism and carcinogenesis.

Methods Employed:

Cultures are made with freshly isolated cells from animals and humans as well as from cell lines which exhibit some of the properties associated with nontransformed cells. To maintain protracted logarithmic multiplication of human foreskin and exocervical epithelial cells, a complete medium, MCDB 153-LB, was devised consisting of variable concentrations of amino acids and hormones but without serum. This medium allows the use of differentiation markers in conjunction with the transformation process. Transfection of mammalian cells is accomplished by calcium phosphate precipitation, DEAE-dextan, protoplast fusion, or electrofusion. Analysis of DNA and RNA in transformed cells is by agarose gel electrophoresis, Southern and Northern blotting, DNA restriction analysis, gene cloning, c-DNA cloning, and DNA sequencing. High resolution prophase and prometaphase banding are used to identify structural chromosome alterations. The molecular *in situ* chromosome hybridization technique devised by this laboratory is being used for gene mapping and to identify proto-oncogene transposition.

Major Findings:

The primary emphasis during the past year has involved (1) investigating specific viral gene products and/or specific interactions with the cellular genome that are crucial for immortalization, and (2) establishing a model utilizing exocervical epithelial cells, negative for human papillomavirus (HPV), since these cells are the target for cervical cancer associated with HPV infection. The importance of integration of HPV DNA for immortalization of foreskin epithelial cells was demonstrated by comparing the results obtained with HPV-1a, -5, -6b, and -11 which are not associated with cancer of the cervix and HPV-16, -18, -31 and -33 which have been found in malignant

cervical cancer. Immortalized lines were obtainable only after transfection with HPV DNA associated with malignancy; the others were ineffective. The immortalized lines contained integrated HPV viral sequences and expressed HPV mRNA, whereas DNA from epithelial cells transfected with noncancerous-associated virus DNA contained only intact HPV sequences. To determine the HPV-16 open reading frames (ORFs) responsible for conferring immortality, various ORFs were cloned into expression vector, pD5, which contained the adenovirus major late promoter and SV-40 enhancer element. Combinations of pD5/E6 and pD5/E7 extended the life span of foreskin epithelial cells.

In cervical adeno- and squamous carcinoma cell lines, HPV-18 sequences were found integrated non-randomly at constitutive or heritable fragile sites and near proto-oncogenes involved in cell growth control or tumorigenesis. The importance of HPV integration into the cellular genome was also demonstrated by the failure of HPV-transfected human foreskin epithelia, negative for HPV-16 DNA, to progress as immortalized cell lines. By in situ hybridization of radiolabeled viral DNA probes, single or multiple HPV-16 DNA sequences were found integrated in immortal lines at the junction of chromosome translocations within homogeneous staining regions (HSR) achromatic lesions or duplicated segments. Thus, for the first time HPV-16 integration into the cellular genome has been shown to be directly responsible for the induction of chromosome rearrangements, including HSR formation. Single grains were detected at the junction of chromosome 16 and an unidentified chromosome region. Multiple grains were identified on duplicated regions of the long arm of chromosome 1 near arg and trk proto-oncogenes in three lines. In two cell lines multiple grains indicative of viral amplification occurred within HSR on chromosome 12 and 20. In the first case HPV-16 integration and subsequent amplification of cellular and viral sequences may have originated at the fragile site, whereas on chromosome 20 the amplification may have affected the src gene located near the amplified region.

Incipient foreskin epithelial lines were analyzed for primary and secondary structural or numerical chromosome alterations. Five immortalized lines examined 120-140 population doublings after HPV-16 DNA transfection exhibited clonal alterations with complex translocations, telomeric associations, achromatic lesions, and deletions common for solid tumors. Partial trisomy of the long arm of chromosome 1, a specific alteration in solid tumors, was observed in three lines and structural changes of chromosome 20 in two lines. Both chromosomes carry proto-oncogenes implicated in several forms of human cancer. In addition, all cell lines had chromosomes with tandem duplications, HSR, and cells with double minutes. These types of alterations are associated exclusively with malignant progression or drug resistance and reflect gene amplification. In two cell lines HSR were identified on chromosomes 12 and 20 as extensions of the telomeric regions of the long arms. In two other lines HSR were on chromosome 14, either as an intercalary segment at the middle of the chromosome or terminal on the long arm. The incidence of cells with double minute chromosomes varied from 1 to 200 to 1 to 50 cells. Also, the number of double minutes per cell varied from a minimum of 8 to as many as 20. The complexity of chromosome alterations and the formation of abnormalities indicative of gene amplification strongly indicate that these genetic alterations were involved in the immortalization of HPV-16-transfected keratinocytes.

An in vitro system has been developed to study the interaction between HPV DNA and normal human exocervical epithelial cells (HCX) because HPV-associated cervical cancer occurs usually at the squamocolumnar junction of the cervix. HCX were isolated from the cervical transformation zone, grown in serum-free medium, and transfected with recombinant HPV-16 or -18 DNA. Although normal HCX senesced after 30 to 40 population doublings, HPV-transfected HCX continue to proliferate (>200 population doublings to date). Eight HPV-immortalized HCX lines were established which contained between 2 to 20 integrated copies of HPV DNA per cell. All eight HPV-immortalized cell lines expressed 1.8 and 4.2bp HPV mRNAs and various other HPV transcripts. Immortalized cells divided rapidly (30 to 46 h) and morphologically resembled normal HCX. In HPV-immortalized HCX, the synthesis of specific keratins (7,8,1,18,19), characteristic of simple epithelia, increased slightly, while synthesis of keratins 6 and 16/17 decreased. Comparison of keratins of immortalized HCX normal endo- and exocervical epithelial cell cultures proved that the lines were of exocervical origin. Alterations in keratin expression were more dramatic in sublines resistant to serum-induced terminal differentiation. Serum-resistant lines were similarly isolated from both immortalized keratinocytes and HCX. Certain simple epithelial keratins (7,8,19) and keratin 13 were up-regulated, while expression of squamous epithelial keratins (5,6,14,16/17) was down-regulated. Sublines resistant to serum-induced terminal differentiation were selected by culture in the presence of serum. These differed in keratin expression from the parent lines. These sublines may represent another stage in the development of carcinogenesis because they have acquired additional characteristics of transformed cells. HPV-immortalized cell lines represent an appropriate model for studying factors that regulate HPV gene expression in normal HCX and for examining the influence of cocarcinogens.

Another area of major investigation in the laboratory has been the study of oncogene activation related to steps in carcinogenesis. Five tumorigenic guinea pig cell lines with mutationally activated N-ras alleles also exhibited up-regulated N-ras mRNA. Mutational activation and mRNA up-regulation were limited to tumorigenic cells; preneoplastic progenitors were unaffected. Therefore, up-regulation occurred at a late stage of carcinogenesis closely associated with acquisition of tumorigenicity. cDNA and S1 protection analysis demonstrated that the polyadenylation site of the short N-ras message and the mRNA start sites were different from that reported for humans. The promoter region contained no canonical TATA or CCAAT boxes, but exhibited GGGCGG and CCGCCC SP1 binding motifs characteristic of growth control genes such as H-ras, K-ras, and the epidermal growth factor (EGF) receptor. Furthermore, the sequence, CCACGTG, was found about 60 bp upstream of the mRNA start sites between two GC boxes. This same motif exists in the adenovirus major late promoter and is the binding site for the E1a protein and cellular transcription factor, AP-1. Because both mutant and wild-type alleles were up-regulated and no mutations were found in the promoter region, up-regulation is modulated by a trans-acting cellular factor, possibly AP-1. Coordinate N-ras mutational activation and up-regulation in five independenttumorigenic lines with unique chromosome constitutions suggest that both events are required for expression of the neoplastic phenotype.

N-<u>ras</u> messages were also observed in the cDNA library in two of ten N-<u>ras</u> cDNA clones. Exon II was spliced directly to exon IV, and exon III was absent. Because exon three is missing, the sequence translates to a truncated <u>ras</u>-related protein that contains the amino acid sequence of exons I and II plus nine new residues; a reading frame change occurs at the II to IV splice. Thus, 40% of the amino acids are missing. The deleted region eliminates domains necessary for guanosine triphosphate binding, transforming activity, and membrane localization. Although its function is not clear, alternatively spliced N-<u>ras</u> represents 5 - 40% of mature N-<u>ras</u> message and probably has an important cellular function.

<u>Publications</u>:

DiPaolo JA. Human papillomavirus 16D DNA and epithelial cell immortalization. In: Feo F, eds. Models and mechanisms in chemical carcinogenesis. New York: Plenum Press (In Press).

DiPaolo JA, Donniger J, Popescu NC. Induction of malignant transformation in hamster fetal cells (HFC) by diverse environmental carcinogens. In: Seemayer NH, Hadnagy W, eds. Environmental Hygiene. Heidelberg-Berlin-New York: Springer Verlag (In Press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04673-17 LB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

The Immunobiology of Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. H. Evans	Chief, Tumor Biology Section	LB	NCI
Others:	P. M. Furbert-Harris	IRTA Fellow	LB	NCI
	E. K. Farley	Visiting Fellow	LB	NCI
	B. A. Gelleri	Visiting Fellow	LB	NCI
	F. D'Alessandro	Visiting Fellow	LB	NCI
	P. D. Baker	Microbiologist	LB	NCI
	A. C. Wilson	Chemist	LB	NCI
	J. R. Ortaldo	Chief	LEI	NCI

COOPERATING UNITS *(if any)*

Laboratory of Neurophysiology, NINCDS, NIH (P. A. Sheehy, J.L. Barker)
Department of Surgery, Duke University (C. Slingluff and H. L. Seigler)

LAB/BRANCH

Laboratory of Biology

SECTION

Tumor Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	4.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Lymphokines, interleukins, and other immunological hormones, i.e., the secretory bioregulatory macromolecules of lymphocytes, macrophages, and other leukocytes, are being studied to define their effective anticarcinogenic and tumor cell growth inhibitory activities. Leukoregulin, a lymphokine recently isolated during the course of this project, can prevent carcinogenesis and inhibit tumor cell growth. Anticarcinogenic action is direct, irreversible and occurs without cytotoxicity. Inhibition of tumor cell growth is primarily reversible but can become irreversible due to increased susceptibility of preneoplastic and neoplastic cells to cytolytic destruction by natural killer cells resulting from leukoregulin target cell interaction. Leukoregulin at very high concentrations is also directly cytolytic for tumor cells. The direct-acting anticarcinogenic activity of leukoregulin is more potent than the tumor cell inhibitory activity; but by also being able to increase target cell sensitivity to the cytoreductive action of naturally cytotoxic lymphocytes, leukoregulin can be an effective homeostatic mechanism for control of carcinogenesis at its later stages of development. Leukoregulin-induced changes in plasma membrane permeability are partially dependent upon extracellular ionic calcium and are accompanied by increased calcium flux, the rapid opening and closing of plasma membrane single ion channels and translocation of protein kinase C from the cytosol to the plasma membrane which may be important events in the molecular pathway resulting in inhibition of tumor and other abnormal cell proliferation by this immunologic hormone. Leukoregulin induces identical changes in target cell plasma membrane permeability as occur during natural killer lymphocyte cytotoxicity, providing strong evidence that it is an intrinsic mediator or element of the natural cytotoxicity reaction and possibly signifying its central role in immunological homeostasis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. H. Evans	Chief, Tumor Biology Section	LB	NCI
F. D'Alessandro	Visiting Fellow	LB	NCI
E. K. Farley	Visiting Fellow	LB	NCI
B. A. Gelleri	Visiting Fellow	LB	NCI
P. M. Furbert-Harris	IRTA Fellow	LB	NCI
P. D. Baker	Microbiologist	LB	NCI
A. C. Wilson	Chemist	LB	NCI
J. R. Ortaldo	Chief	LEI	NCI

Objectives:

This project provides a means to study the potential of the normal immune system to prevent, suppress, inhibit or enhance the growth of incipient tumor cells during carcinogenesis. Natural cytotoxicity of macrophages, lymphocytes and lymphokines, alone or in combination, are being studied at various stages of carcinogenesis to provide insight 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 natural and induced immunity may be augmented to suppress and even prevent the final aspects of carcinogenesis--the transition from the preneoplastic to the neoplastic state.

The primary objective of this project is to elucidate, at the target cell level, the interactions between cell surface alterations accompanying the development of carcinogenesis and host mechanisms able to prevent, inhibit, or 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. Particular emphasis is placed on natural and induced cellular and humoral immunobiological interactions due to the frequent occurrence of neoantigens, re-expression of fetal antigens, and alterations in alloantigens on pre-neoplastic and tumor cells.

Methods Employed:

Normal and malignant animal and human cells in culture, including chemical and physical carcinogen-treated cells at progressive stages in the transformation process, are studied for somatic cell changes such as altered morphology, morphological transformation, anchorage-independent growth and tumorigenicity in relation to their interaction and response to components of the immune system. Immunobiological techniques including direct and indirect immunofluorescence, flow cytometry, complement fixation, colony inhibition, radionuclide uptake and release, delayed hypersensitivity skin reactions, and tumor transplantation rejection are employed in analyzing cell membrane changes and in assessing host interactions to the changes. A major emphasis

is placed upon flow cytometry and cell sorting to identify plasma membrane and intracellular alterations responsible for regulation of cell proliferation and carcinogenesis.

Major Findings:

Characterization of the molecular and biological pathways of leukoregulin action this past year focused upon further elucidation of the mechanism of action and potential usefulness of this direct-acting anti-cancer lymphokine. Examination of the physiologic role of leukoregulin demonstrates that its ability to up-regulate target cell sensitivity to natural killer cell immunocytotoxicity extends to lymphokine activated killer (LAK) lymphocyte cytotoxicity and to T lymphocyte killing of allogeneic human tumor cells. Continued evaluation of leukoregulin's ability to facilitate the target cell uptake of pharmacologically active molecules indicates that the leukoregulin membrane channel freely admits molecules as large at 20,000 daltons. In the third area concentrating upon the biotechnological production of leukoregulin, clones of the RPMI 1788 leukoregulin-producing human lymphocyte cell line have been developed that produce large amounts of the lymphokine required for cloning of the leukoregulin gene and expression of recombinant human leukoregulin.

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cell lysis. Molecular sizing and calcium dependence biochemical studies indicate that the two lymphokines are distinct. In collaboration with J. R. Ortaldo, Chief of the Laboratory of Experimental Immunology, NCI, antibody neutralization studies employing polyclonal and monoclonal anti-NKCF antibodies demonstrate no inhibition of leukoregulin activity, providing further evidence that leukoregulin and NKCF are distinct molecules.

The up-regulation of target cell sensitivity to natural lymphocyte cytotoxicity occurs concurrently with an increase in target cell plasma membrane permeability and increased uptake of pharmacologically active macromolecules. The uptake of metabolic inhibitory antibiotics as large as 4000 daltons is augmented as much as 30-fold within 60 minutes after leukoregulin target cell interaction. Various sized preparations of fluorescein-labelled dextran demonstrate that molecules as large as 20,000 daltons are taken up more rapidly by leukoregulin- treated cells. Leukoregulin also facilitates tumor cell uptake of insulin and rapidly modulates growth factor receptors as indicated flow cytometrically by down-regulation of transferrin uptake in K562 leukemia cells. Modulation of target cell drug uptake and growth factor binding in combination with lymphocyte cytotoxicity provides new and potentially potent methods for controlling target cell function and, in particular, for more specific and potent tumor cell destruction.

New sources for improved biotechnological production of leukoregulin necessary for isolation and molecular cloning of the leukoregulin gene have been developed this past year. Leukoregulin is not constitutively synthesized; it must be induced following antigen or other ligand membrane interaction or acute membrane perturbation which occurs in cells treated with the phorbol diester 12-O-tetradecanoylphorbol 13-acetate (TPA). Stimulated lymphocytes secrete only small quantities of labile leukoregulin along with large amounts of many other lymphokines. Molecularly homogeneous leukoregulin, with a molecular weight of 50,000 daltons at pH 7.4 and conditions approximating physiological ionic strength and a pI of 5.1, has been isolated from both phytohemagglutinin-stimulated human peripheral blood lymphocytes and from TPA-stimulated RPMI 1788 lymphocytes using sequential tangential ultrafiltration, anion exchange chromatography, preparative immobiline polyacrylamide flat bed gel electrophoresis and preparative spherical matrix high performance liquid chromatography (HPLC). Quantitative protein and activity measurements indicate that leukoregulin has a very high specific activity, estimated at greater than one million cytostatic K562 units/picogram. This approximates 5 x 10E19 units/mole and indicates that one molecule of leukoregulin/target cell is sufficient to modulate target cell function.

As a result of the high specific activity of leukoregulin, lymphokine preparations produced by either fresh human peripheral blood lymphocytes or by lymphocyte cell lines contain extremely small quantities of the lymphokine. This necessitates pilot scale biotechnological production of leukoregulin in hundreds of liters of unfractionated lymphokine to generate sufficient leukoregulin for molecular cloning of the gene. Cloned RPMI 1788 cells lines have been developed that proliferate rapidly in chemically defined serum-free medium and high producer clones have been isolated. The availability of larger quantities of native, biologically active leukoregulin from cloned cell

lines will accelerate production of antibodies to leukoregulin and cloning of the leukoregulin gene necessary for expression and production of recombinant human leukoregulin.

Publications:

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01CP05499-02 LB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)
Chromosome Alterations and Proto-Oncogene Transposition in Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	N. C. Popescu	Research Microbiologist	LB	NCI
Others:	J. A. DiPaolo	Chief	LB	NCI
	K. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
	S. Y. Cheng	Research Chemist	DCBD	NCI
	S. Eva	Visiting Scientist	LCMB	NCI
	S. Tronick	Chief, Gene Structure Section	LCMB	NCI

COOPERATING UNITS (if any)

Fox Chase Cancer Center, Philadelphia, PA (P. Lazo)

LAB/BRANCH
Laboratory of Biology

SECTION
Somatic Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	0.9	0.5

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

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

Fyn, a novel human proto-oncogene of the src gene family, has been localized by in situ hybridization on chromosome 6. This localization shows that src genes are located on different chromosomes and represent distinct loci. Another proto-oncogene, dbl, isolated from a diffuse B-cell lymphoma, was localized on the X chromosome. Sex chromosomes, therefore, like autosomes, have oncogenic potential. A gene coding for a human cellular thyroid hormone-binding protein (P55) was assigned on chromosome 17. The precise localization of these three genes will permit the evaluation of possible effects of chromosome changes on their structure and activity in malignancies and chromosome syndromes. Cellular sequences flanking an integrated human papillomavirus (HPV)-18 DNA copy was localized on chromosomes from normal and HeLa cells to chromosome 8 near the myc gene. The integration at this site was accompanied by amplification of viral sequences and increased myc RNA expression. These alterations may have contributed to the development of this malignancy.

Z01CP05499-02 LB

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. C. Popescu	Research Microbiologist	LB	NCI
J. A.DiPaolo	Chief	LB	NCI
K. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
S. Y. Cheng	Research Chemist	DCBD	NCI
S. Eva	Visiting Scientist	LCMB	NCI
S. Tronick	Chief, Gene Structure Section	LCMB	NCI

Objectives:

This project is directed toward understanding the role of chromosome alterations in malignant transformation. The localization of proto-oncogenes and breakpoints on specific chromosome rearrangements or deletions may lead, at the molecular level, to the identification of new recombination mechanisms in human carcinogenesis.

Methods Employed:

High resolution prophase and prometaphase banding is used to identify structural alterations. Chromosome changes are further characterized by specific methods for visualization of constitutive heterochromatin (C band), ribosomal genes (N-band) and immunochemical methods using antinucleoside antibodies on denatured chromosomes. The molecular in situ chromosome hybridization technique and banding procedure devised by us are being used for gene mapping and proto-oncogene localization on cancer cells, as well as for assigning the integration site of viral sequences on chromosomes from in vitro transformed cells and human cancers.

Major Findings:

Human cancer cytogenetic and molecular evidence implicate chromosomal changes and proto-oncogene alterations in neoplastic development. Fyn gene, a transforming gene, was cloned from human C-DNA libraries in the Laboratory of Cellular and Molecular Biology. The protein products of this gene share structural features with src genes. With a DNA probe representing the coding sequences, the Fyn gene was localized by in situ hybridization at 6q21 within the same region of ros and myb proto-oncogenes raising the possibility that these genes may be closely linked at the molecular level. The existence of a fragile site at 6q21 may, in part, account for the large number of hematological malignancies and solid tumors with chromosome alterations at this region that could involve the Fyn proto-oncogene.

A cDNA for the gene that encodes for a human cellular thyroid hormone binding protein (P55) has been isolated in the Laboratory of Molecular Biology. P55 is the key enzyme responsible for the post-translational modification in the synthesis of collagen and may be involved in the disulfide bond formation of proteins. The gene for P55 was localized on chromosome 17 at band q25. The

localization at this site shows that the P55 gene is not linked to other nuclear thyroid hormone binding protein genes, as erbA1 and erbA2 are located at distant sites on chromosome 17q11-21 and 3p21-pter, respectively. Gene balance in chromosome 17 is considered essential for normal embryonic development. The chromosome syndrome associated with the trisomy 17q-ter may be the result of an altered dosage of genes located at this region. The location of P55 gene and Hu-ets-1 proto-oncogene correspond to the breakpoints of a 11; 17 (q22; q25) translocation identified in acute non-lymphocytic leukemia. The influence of chromosome alterations on the expression and activity of P55 gene remains to be investigated in neoplasia and chromosome syndromes such as partial trisomy 17 syndrome.

The dbl proto-oncogene was localized by in situ hybridization on the long arm of chromosome X at band q27. Related sequences were also identified on the long arm of chromosome 3. The in situ localization places the dbl gene at the fragile site associated with the most frequent form of mental retardation. In addition, anti-hemophilic factor IX is located within the same region of major importance on the X chromosome.

HeLa, a cervical carcinoma cell line, has 10-50 copies of human papillomavirus (HPV) type 18 DNA. Three integrated copies of HPV-18 were isolated from HeLa cell DNA and identified as Hind III bands with a common 5′ flank. Cellular sequences flanking an integrated HPV-18 DNA copy were localized by in situ hybridization of metaphases or prometaphases from normal and HeLa cells on chromosome 8 band q24. This site is one of the four HPV-18 DNA integration sites on HeLa cells and coincides with the locations of a constitutive fragile site and the c-myc proto-oncogene. The integration of HPV DNA may have been facilitated by the fragile site near the c-myc proto-oncogene. With viral probes, multiple copies of viral DNA sequences were demonstrated; E6, E7 AND E1 open reading frames are amplified fivefold, and the late viral DNA region, the viral long control region and cellular flanking sequences 15-fold. The amplification of cellular sequences does not include the myc locus since this gene is neither rearranged nor amplified. However, myc RNA level normalized to the level of actin and B2-microglobulin RNA is increased 3- to 20-fold, respectively. The amplified HPV-18 DNA sequences near the myc gene may account for immortalization and/or proliferative capacity of this malignancy. The specificity of the flanking sequences by in situ hybridization at 8q24 suggests that viral integration occurred independently at multiple sites. Two other HPV sites overlap to the location of c-abl and c-sis proto-oncogenes. Integrations at these sites may have also contributed to the neoplastic development of these cells.

Publications:

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Popescu NC, Chahinian AP, DiPaolo JA. Nonrandom chromosome alterations in human malignant mesothelioma. Cancer Res 1988;48:142-8

Popescu NC, Cheng S, Pastan I. Chromosomal localization of the gene for a human thyroid binding protein. Am J Hum Genet 1988;42:560-65

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05552-01 LB

PERIOD COVERED

October 1, 1987 to Sept 30, 1988

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

Lymphokine Modulation of Human Cervical Epithelial Cell Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. H. Evans	Chief, Tumor Biology Section	LB	NCI
Others:	P.M. Furbert-Harris	IRTA Fellow	LB	NCI
	C.D. Woodworth	Senior Staff Fellow	LB	NCI
	J.A. DiPaolo	Chief	LB	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Biology

SECTION

Tumor Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Human papillomavirus (HPV)-immortalized cervical epithelial cells and HPV-positive cervical carcinomas are being evaluated as a model of lymphokine modulation of epithelial cell sensitivity to natural immunologic cytotoxicity. Epithelial cells, immortalized by transfection with HPV-16 DNA, are treated with 2.5 U/ml leukoregulin for 1 hr and mixed with natural killer (NK) cells or interleukin 2 (IL2)-induced lymphokine activated killer (LAK) cells at E:T ratios up to 50:1 in a 4 hr 51 Cr release assay. Transfected exocervical cells are evaluated at early and late passages to determine stages at which they can be destroyed by NK and/or LAK cell cytotoxicity. Both early and late passage HPV-16-immortalized cells are resistant to NK but sensitive to LAK cell cytotoxicity. Leukoregulin treatment of the target cells induces a modest sensitivity to NK ($p<.05$) and markedly up-regulates LAK sensitivity 1.5 to fourfold. QG-U and C4-1 cervical carcinoma cells possessing integrated HPV-16 and HPV-18 DNA, respectively, are resistant to NK but sensitive to LAK. The response of leukoregulin-treated QG-U and C4-1 cells is similar to HPV-16-immortalized targets, i.e., leukoregulin confers sensitivity to the once NK-resistant tumor target cells and dramatically increases their sensitivity to LAK. Although the HPV-immortalized exocervical cells containing integrated HPV DNA are not yet tumorigenic, they mimic the response of established HPV-16 or -18-positive cervical carcinoma cells. These observations provide a foundation for the usefulness of this model in evaluating the therapeutic potential of leukoregulin alone, or in combination with other cytokines or chemotherapeutic drugs in the prevention and treatment of cervical dysplasia and neoplasia.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Furbert-Harris	IRTA Fellow	LB	NCI
C. H. Evans	Chief, Tumor Biology Section	LB	NCI
C. D. Woodworth	Senior Staff Fellow	LB	NCI
J. A. DiPaolo	Chief	LB	NCI

Objectives:

The overall objective of this project is 1) to evaluate the susceptibility of human papillomavirus (HPV)-immortalized human cervical epithelial (HCX) cells at different stages of cocarcinogenesis and tumorigenesis to natural killer lymphocyte (NK) and lymphokine-activated killer (LAK) cytotoxicity and 2) to investigate the NK and LAK responses to these cells after treatment with immunoregulatory substances, e.g., lymphokines, and other agents regulating cell growth and differentiation.

Papillomaviruses are known to infect squamous epithelium and are associated with benign and malignant epithelial tumors. HPV-16 and -18 are the predominant viral types found associated with premalignant and malignant cervical lesions. HPV DNA exists in an episomal form in most benign and premalignant cervical lesions, while in cervical cancers it is found integrated into the cellular genome. HPV-16 DNA immortalized human cervical cell lines have been developed which have HPV DNA integrated within the host genome. However, these HPV-immortalized cell lines are not tumorigenic in nude mice. These cell lines are being used to study the role of HPV as a cofactor in the multistep tumorigenesis process, resulting in the development of cervical cancer.

Although the susceptibility of HPV DNA-associated cervical carcinoma cells to NK cytotoxicity has been studied, no information exists regarding their susceptibility to LAK cell cytotoxicity, nor has lymphokine modulation of cervical carcinoma cell sensitivity to either NK or LAK lymphocyte cytotoxicity been reported. Preliminary studies on the susceptibility of HPV-immortalized human cervical cells to NK and LAK cell cytotoxicity are in progress. Additional aims of this project are (1) to study the action of lymphokines on the surface antigens of HPV-transfected human cervical (HCX) cells including measurement of HLA class 1 and II antigens and other antigen marker expression, and to determine whether interferon (IFN), tumor necrosis factor (TNF), leukoregulin (LR), interleukin-1 (IL-I), and transforming growth factor β (TGFβ), alone or in combination, modulate the expression of these antigens; (2) to evaluate the reactivity of NK and LAK cells after cytokine interaction; (3) to assess the effect of cytokine treatment on HPV mRNA, HPV DNA integration, HLA mRNA and mRNA of other antigens; (4) to determine whether cytokine interaction affects the state of differentiation; (5) to determine whether other agents such as hormones, butyrate, or retinoic acid, induce differentiation; and (6) to evaluate the reactivity of NK and LAK after treatment with agents affecting the differentiated state/function of HPV-transfected HCX cells.

Methods Employed:

HPV-16 DNA-immortalized cervical epithelial cells are evaluated by flow cytometry for the presence of surface antigens, e.g., HLA , transferrin receptor and other growth factor receptors, and cell surface markers of differentiation. The cells are treated with lymphokines, e.g., IFN, TNF, IL-1, leukoregulin, and TGF beta, alone and in various combinations, and their effect on the expression of cell surface antigens and growth factor receptors analyzed. Additionally, NK and LAK cell cytotoxicity against these lymphokine-treated cells is determined using a 4 hr chromium release assay. Molecular analysis (Southern and Northern blot profiles) of viral mRNA, surface antigen mRNAs and viral DNA modulation is performed after lymphokine treatment. During the development of carcinogenesis and tumorigenesis, HPV-16 DNA-immortalized cells are also being evaluated for cellular differentiation properties such as changes in the keratin profile. Agents known to induce cellular differentiation (butyrate, retinoic acid, hormones) are used to treat HPV-transfected HCX cells and the cells are evaluated for changes in differentiation properties. Lymphokines, alone and in combination, are also being studied for their differentiation-inducing capabilities, and NK and LAK cell activity against these different stages are under investigation.

Major Findings:

Several HPV-16 DNA-immortalized HCX lines have been examined with regards to their sensitivity to both NK and LAK cell cytotoxicity. The modulation of NK and LAK sensitivity by leukoregulin has also been evaluated. HPV-16 DNA HCX lines are resistant to NK and sensitive to LAK cell cytotoxicity. Leukoregulin treatment of these target cells induces and/or up-regulates their sensitivity to NK and LAK cell cytotoxicity, with LAK cell cytotoxicity being up-regulated more than NK cell cytotoxicity. When the HPV-16 DNA immortalized cell lines are evaluated according to their length of time in culture, early passage cells are less sensitive to the enhancing effect of leukoregulin than are the late passage cells. The early passage cells react similarly to the control non-transfected cervical cells. These observations are the first demonstration that leukoregulin up-regulates the sensitivity of papilloma viral DNA-immortalized human epithelial cells to different forms of natural lymphocyte cytotoxicity.

ANNUAL REPORT OF

THE LABORATORY OF CELLULAR CARCINOGENESIS AND TUMOR PROMOTION
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

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; and (5) elucidate the mechanism by which certain pharmacologic agents inhibit carcinogenesis.

The Laboratory is composed of three sections, and each 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 (IVP) 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; and (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 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 and Relationship to Early Events in Epidermal Carcinogenesis: Cellular and molecular aspects of chemical carcinogenesis in lining epithelia are studied in mouse epidermis by in vivo and in vitro techniques. The initiation event in skin carcinogenesis is highly correlated to an alteration in the program of terminal differentiation of epidermal cells. In cell culture, epidermal differentiation is regulated by the concentration of extracellular calcium. Induction of terminal differentiation by increasing the calcium concentration in the culture medium causes a two- to

threefold increase in the level of intracellular-free calcium. Cells of initiated lines which survive in medium with high calcium showed an altered response to increased external calcium, with a sharp four- to ninefold peak of intracellular-free calcium in all cells within 2 minutes. These differences in intracellular calcium between normal and initiated keratinocytes may be related to alterations in phosphoinositide metabolism. The ras oncogene is highly correlated to the initiated phenotype in epidermis. Introduction of an activated ras gene into normal keratinocytes alters their phenotype to that of papilloma cells. Chemically induced papillomas yield an activated ras oncogene with a mutation at codon 61. Papilloma cells and initiated cells are resistant to the differentiation-inducing effects of phorbol ester tumor promoters. Since phorbol esters induce differentiation in normal cells, papilloma cells can be selected among an excess of normal cells in culture by their ability to continue to proliferate in culture medium containing phorbol esters. In culture, introduction of the v-fos oncogene into initiated cells with an activated ras results in their conversion to malignancy. Transforming growth factor-β (TGF-β) is elaborated by normal keratinocytes induced to differentiate by 12-O-tetradecanoylphorbol-13-acetate (TPA) or by increasing external calcium. This secretion is not altered by introduction of v-ras into normal keratinocytes. In vivo, several classes of benign tumors can be induced by initiation and promotion. Papillomas with a high risk for spontaneous conversion to carcinomas are also most responsive to chemical-converting agents. Malignant conversion can be accomplished by a single injection of cisplatin.

Molecular Regulation of Epidermal-Specific Differentiation Products: cDNA clones corresponding to the major proteins expressed in mouse epidermis have been isolated and characterized. Using a combination of *in situ* hybridization with RNA probes (which are specific for individual mRNAs) and indirect immunofluorescence with monospecific antisera (which were elicited with synthetic peptides corresponding to unique sequences within each protein), it is possible to show that these genes belong to at least four subsets: those expressed predominantly in the proliferating basal layer of the epidermis; those expressed predominantly in the differentiated suprabasal spinous layers and, to a lesser extent, in the granular layer; those expressed only in the granular layer; and those expressed only under hyperproliferative conditions. Genes representing each subset have been isolated and sequenced. Various strategies have been employed to identify sequences regulating the expression of these genes, including vector constructs using different regions of the genomic clones to drive expression of the chloramphenicol acetyltransferase gene and the production of transgenic mice which express a human differentiation-associated keratin gene in a tissue- and developmental-specific pattern. A gene encoding a cysteine-rich protein, which is a major component of the cornified envelope, has been isolated and shown by *in situ* hybridization experiments to be expressed in the granular layer of the epidermis. A monospecific antiserum has been used to demonstrate that the C-terminal portion of this protein is detectable only on the inner surface of mature envelopes. Monospecific antisera that have been produced against mouse and human keratins and other epidermal-specific differentiation products have been used to study various stages of carcinogenesis, gene expression in mutant mice exhibiting developmental defects in epidermal differentiation, the induction of terminal differentiation in malignant cell lines by pharmacological agents, the in vivo kinetics of expression of the differentiation-associated keratins with respect to cell division, and requirements for the induction of terminal differentiation products in vitro.

Determinants for Susceptibility to Carcinogenesis: In vivo studies show that the SENCAR mouse is unusually sensitive to skin carcinogenesis by initiation and promotion, while the BALB/c mouse is resistant. We have developed a technique of grafting epidermal and dermal cells to athymic nude mice to form a reconstituted skin. Initiated cell lines 308 and SP-1, derived from initiated skin of BALB/c and SENCAR mice, respectively, have been developed and characterized. These lines form benign squamous papillomas in grafts and have an activated ras^{Ha} oncogene. We can thus reconstruct an "initiated" skin using mixtures of papilloma-forming cells with primary epidermal cells and dermal fibroblasts. Suppression of papilloma size occurs when normal SENCAR primary epidermal cells are grafted along with small numbers of SP-1 cells. This suppression is specific for SENCAR primary epidermal cells and does not occur with SENCAR primary dermal fibroblasts, BALB/c primary epidermal cells, or an initiated cell line derived from SENCAR skin which makes an apparently normal skin when grafted. BALB/c primary epidermal cells fail to suppress papilloma formation either from grafted SP-1 cells or the BALB/c-derived 308 cells. Papillomas formed on SENCAR mice by treatment with promoter alone, i.e., without exogenous chemical initiation, did not have an activated ras^{Ha} oncogene at codon 61. These papillomas presumably resulted from endogenously initiated cells. Cell lines being developed from these papillomas will presumably be useful in identifying the molecular lesions in endogenously initiated cells.

Immunological Techniques to Study the Interaction of Carcinogens with DNA: Antibodies specific for carcinogen-DNA adducts have probed the nature, extent, and consequences of in vitro and in vivo DNA modification. Biological samples substituted with 2-acetylaminofluorene (AAF), *cis*-diamminedichloroplatinum II (*cis*-DDP) and benzo(a)pyrene (BP) have been analyzed by quantitative immunoassays and by immunohistochemical procedures developed to localize adducts *in situ*. In hepatic DNA of rats chronically fed a carcinogenic dose of AAF, adduct removal was biphasic, suggesting two genomic compartments -- one from which adducts are removed rapidly and another from which they are removed slowly. These kinetics are not due to different liver cell types or DNA associated with more or less tightly-bound chromatin regions including the nuclear matrix. Comparison of adduct and tumor dose responses in bladder and liver of mice fed AAF chronically showed linear adduct and liver tumor formation, but non-linear bladder tumor formation. Immunohistochemical localization of aminofluorene-DNA (AF-DNA) adducts in livers of rats fed AAF demonstrated a non-uniform adduct distribution and no adducts in preneoplastic foci induced by several different hepatocarcinogenesis protocols. *Cis*-DDP-DNA adducts were measured in DNA from nucleated peripheral blood cells and several tissues obtained from cancer patients at multiple times during courses of *cis*-DDP therapy and/or at autopsy. Adduct accumulation occurred as a function of total cumulative dose. Disease response data on 55 ovarian cancer patients and 17 testicular cancer patients indicated that individuals with high adduct levels have a high rate of complete response to therapy. BP-DNA antigenicity was measured in enzyme-linked immunosorbent assays (ELISA) blood cell DNA of 43 firefighters and 40 controls. In both groups about 30% of the samples were positive, but mean adduct levels were higher in samples from firefighters. Other factors which contributed to sample positivity were the ingestion of charcoal broiled foods and smoking.

DIFFERENTIATION CONTROL SECTION: The Differentiation Control Section: (1) studies the biological and biochemical factors involved in normal differentiation of epithelial tissues; (2) uses pharmacological techniques to alter differentiation of normal, preneoplastic, and neoplastic epithelial cells to determine the

relevance of differentiation to carcinogenesis and to determine methods to intervene in preneoplastic progression; (3) studies the relationship between differentiation and growth control; (4) focuses on cell surface changes in differentiation and neoplasia.

Vitamin A and its derivatives, the retinoids, are of interest in cancer research because they play an essential role in the maintenance of normal differentiation in most epithelial tissues under normal physiological conditions and modulate growth by inducing differentiation of certain neoplastic cells. Vitamin A deficiency causes loosening of adhesive strength between the tracheal epithelium and the underlying connective tissue. Conversely, we have recently found that retinoic acid (RA) and other retinoids with biological activity in maintaining normal epithelial differentiation enhance the adhesiveness of cultured fibroblastic cells (NIH-3T3) to plastic dishes coated with specific extracellular matrix proteins, such as laminin and type IV collagen. Thus, the study of fibroblast cell adhesion induced by RA has become relevant to our understanding of the mechanism by which retinoids maintain epithelial cell differentiation.

In our attempts to investigate the possibility that RA may act by second messenger systems involving phosphatidylinositol turnover, we have discovered that the enhanced NIH-3T3 cells adhesiveness is accompanied by a marked (70%) reduction in the carrier-mediated accumulation of inositol in these cells. The effect of RA was relatively rapid (6 to 14 hr), cell density-dependent, reversible and it involved a reduction in Vmax with no change in the affinity. It also was specific for inositol in that the transport of other monosaccharides such as glucose, mannose, fucose and galactose was not affected in an appreciable manner. The possibility that this effect is the result of RA-induced structural modifications of cell surface carriers is presently being investigated.

In a parallel study intended to study the effect of the tumor-promoting agent phorbol-12-myristate, 13-acetate (PMA) on cell adhesiveness we discovered that PMA causes a dose-dependent enhancement in 3T3 cell attachment to plastic dishes coated with laminin and type IV collagen. This effect was very rapid (30 to 60 min) and correlated with structures known to exert tumor-promoting activity. This effect of PMA differs from the RA effect in time course and appears to involve activation of the receptors for laminin and type IV collagen, possibly through phosphorylation.

We are presently investigating compositional changes of the cell surface as caused by RA and PMA utilizing various probes for laminin, type IV and fibronectin receptors and the binding activity of various lectins.

MOLECULAR MECHANISMS OF TUMOR PROMOTION SECTION: The efforts of the Molecular Mechanisms of Tumor Promotion Section are directed toward understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. Particular attention is being devoted to the analysis of the major phorbol ester receptor, protein kinase C. The bryostatins, macrocylic lactones, activate protein kinase C and compete for phorbol ester binding. They only induce a subset of the typical phorbol ester responses, however. In Friend erythroleukemia cells, they restore differentiation inhibited by the phorbol esters. In primary mouse epidermal cells, they induce markers of the proliferative response but block phorbol ester induction of markers of differentiation. Part of the difference in response pattern can be explained by the bryostatins functioning to activate protein kinase C transiently followed by suppression of the pathway. Thus, both

for cell-cell communication and epidermal growth factor binding, the bryostatins initially act like the phorbol esters but subsequently block phorbol ester responsiveness. In addition, the bryostatins intrinsically differ from the phorbol esters in their stimulatory activity for some responses; for example, they fail to induce arachidonic acid release in C3H10T1/2 cells even at very early times. The biochemical mechanisms for these differences are being explored through immunoblotting of protein kinase C, comparison of phosphorylation patterns, tritiated bryostatin binding analysis, and comparison of effects on cloned and chromatographically separated protein kinase C isozymes. Structure-activity analysis suggests that bryostatin derivatives differ in the degree to which they are bryostatin-like in their actions rather than phorbol-ester like. Computer modeling indicates excellent fit to the previously derived phorbol ester pharmacophore and is consistent with the structure-activity relations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04504-16 CCTP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

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

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. H. Yuspa	Chief	LCCTP	NCI
Others:	H. Hennings	Research Chemist	LCCTP	NCI
	M. Poirier	Research Chemist	LCCTP	NCI
	D. Roop	Microbiologist	LCCTP	NCI
	J. Strickland	Research Chemist	LCCTP	NCI
	D. Greenhalgh	Visiting Fellow	LCCTP	NCI
	U. Lichti	Guest Researcher	LCCTP	NCI

COOPERATING UNITS (if any)

ImmuQuest Laboratories, Rockville, MD (G. B. Cannon); Johns Hopkins, Baltimore, MD (R. Tucker); Univ. of Arizona, Tucson, AZ (G. T. Bowden); Osaka Univ., Osaka, Japan.

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
9.0	6.0	3.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Cellular and molecular aspects of chemical carcinogenesis in lining epithelia are studied in mouse epidermis by in vivo and in vitro techniques. The initiation event in skin carcinogenesis is highly correlated to an alteration in the program of terminal differentiation of epidermal cells. In cell culture, epidermal differentiation is regulated by the concentration of extracellular calcium. Induction of terminal differentiation by increasing the calcium concentration in the culture medium causes a two- to threefold increase in the level of intracellular-free calcium. Cells of initiated lines which survive in medium with high calcium showed an altered response to increased external calcium, with a sharp four- to ninefold peak of intracellular free calcium in all cells within 2 minutes. These differences in intracellular calcium between normal and initiated keratinocytes may be related to alterations in phosphoinositide metabolism. The ras oncogene is highly correlated to the initiated phenotype in epidermis. Introduction of an activated ras gene into normal keratinocytes alters their phenotype to that of papilloma cells. Chemically induced papillomas yield an activated ras oncogene with a mutation at codon 61. Papilloma cells and initiated cells are resistant to the differentiation-inducing effects of phorbol ester tumor promoters. Since phorbol esters induce differentiation in normal cells, papilloma cells can be selected among an excess of normal cells in culture by their ability to continue to proliferate in culture medium containing phorbol esters. In culture, introduction of the v-fos oncogene into initiated cells with an activated ras results in their conversion to malignancy. TGF-β is elaborated by normal keratinocytes induced to differentiate by TPA or by increasing external calcium. This secretion is not altered by introduction of v-ras into normal keratinocytes. In vivo, several classes of benign tumors can be induced by initiation and promotion. Papillomas with a high risk for spontaneous conversion to carcinomas are also most responsive to chemical-converting agents. Malignant conversion can be accomplished by a single injection of cisplatin.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

S. Yuspa	Chief	LCCTP NCI
H. Hennings	Research Chemist	LCCTP NCI
M. Poirier	Research Chemist	LCCTP NCI
D. Roop	Microbiologist	LCCTP NCI
J. Strickland	Research Chemist	LCCTP NCI
U. Lichti	Guest Researcher	LCCTP NCI
W. Weinberg	Guest Researcher	LCCTP NCI
D. Greenhalgh	Visiting Fellow	LCCTP NCI
E. Lee	Guest Researcher	LCCTP NCI
F. Kruszewski	Special Volunteer	LCCTP NCI
M. Sporn	Chief	LC NCI
A. Roberts	Research Chemist	LC NCI
D. Lowy	Chief	LCO NCI
S. Aaronson	Chief	LCMB NCI

Objectives:

To study cellular and molecular changes during stages of chemical carcinogenesis through the development and use of cultures of epithelial lining cells which are the major target site for cancer in humans. Studies are directed to give insight into general changes occurring in specialized mammalian cells during malignant transformation and specific molecular events that may be causative to the transformation process. Specific markers of the transformed phenotype of epithelia are also being sought and mechanisms to prevent or reverse transformation are being studied.

Methods Employed:

This laboratory has developed and utilized mouse epidermal cell culture as an appropriate model to approach the stated objectives. Previous studies have shown that this model functions biologically in a fashion highly analogous to mouse skin in vivo. In vivo studies utilizing the initiation-promotion model for mouse skin carcinogenesis and grafts of human or mouse skin or cultured cells onto nude mice are also employed. A number of laboratory techniques are required to pursue the objectives. Morphology is followed by light and electron microscopy and immunohistochemical staining. Macromolecular synthesis and growth kinetics are studied by biochemical and autoradiographic procedures and flow cytometry. Intracellular-free calcium levels are determined by digital imaging analysis of cells loaded with the calcium-sensitive probe, Fura 2. Cellular metabolic functions, including the production of specific differentiation products, are monitored by enzyme assays, one- and two-dimensional gel electrophoresis, amino acid analysis, and radioimmunoassay. Protein purification and phospholipid studies employ column chromatography, fast protein liquid chromatography, and high pressure liquid chromatography. The progression to the malignant phenotype is monitored by growth rates, soft agar assay, karyotypic abnormalities,

enzymatic changes, changes in gene expression at the level of mRNA and injection or grafting of cells into nude or newborn mice. Genetic aberrations are studied by DNA transfection, gene cloning and sequencing, and nucleic acid hybridization and restriction analysis.

Major Findings:

A. Mechanism of Ca^{2+} action. Previous studies in this laboratory have shown that medium with 0.02-0.1 mM Ca^{2+} (low Ca^{2+}) selects for the growth of proliferating basal cells and that the shift to medium with 1.2 mM Ca^{2+} (high Ca^{2+}) concomitantly inhibits proliferation and induces terminal differentiation. Cell lines established from papillomas, from initiated skin, or after carcinogen exposure in culture (putative initiated cell lines) survive in high Ca^{2+} medium. In collaboration with Dr. Robert Tucker of Johns Hopkins University, intracellular-free calcium (Ca_i) levels were measured by use of a calcium-sensitive probe, Fura 2, in normal and initiated cells. Most normal keratinocytes respond to increased extracellular calcium by a gradual two- to threefold increase in Ca_i lasting for at least 28 minutes. A subpopulation displays a sharp peak of Ca_i at 2 minutes. In the initiated cells, the Ca_i level in low calcium medium was two- to threefold higher than that in normal cells, and all cells showed a four- to ninefold increase in Ca_i 2 minutes after external calcium was increased. As in the normal cells, the plateau level of Ca_i was 2-3 times the initial level.

Treatment of initiated keratinocyte cell lines with the Ca^{2+} ionophore ionomycin in high Ca^{2+} medium induces a prolonged elevation of Ca_i followed by terminal differentiation of the cells. If ionomycin should act similarly in vivo, initiated cells could be eliminated by this reversal of their neoplastic phenotype. When a non-necrogenic dose of ionomycin was applied after initiation by 7,12-dimethlybenz[a]anthracene (DMBA), but before promotion by mezerein, a substantial reduction in tumor yield was found. Ionomycin may act by inducing terminal differentiation of some of the initiated cells in vivo as well as in vitro.

The specificity of the Ca^{2+} signal is emphasized by the finding that the expression of particular gene products associated with differentiation is dependent on a particular concentration of Ca^{2+} in the medium. Keratins are expressed within 24 hours of exposure to 0.1 mM Ca^{2+}, but not to 1 mM. Genes expressed later in terminal differentiation in vivo are expressed later in vitro at particular concentrations of external Ca^{2+}. For example, filaggrin and a cornified envelope precursor are expressed at 48 hours in 0.12 mM Ca^{2+} but not in 1.0 mM. The regulation is transcriptional.

Ca^{2+} may act both by increasing intracellular Ca^{2+} and by stimulating phosphotidylinositol turnover. Inositol turnover is stimulated with both 1 mM and 0.15 mM Ca^{2+} but is not sustained at the lower concentration of Ca^{2+} in normal cells. The profile of inositol metabolism is altered in initiated cells; in particular, the metabolite IP_3 is not increased substantially.

B. Malignant conversion in vivo and in vitro. The stage of malignant conversion, the conversion of a papilloma to a carcinoma, was defined by experiments from this laboratory. The tumor promoter 12-O-tetradecanolyphorbol-13-acetate (TPA)

was inactive as a converting agent, while repeated exposure to either 4-nitroquinoline-N-oxide (4-NQO) or urethane was required to accomplish malignant conversion. The persistent papillomas which arise after short-term application of TPA are also the most sensitive to malignant conversion when either urethane or 4-NQO is used to enhance conversion. The chemotherapeutic agent cisplatin has been reported to be an initiating agent for mouse skin. When tested for activity in the malignant conversion stage, cisplatin was active when given to papilloma-bearing mice as a single injection. In contrast to urethane or 4-NQO, which require 10-30 treatments, cisplatin was less effective after 10 injections and inhibitory with 20-30 injections. The single injection of cisplatin was as effective as 30 injections of urethane. The sensitivity of persistent papillomas to cisplatin is now being tested. Trans-platin, which is inactive as a chemotherapeutic agent and differs from cisplatin in DNA adducts produced, is being tested for activity in the malignant conversion stage.

Activation of the ras oncogene is sufficient to initiate epidermal cells and produce papillomas. In order to study genes which may complement ras in producing malignant conversion, an assay has been developed which uses primary keratinocytes in which an activated ras gene has been introduced by a defective retroviral vector. When these cells are switched to high Ca^{2+} medium, they are blocked in their differentiation program but can no longer proliferate, leaving a lawn of non-proliferating, partially-differentiated cells in culture. When introduction of the ras oncogene is followed by exposure to carcinogens, foci evolve which can proliferate in high calcium medium. The number of these foci increases with increasing dose of carcinogen and with exposure to several different carcinogens which are known to cause malignant conversion in vivo. Cisplatin is currently being tested in this assay. Exposure to the tumor promoter TPA does not enhance focus formation in analogy to its action in vivo. Currently, foci which proliferate in high Ca^{2+} medium have been isolated and are being tested to determine whether they have been converted to malignancy.

C. Genes complementing the ras oncogene. Four mouse epidermal cell lines have been developed which form squamous papillomas when grafted to athymic nude mouse hosts along with primary dermal fibroblasts as a reconstituted skin (see Project Z01CP05178-07 CCTP). Each cell line has an activated ras^{Ha} oncogene with an A -> T transversion in codon 61, and each line persists in culture in high Ca^{2+} medium. Using transfection methodology developed in this laboratory, genes to complement the constitutively activated ras gene have been analyzed. Addition of an exogenous ras gene to increase the level of expression of mutated gene products causes malignant conversion. Introduction of the v-fos oncogene can convert benign cells to malignancy. The tumors produced by two distinct v-fos constructs in two different papilloma cell lines are squamous cell carcinomas. A rearranged c-myc gene from a mouse plasmacytoma and the adenovirus E1A gene did not convert, although mRNA for these genes, as well as the fos constructs, was highly expressed in the tumors and cell lines. The human c-fos proto-oncogene did not cause malignant conversion nor did the genes for a secreted protease, transin, or for a mutated form of human β-actin.

D. Initiation not involving ras. Keratinocyte cell lines derived by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) initiation protocols do not have an activated

ras oncogene, in contrast to those which result from DMBA-initiated epidermis or DMBA-treated cultured keratinocytes. These lines allow the study of other mechanisms of initiation. Addition of an activated ras gene leads to malignant conversion of one of these cell lines, implying that the MNNG-induced initiating lesion is complementary to ras for conversion. There is no evidence for over-expression of any of several oncogenes, including myc, fos, myb, and abl. Papillomas produced in SENCAR mice by treatment with tumor promoter alone presumably originate from endogenously or constitutively initiated cells. We have shown that 9 of 9 such papillomas do not have an activated ras gene; cell lines are being derived from these papillomas to determine the molecular lesion leading to their initiation.

E. Hair follicles in culture. A culture system for hair follicles is currently being developed as an in vitro model to parallel current studies on epidermal carcinogenesis. Methods of isolation have been improved by using collagenase without trypsin or by physical disruption, resulting in relatively intact follicle preparations which include the dermal papilla. Follicle-specific proteins, determined by 2-dimensional gel analysis, are synthesized by these cultured intact follicle organoids. The biological potential of freshly isolated hair follicles is assessed by grafting to nude mice and examining hair growth in the graft site. Combination with freshly isolated fibroblasts in the graft bed appears to be essential for hair formation. If the dermal fibroblasts are cultured for one week prior to grafting, then hair growth does not occur. The introduction of the transforming growth factor-α (TGF-α) gene by a defective retrovirus stimulates follicle growth and causes release of collagenase in vitro. Introduction of v-ras into cultured hair follicles results in papillomas when the follicles are grafted to nude mice. These tumors are phenotypically identical to tumors produced by introducing v-ras into epidermal cells.

F. Reconstitution experiments in vitro. A cell culture analogue of initiated mouse epidermis has been established in which a small number of putative initiated cells are co-cultured with a large excess of normal keratinocytes. In medium with high Ca^{2+} (but not in medium with low Ca^{2+}), growth of foci of the initiated cells was inhibited by normal keratinocytes. This inhibition was overcome by treatment with tumor promoters such as TPA, 12-O-retinoylphorbol-13-acetate (RPA), mezerein or benzoyl peroxide. The action of tumor promoters in this model is inhibited by treatment with inhibitors of promotion such as retinoic acid and fluocinolone acetonide. Bryostatin 1 was shown to inhibit TPA-induced focus formation in this assay which predicted its action as an antipromoter in vivo. This in vitro model may be valuable as a screening test for promoters or inhibitors of promotion as well as for mechanistic studies. After microinjection of the fluorescent dye, lucifer yellow, gap junctional communication can be studied by observing transfer of the dye from the injected cell to an adjacent cell. Normal and initiated cells were found to communicate only with homologous cells. Thus, inhibition of growth of initiated cells by normal cells does not appear to result from heterologous transfer of inhibitor molecules.

G. TGF-β effects. The influence of transforming growth factor-β (TGF-β) on normal and initiated keratinocytes is being explored in collaboration with

Drs. Michael Sporn and Anita Roberts of the Laboratory of Chemoprevention. TGF-β inhibits growth of both cell types, and this can be reversed by adding anti-TGF-β antibody to the medium. TGF-β is elaborated by keratinocytes induced to differentiate by Ca^{2+} or TPA. In addition, retinoic acid increases TGF-β secretion in normal and initiated cells. The introduction of v-ras into normal cells does not alter their elaboration of TGF-β. Since this oncogene blocks cells in a late basal cell stage of differentiation, the increase in TGF-β elaboration must be an early change in the differentiated phenotype.

H. Octahydromezerein activity as a tumor promoter. Mezerein is less active than TPA as a complete tumor promoter, perhaps because of its toxicity to the skin, its inability to induce a sustained hyperplasia, or to differences in receptor binding. A mezerein derivative, octahydromezerein (OHM), was synthesized by Dr. Peter Blumberg (see Project Z01CP05270-07 CCTP) because of its potential utility in proposed receptor studies. In order to test its activity as a promoter, appropriate doses of OHM were chosen based on the hyperplastic and inflammatory effect of topical application to the backs of SENCAR mice. At doses which produced an equivalent hyperplasia, OHM was found to be as effective as TPA and much more effective than mezerein in the promotion of papillomas.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04798-18 CCTP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Metabolism and Mode of Action of Vitamin A

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. M. De Luca	Research Chemist	LCCTP NCI
Others:	M. M. Webber	IPA Appointee	LCCTP NCI
	D. Joel	IRTA Appointee	LCCTP NCI
	R. Sinha	Visiting Fellow	LCCTP NCI
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COOPERATING UNITS (if any)

University of South Carolina, Columbia, SC (K.E. Creek); University of Maryland, Baltimore, MD (E.M. McDowell); St. Marianna University, Tokyo, Japan (S. Kato).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

Differentiation Control Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	5.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Our studies have focused on the effects of retinoids and tumor promoting agents on the cell surface. Retinoids were shown to enhance the adhesiveness of NIH 3T3 cells to plastic substrates coated with laminin in a reversible manner and with maximal effect at 48hr. Simultaneous with its effect on cell attachment retinoic acid (RA) caused a marked (70%) decrease in the uptake of inositol. The onset of RA inhibition of [3H]inositol uptake was rapid, with a 10-15% decrease after 2-3h and 60-70% at 16h of RA. A decrease in uptake was found as RA increased from 10-8 to 10-5 Molar (M) and was reversible 48h after RA removal. Further maximal effect on inositol uptake was dependent on RA treatment of the cells after they reached saturation density. RA inhibition of inositol uptake was also observed in 3T3-Swiss and Balb/3T3 cells but not in two virally transformed 3T3 cell lines, previously shown not to respond to RA by increased adhesion. Treatment of NIH 3T3 cells with phorbol 12-myristate 13-acetate (PMA) enhanced cell attachment to laminin and type IV collagen. The effect was dose-dependent, as early as 30 min. and reached a maximum at 2 hrs. These results suggest that PMA may enhance NIH 3T3 cell adhesion through effects on laminin and type IV collagen receptors. Retinoic acid, which itself requires at least 6 hours to show an effect on attachment, did not have any effect on cell attachment in 2 hours and, if anything, slightly inhibited PMA-enhanced cell attachment to laminin and type IV collagen substrates.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

L. M. De Luca	Research Chemist	LCCTP NCI
M. M. Webber	IPA Appointment	LCCTP NCI
D. Joel	IRTA Appointee	LCCTP NCI
R. Sinha	Visiting Fellow	LCCTP NCI
D. Cai	Visiting Fellow	LCCTP NCI

Objectives:

To investigate whether and how retinoids and phorbol ester tumor promoters modify cell surface properties, such as cell adhesiveness and transport properties.

Methods Employed:

The ability of fibroblasts to attach to microwell plates was measured after coating the plates with fibronectin, laminin, type IV collagen, or gelatin. Retinoid pretreatment for 6 hr to 2 days in culture caused an enhanced ability of the trypsinized retinoid-treated (10-6 to 3x10-8 M RA) cells to attach to laminin and type IV collagen in a short time (60 to 90 minutes). Since none of the cell lines used for the assay attached in control, solvent-treated wells, the number of unattached cells in control wells was used for the calculation of percent attachment in the following equation:

$$\% \text{ attachment} = \left(1 - \frac{[\text{unattached cells in experimental wells}]}{[\text{unattached cells in control wells}]}\right) \times 100$$

When the attached cells were counted after trypsinization, the results were basically the same.

Similar techniques were utilized to study the effects of PMA on cell attachment except that a shorter exposure (30-60 min) was necessary for the effect to occur.

[^{3}H]-Inositol Uptake Assay

The cells were washed with 2 ml of Dulbecco minimal essential medium (DMEM). One ml of supplemented DMEM containing [3H]inositol (2.0 uCi) was added and the cells inbubated at 37C for the times indicated.

The cells were then washed 3 times with 2 ml of ice-cold DMEM, scraped in 1 ml of methanol and the plate washed with another ml of methanol. Radioactivity in 1 ml of the 2 ml extract was determined in 15 ml of Aquasol (New England Nuclear, Boston MA). Cell numbers were determined by detaching cells with trypsin and counting them in a Coulter Counter (Coulter Electronics, Inc. Hialeah, FL).

Major Findings:

A. Effect of RA on inositol uptake. Treatment of 3T3 fibroblasts with biologically active retinoids, especially RA, substantially decreased the uptake of [3H]inositol. The onset of the inhibitory effect was rapid and could be observed as early as 2-6 h following RA treatment with maximal effect occurring between 15-24 h.

Various retinoids were used to examine their effect on inositol uptake. The relative activities of these compounds were similar to those observed previously in the hamster trachea assay except for 4-hydroxyphenyl retinamide (4HPR) which is active in the trachea system. RA was the most active retinoid of those tested and inhibited the uptake of inositol by 39% at 10-8 M while 5,6-dihydroxyretinoic acid methyl ester and 4HPR had very little activity (inhibited 16 to 20%).

Contrary to this effect on inositol, the uptake of fucose, mannose, galactose, and glucose was either not affected or enhanced (for mannose and fucose). RA lowered the Vmax of the uptake process without effect on the Kt.

B. Effect of PMA on attachment of NIH 3T3 cells to extracellular matrix macromolecules. Our data show, for the first time, that PMA enhances the attachment of NIH 3T3 fibroblast cells specifically to laminin and type IV collagen substrates. The data also suggest that PMA may induce receptors for laminin and type IV collagen in 3T3 cells, possibly in a manner similar to its stimulation of the expression of laminin receptors in neutrophils. The effects of PMA on NIH 3T3 cell attachment are apparently similar to those of retinoic acid. However, the fact that the effect of PMA (2 hours for the maximum) is much quicker that that of retinoic acid (48 hours for the maximum) suggests different mechanisms.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05177-07 CCTP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Use of Immunological Techniques to Study Interaction of Carcinogens with DNA

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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	S. Gupta-Burt	Biotechnology Fellow	LCCTP NCI
	S. Yuspa	Chief	LCCTP NCI
	E. Reed	Senior Investigator	MP NCI
	R. Ozols	Chief	MB NCI
	A. Weston	Visiting Associate	LHC NCI

COOPERATING UNITS (if any)

MIT, Boston, MA (L. Lippart); NCTR, Jefferson, AR (F. A. Beland); Natl. Hosp., Oslo, Norway (H. Huitfeldt); McArdle, Madison, WI (H. Pitot); U. of MD Med. Sch., Baltimore, MD (P. Strickland); Columbia Univ., New York, NY (R. Santella).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.25	4.75	1.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Antibodies specific for carcinogen-DNA adducts have probed the nature, extent, and consequences of in vitro and in vivo DNA modification. Biological samples substituted with 2-acetylaminofluorene (AAF), cis-diamminedichloroplatinum II (cis-DDP) and benzo(a)pyrene (BP) have been analyzed by quantitative immunoassays and by immunohistochemical procedures developed to localize adducts in situ. In hepatic DNA of rats chronically fed a carcinogenic dose of AAF, adduct removal was biphasic, suggesting two genomic compartments - one from which adducts are removed rapidly and another from which they are removed slowly. These kinetics are not due to different liver cell types or DNA associated with more or less tightly bound chromatin regions including the nuclear matrix. Comparison of adduct and tumor dose responses in bladder and liver of mice fed AAF chronically showed linear adduct and liver tumor formation, but non-linear bladder tumor formation. Immunohistochemical localization of 2-aminofluorene-DNA (AF-DNA) adducts in livers of rats fed AAF demonstrated a non-uniform adduct distribution and no adducts in preneoplastic foci induced by several different hepatocarcinogenesis protocols. Cis-DDP-DNA adducts were measured in DNA from nucleated peripheral blood cells and in several tissues obtained from cancer patients at multiple times during courses of cis-DDP therapy and/or at autopsy. Adduct accumulation occurred as a function of total cumulative dose. Disease response data on 55 ovarian cancer patients and 17 testicular cancer patients indicated that individuals with high adduct levels have a high rate of complete response to therapy. BP-DNA antigenicity was measured in enzyme-linked immunosorbent assays (ELISA) blood cell DNA of 43 firefighters and 40 controls. In both groups about 30% of the samples were positive, but mean adduct levels were higher in samples from firefighters. Other factors which contributed to sample positivity were the ingestion of charcoal broiled foods and smoking.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

M. C. Poirier	Research Chemist	LCCTP	NCI
O. Olivero	Fogarty Fellow	LCCTP	NCI
S. Gupta-Burt	Biotechnology Fellow	LCCTP	NCI
S. H. Yuspa	Chief	LCCTP	NCI
E. Reed	Senior Investigator	MB	NCI
R. Ozols	Section Head	MB	NCI
A. Weston	Visiting Associate	LHC	NCI

Objectives:

To develop specific and sensitive quantitative and morphological immunoassays for the investigation of carcinogen-DNA interactions. Studies are directed toward quantitative and qualitative analyses of covalent DNA adduct formation and removal, and localization of adducts at the cellular and subcellular levels. These data are correlated with biological consequences of chemical carcinogen exposure, including cell transformation and tumorigenesis. In the case of the chemotherapeutic agent, cis-diamminedichloroplatinum (II) (cis-DDP), biological end points include chemotherapeutic efficacy and short- and long-term toxicity.

Methods Employed:

Both in vivo carcinogen exposure of experimental animals and carcinogen treatment of cultured cells are employed to pursue the objectives. Tissues and cells obtained from individuals environmentally exposed to carcinogens or from patients given cancer chemotherapeutic agents are also utilized. The chemical synthesis of radiolabeled and unlabeled DNA-carcinogen adducts and their purification by column chromatography are currently performed. Isolation of macromolecules utilize density gradient centrifugation and phenol extraction. 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), enzyme-linked immunosorbent assay (ELISA) and micro-fluorometry. Liver cells are separated by centrifugal elutriation, and chromatin fractions prepared by high and low salt extractions. Cisplatin-DNA adducts are determined by atomic absorbance spectroscopy as well as ELISA. Messenger RNA is prepared from rat liver RNA by oligo-dT columns and hybridized to genomic DNA. The hybrids are separated by hydroxylapatite-FPLC. DNA sequences coding for the DHFR gene are separated from genomic DNA by restriction and gel electrophoresis, and localized, after Southern blotting, by 32P-labeled c-DNA probe.

Major Findings:

Interactions of various carcinogens with DNA have been studied in cultured cells, animal organs, and human tissues by a unique immunotechnology pioneered in this laboratory. Rabbit antibodies have been elicited against carcinogen-nucleoside

adducts or protein-complexed modified DNAs. The high-affinity antisera obtained have been used to develop RIA and ELISA which are able to detect as little as one adduct in 10^7 nucleotides. The areas of ongoing intensive investigation in this laboratory are as follows: (1) elucidation of mechanisms by which the major rat liver DNA adducts are formed and removed during chronic feeding of a carcinogenic 2-acetylaminofluorene (AAF) regimen; (2) cis-DDP-DNA adduct formation, removal and persistence in nucleated blood cells and tissues of cancer patients receiving platinum drug-based chemotherapy and in tissues of animal models exposed to cisplatin; and (3) monitoring of DNA from human samples for the presence of hydrocarbon-DNA adducts.

Rabbit antisera specific for guanosin-(8-yl)-2-acetylaminofluorene (G-8-AAF) and guanosin-(8-yl)-2-aminofluorene (G-8-AF) have been utilized in competitive RIAs to obtain profiles of liver DNA adducts, while feeding a carcinogenic level of AAF to male rats. These studies demonstrated biphasic adduct removal during 4 weeks of feeding control diet. A pharmacokinetic model, constructed from the data, postulated two compartments: one susceptible to fast C-8 adduct removal and another from which these adducts are removed slowly. The nature of the two compartments was found not to be associated with different cell types within the liver. Experiments to fractionate DNA associated with differentially-bound chromatin are being performed in collaboration with Dr. F. Beland (National Center for Toxicological Research) and involve extraction by high and low salt to obtain DNA postulated to be located at varying distances from the nuclear matrix. These studies have demonstrated that the 5% of total DNA associated with the nuclear matrix contains fewer DNA adducts and removes them more slowly than the other fractions. The profile of adduct removal in each of these chromatin fractions is biphasic, similar to that exhibited by the liver as a whole. Therefore, the biphasic adduct removal kinetics may be indicative of some very fundamental aspect of DNA organization. Current studies are designed to investigate adduct removal in expressed and non-expressed genes in the liver.

In an attempt to elucidate the relationship between AAF-DNA adduct formation and tumorigenesis, adducts have been measured in livers and bladders of mice after 28 days of continuously feeding 0.02% AAF in the diet. These studies, done in collaboration with Dr. F. Beland, were designed to model lifetime feeding studies in which mice were sacrificed at 18, 24 and 33 months. In both liver and bladder, adduct formation was linear over a dose range between 5 and 150mg/kg diet, although adducts in bladder were at least three times higher than those in liver. Tumorigenesis in the liver was also linear with a slope similar to that found for adduct formation. The tumor incidence in the bladder was not significantly higher than that in unexposed controls at doses lower than 60mg/kg, but above this dose the slope of the line for tumor formation, as compared to dose, was much steeper than that for adduct formation compared to dose. Thus, tumor response in the same animal can vary in different tissues and is not necessarily linear, in spite of linear adduct formation.

In collaboration with Dr. H. Huitfeldt (University of Oslo), immunohistochemical localization of DNA adducts has been studied in frozen sections of livers from male Fischer rats fed AAF. The pattern of adduct formation was nonuniform, with the highest adduct concentration in the periportal areas and a decreasing

gradient through the midzonal to the centrilobular areas. This pattern did not change during a month of chronic AAF feeding. The lobular localization obtained during 28 days of AAF feeding was confirmed in a semi-quantitative fashion by microfluorometry. Immunohistochemical studies were further utilized to examine preneoplastic enzyme-altered foci for the presence of AAF-DNA adducts. Foci were induced by four different hepatocarcinogenesis regimens, AAF was fed for 5-6 days prior to sacrifice and liver sections were stained for AAF-DNA adducts by immunofluorescence. The localization of areas positive for GGT or GST and/or negative for glucose-6-phosphatase (G-6-Pase) or adenosine triphosphatase (ATPase) was determined, in collaboration with Dr. H. Pitot (McArdle Laboratory), by computer analysis. There were 21 different phenotypes and 572 different foci examined, and all were shown to be negative for dG-8-AF adduct formation.

cis-DDP is a potent chemotherapeutic agent which induces the formation of intrastrand N^7-deoxy(GpG)-and N^7-deoxy(GpA)-diammineplatinum adducts as a major fraction of total platinum bound to DNA. An ELISA (established in collaboration with Dr. S. Lippard of MIT) has been used to quantitate adducts in tissues of cancer patients and animal models. DNA samples extracted from nucleated peripheral blood cells of controls and testicular and ovarian cancer patients receiving cis-DDP therapy (collaboration with Dr. R. Ozols, Medicine Branch, NCI) have been monitored. About half of the patients on their first course of chemotherapy receiving cis-DDP for 5 days of drug infusion followed by 2 or 3 drug-free weeks accumulated DNA adducts as a function of dose, while others did not form measurable adducts. Disease response data for 55 ovarian cancer patients and 17 poor prognosis testicular cancer patients showed that individuals forming high levels of cis-DDP adducts were more likely to undergo complete remission than those forming fewer adducts or no adducts at all. When several tissues (brain, bone marrow, peripheral nerve, kidney, liver, lung, spleen and adrenal) and an ovarian tumor were obtained at autopsy from the same individual, adducts were present in all tissues of the same individual, including tumor, and appeared to be persistent for many months after the last treatment. A collaborative study with Dr. M. Egorin (U. of Md. Medical School) has shown that similar adduct levels can be found in blood cell DNA and cervical tumor 24hr after treatment with carboplatinum. All of our human DNA samples are being assayed by both ELISA and atomic absorption spectrophotometry (AAS) (a collaboration with Dr. E. Reed of the Medicine Branch, NCI).

An interlaboratory comparison and standardization of the benzo(a)pyrene-DNA ELISA was completed with contributions by this Laboratory, Dr. A. Weston (LHC, NCI) and Dr. R. Santella (Columbia University). The new assay gives a more accurate evaluation of biological samples, and has been shown to be specific for several adducts formed between polycyclic aromatic hydrocarbons and DNA. Thus, the results are being expressed as fmoles BP-DNA antigenicity. This ELISA was used to monitor the DNA of nucleated blood cells obtained from firefighters and age- and sex-matched controls in the Baltimore-Washington area. The samples were obtained by Dr. P. Strickland of Johns Hopkins Medical School. In this study, 15 out of 43 firefighters were positive for adducts with a mean value of 1.87 fmoles adduct/μg DNA, and 13 out of 40 controls were also positive with a mean adduct value of 1.0 fmoles adduct/μg DNA. Factors which also contributed to adduct

formation as independent variables were consumption of charcoal broiled foods and smoking.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05178-07 CCTP

PERIOD COVERED

October 1, 1987, to September 30, 1988

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

Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. E. Strickland	Research Chemist	LCCTP NCI
Others:	S. H. Yuspa	Chief	LCCTP NCI
	H. Hennings	Research Chemist	LCCTP NCI
	M. Ueda	Guest Researcher	LCCTP NCI
	D. Greenhalgh	Visiting Fellow	LCCTP NCI

COOPERATING UNITS (if any)

ImmuQuest Laboratories, Inc., Rockville, MD (G. Cannon)

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Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	1.7	1.7

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

In vivo studies show that the SENCAR mouse is unusually sensitive to skin carcinogenesis by initiation and promotion while the BALB/c mouse is resistant. We have developed a technique of grafting epidermal and dermal cells to athymic nude mice to form a reconstituted skin. Initiated cell lines 308 and SP-1, derived from initiated skin of BALB/c and SENCAR mice, respectively, have been developed and characterized. These lines form benign squamous papillomas in grafts and have an activated ras oncogene. We can thus reconstruct an "initiated" skin using mixtures of papilloma-forming cells with primary epidermal cells and dermal fibroblasts. Suppression of papilloma size occurs when normal SENCAR primary epidermal cells are grafted along with small numbers of SP-1 cells. This suppression is specific for SENCAR primary epidermal cells and does not occur with SENCAR primary dermal fibroblasts, BALB/c primary epidermal cells, or an initiated cell line derived from SENCAR skin which makes an apparently normal skin when grafted. BALB/c primary epidermal cells fail to suppress papilloma formation either from grafted SP-1 cells or the BALB/c-derived 308 cells. Papillomas formed on SENCAR mice by treatment with promoter alone, i.e., without exogenous chemical initiation, did not have an activated ras oncogene at codon 61. These papillomas presumably resulted from endogenously initiated cells. Cell lines being developed from these papillomas will presumably be useful in identifying the molecular lesions in endogenously initiated cells.

PHS 6040 (Rev. 1/84)

GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

James E. Strickland	Research Chemist	LCCTP	NCI
Stuart H. Yuspa	Chief	LCCTP	NCI
Henry Hennings	Research Chemist	LCCTP	NCI
Masato Ueda	Guest Researcher	LCCTP	NCI
David Greenhalgh	Visiting Fellow	LCCTP	NCI

Objectives:

To elucidate the cellular and molecular mechanisms of enhanced sensitivity to carcinogenesis in genetically derived susceptible mouse strains.

Methods Employed:

The SENCAR mouse was developed by a selective breeding protocol for enhanced susceptibility to skin carcinogenesis by initiation and promotion. Comparisons are made to BALB/c mice as a representative resistant strain. The epidermis can be separated from the dermis by flotation of the skin, dermis side down, on a solution containing trypsin. The separated epidermal cells are cultured in medium containing Ca^{2+} levels <0.1 mM to select for basal cells or >0.1 mM to induce terminal differentiation. Initiated cells can be selected from an excess of normal cells on the basis of the resistance of the former to Ca^{2+}-induced terminal differentiation. Papilloma cell lines were developed from papillomas produced on adult SENCAR and BALB/c mouse skin by initiation with 7,12-dimethlybenz[a]anthracene (DMBA) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and promotion with TPA. Papillomas were removed, minced, and cells were dissociated by treatment with collagenase, followed by trypsin, and cells were cultured in medium with low $[Ca^{2+}]$. A grafting system has been developed in athymic nude mice which can produce normal skin from cultured primary epidermal and dermal cells of newborn mice. A vascularized graft bed is produced by subcutaneous implantation of a glass disk (3 mm thick X 17 mm diam.) one week prior to grafting. This system makes possible grafting of mixtures of normal with initiated cells as well as epidermal and dermal cells from different mouse strains in the same graft. Cultured cells are released from flasks or dishes by treatment with trypsin, and a mixture of epidermal and dermal cells is centrifuged and the cell pellet is applied to the graft bed within a silicone chamber which separates the graft from the host skin. After one week, the chamber is removed and the graft is bandaged for an additional week, following which the bandage is removed and the wound allowed to heal. Grafts are examined both grossly and histologically at sacrifice.

Major Findings:

We have studied interactions between normal epidermal cells and dermal fibroblasts from both SENCAR and BALB/c mice with initiated cell lines. We have primarily utilized two murine epidermal cell lines which form squamous papillomas

when grafted to athymic nude mice as part of a reconstituted skin. Line SP-1 was derived from papillomas produced on SENCAR mouse skin by initiation with DMBA and promotion with TPA. Line 308 was derived from BALB/c mouse skin which was initiated in vivo with DMBA followed by culture of the epidermal cells and selection of cells resistant to Ca^{2+}-induced terminal differentiation, a property which we have previously shown to be associated with initiated cells.

Both SP-1 and 308 cells have an activated ras^{Ha} oncogene with a mutation in codon 61 as shown by restriction endonuclease analysis.

Papilloma formation by SP-1 cells in grafts is strongly suppressed by normal SENCAR primary epidermal cells, but not by primary dermal fibroblasts. However, BALB/c primary epidermal cells do not suppress papilloma formation by 308 cells or by LC14 cells, which are also derived from BALB/c skin, have an activated ras^{Ha} oncogene, and form papillomas when grafted. In heterologous strain experiments, BALB/c primary epidermal cells also fail to suppress papilloma formation by SP-1 cells. Initiated cell line SCR722, which does not have an activated ras gene and makes an apparently normal epidermis when grafted, does not suppress papilloma formation by SP-1 cells. SCR722 was derived from SENCAR mice initiated with N-methyl-N'-nitro-N-nitrosoguanidine. The biological behavior observed from grafting studies suggests various interactions among initiated cells and primary epidermal and dermal cells. In vitro studies may help to elucidate the mechanisms of these interactions.

Spontaneous activation of the ras^{Ha} oncogene occurs with apparent equal frequency in cultures of primary epidermal cells from SENCAR and BALB/c mice.

Both in vivo tumorigenesis studies and in vitro focus formation studies have suggested that a population of endogenously initiated cells exists in SENCAR skin. To study the nature of the molecular lesion in these cells, we have examined DNA from papillomas derived from SENCAR mouse skin treated with TPA alone, i.e. without exogenous chemical initiation. None of the nine papillomas examined has an activated ras^{Ha} gene with an A to T transversion at codon 61. Development of cell lines from these papillomas is in progress.

Publications:

Strickland JE, Greenhalgh DA, Koceva-Chyla A, Hennings H, Restrepo C, Balaschak M, and Yuspa SH: Development of murine epidermal cell lines which contain an activated ras-Ha oncogene and form papillomas in skin grafts on athymic nude mouse hosts. Cancer Res 1988;48:165-69.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05270-07 CCTP

PERIOD COVERED

October 1, 1987 to November 30, 1988

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

Molecular Mechanism of Action of Phorbol Ester Tumor Promoters

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Peter M. Blumberg	Research Chemist	LCCTP	NCI
Others:	M. Dell'Aquila	Staff Fellow	LCCTP	NCI
	B. Warren	IRTA Fellow	LCCTP	NCI
	H. Nakakuma	Visiting Fellow	LCCTP	NCI
	D. de Vries	Visiting Fellow	LCCTP	NCI
	L. Schuman	IRTA Fellow	LCCTP	NCI
	A. Szallasi	Visiting Fellow	LCCTP	NCI

COOPERATING UNITS (if any)

Boston U. School of Med., Boston, MA (A.I. Tauber); Arizona St. U. (G.R. Pettit, C.L. Herald, Y. Kamano); Ciba-Geigy, Summit, NJ (A.Y. Jeng); Upjohn Co., Kalamazoo, MI (K.L. Leach); U. of California, Riverside (J.A. Traugh, D. Murray).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

Molecular Mechanisms of Tumor Promotion Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.0	6.25	1.75

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The efforts of the Molecular Mechanisms of Tumor Promotion Section are directed toward understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. Particular attention is being devoted to the analysis of the major phorbol ester receptor, protein kinase C. The bryostatins, macrocylic lactones, activate protein kinase C and compete for phorbol ester binding. They only induce a subset of the typical phorbol ester responses, however. In Friend erythroleukemia cells, they restore differentiation inhibited by the phorbol esters. In primary mouse epidermal cells, they induce markers of the proliferative response but block phorbol ester induction of markers of differentiation. Part of the difference in response pattern can be explained by the bryostatins functioning to activate protein kinase C transiently, followed by suppression of the pathway. Thus, both for cell-cell communication and epidermal growth factor binding, the bryostatins initially act like the phorbol esters but subsequently block phorbol ester responsiveness. In addition, the bryostatins intrinsically differ from the phorbol esters in their stimulatory activity for some responses; for example, they fail to induce arachidonic acid release in C3H10T1/2 cells even at very early times. The biochemical mechanisms for these differences are being explored through immunoblotting of protein kinase C, comparison of phosphorylation patterns, tritiated bryostatin binding analysis, and comparison of effects on cloned and chromatographically separated protein kinase C isozymes. Structure-activity analysis suggests that bryostatin derivatives differ in the degree to which they are bryostatin-like in their actions rather than phorbol-ester like. Computer modeling indicates excellent fit to the previously derived phorbol ester pharmacophore and is consistent with the structure-activity relations.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter M. Blumberg	Research Chemist	LCCTP	NCI
M. Dell'Aquila	Staff Fellow	LCCTP	NCI
B. Warren	IRTA Fellow	LCCTP	NCI
H. Nakakuma	Visiting Fellow	LCCTP	NCI
D. de Vries	Visiting Fellow	LCCTP	NCI
L. Schuman	IRTA Fellow	LCCTP	NCI
A. Szallasi	Visiting Fellow	LCCTP	NCI
S. Yuspa	Chief	LCCTP	NCI
H. Hennings	Research Chemist	LCCTP	NCI
M.C. Willingham	Research Biologist	LMB	NCI
T. Fleming	Staff Fellow	LCMB	NCI
S. Aaronson	Chief	LCMB	NCI

Objectives:

The early events in the interaction of the phorbol esters and related compounds with cells and tissues are being characterized. Specific aims are as follows: (1) elucidation of mechanistic differences between different classes of protein kinase C activators; (2) determination of the biological role of intact protein kinase C and its functional domains in cellular regulation; (3) characterization of the interactions of protein kinase C activators with the reconstituted enzyme; (4) analysis of the specificity and mechanism of action of protein kinase C inhibitors; and (5) identification of targets for the phorbol esters and related compounds in addition to protein kinase C.

The major phorbol ester receptor, protein kinase C, is postulated to mediate one of the two pathways activated by a large class of hormones for which receptor occupancy is associated with enhanced phosphatidylinositol turnover. Several oncogenes may also function, in part, through this pathway. Understanding of the mechanisms of endogenous modulation of the phorbol ester receptors may thus provide insights into both basic biochemical mechanisms and the process of human carcinogenesis as well as identify biochemical steps suitable for intervention.

Methods Employed:

This Section uses a wide range of techniques to pursue the above aims. Phorbol and derivatives are isolated from natural sources. Semisynthetic derivatives of phorbol and the bryostatins for use in affinity labeling, structure-activity analysis, and binding studies are prepared and radioactively labeled as necessary. Binding studies are carried out using the ligands and methodologies developed by us. Analysis of receptors utilizes both photoaffinity labeling and standard biochemical membrane methodology. The systems analyzed are chosen as optimal for the specific questions being examined. Brain homogenates, because of their richness in receptors, are being used for receptor

purification and biochemical analysis. Mouse skin and cultured keratinocytes are being used to dissect subclasses of receptors and to study the relation between protein kinase C, cell differentiation, and tumor promotion. A variety of intact cells, including 3T3 and C3H10T1/2 fibroblasts, HL-60 promyelocytic leukemia cells, and human neutrophils, are being utilized to elucidate the coupling between receptor occupancy and biological responses. Microinjection of receptor domains is employed to clarify their cellular function. A variety of whole animal physiological endpoints are analyzed to characterize unique responses to the resiniferatoxin class of phorbol-related diterpenes.

Major Findings:

A central issue in understanding the mechanism of phorbol ester action is the discrepancy between a single major receptor, protein kinase C, and both biological and binding data indicative of heterogeneity in phorbol ester response. A strategy for studying heterogeneity that is receiving substantial current emphasis is to compare the behavior of structurally different classes of protein kinase C activators. Bryostatin, a recently described macrocyclic lactone isolated from a marine bryozoan on the basis of its anti-leukemic activity, has proven to be of particular interest. Although bryostatin inhibits phorbol ester binding and activates protein kinase C, bryostatin functionally antagonizes some but not other phorbol ester responses.

We have been involved in a close collaboration with Dr. G.R. Pettit of Arizona State University in characterizing differences in the patterns of response to phorbol esters and bryostatins and in elucidating biochemical mechanisms responsible for these differences.

In C3H10T1/2 mouse fibroblasts, bryostatin 1 causes long-term inhibition of epidermal growth factor-binding (EGF) but induces only 15% of the level of arachidonic acid release found for the phorbol esters. The combination of bryostatin 1 together with phorbol ester induces no more release than treatment with bryostatin 1 alone. Structure-activity relations reveal that different bryostatin congeners differ in the maximal extents of arachidonic acid release which they are able to induce. Levels of release range from 15% for bryostatins 1 and 4 to 60% for bryostatin 3. As with bryostatin 1, the level of release by the combination of bryostatin congener and phorbol ester is the same as for the bryostatin derivative alone. We conclude that different bryostatins differ in the degree to which they are "bryostatin-like" or "phorbol ester-like." The results from the combined treatments most readily suggest that the bryostatins all fully activate protein kinase C but variably inhibit that pathway in a dominant fashion.

Computer comparison of different classes of protein kinase C activators has suggested a specific 3-dimensional model for the phorbol ester pharmacophore. Evaluation of the bryostatins in this model yields an excellent correlation. Structure-activity analysis, comparing binding affinities of different derivatives, indicates lack of influence of the ester residues at C-7 and C-20 and of the double bond at C-13,30 of bryostatin. In contrast, either esterification or epimerization of the C-26 hydroxyl causes dramatic loss of

activity. These results fully support the pharmacophore model, which predicts that the carboxyl at C-1 and the hydroxyl groups at C-19 and C-26 represent the critical homologies to phorbol. The modeling studies are of great importance because they afford guidance in the development of semi-synthetic derivatives to further clarify structural requirements and of novel classes of synthetic protein kinase C modulators.

Radioactively labeled bryostatin 4 was synthesized in order to characterize bryostatin binding to protein kinase C. Assay conditions were developed which permit equilibrium analysis. Under these conditions, bryostatin and phorbol esters each block binding of the other, and the inhibition of bryostatin binding by phorbol esters is competitive. These results imply that both classes of compound interact at the same site on the enzyme.

Although phorbol esters and bryostatin qualitatively bind to protein kinase C in a similar fashion, quantitatively they show major differences. When protein kinase C is reconstituted in phosphatidylserine, bryostatin binds with pM affinity, in contrast to the nM affinity of the phorbol esters. This result implies that nM concentrations of bryostatin may be able to interact with secondary targets which have only weak (μM) affinity for phorbol esters. In support of this model, some bryostatin-like effects are mimicked by μM phorbol ester concentrations. Secondly, bryostatins display a very slow (several hours) off-rate from protein kinase C, in contrast to the rapid off-rate for the phorbol esters. These results suggest that the bryostatins may anchor protein kinase C, preventing re-equilibration between membrane compartments.

Protein kinase C exists as a family of isozymes. The interaction of the bryostatins with the individual isozymes, obtained either by isolation from rat brain or by cloning, is being characterized. Preliminary results suggest that the bryostatins display considerable selectivity between isozymes. The bryostatins represent the first class of potent protein kinase C modulators to display such selectivity.

The patterns of response to bryostatins in mouse keratinocytes had suggested that a transient duration of action could account for the differences with the phorbol esters in that system. Using immunoblotting, we have demonstrated that the bryostatins cause accelerated down regulation of protein kinase C in the keratinocytes, as well as in C3H10T1/2 cells and Friend erythroleukemia cells.

This accelerated breakdown could contribute to the transient duration of action, although quantitatively it may not be sufficient to fully explain the biological results.

Proteolysis of protein kinase C, cleaving the regulatory domain and generating an activated catalytic domain, termed "protein kinase M," represents an alternative scheme for activation of kinase. Because protein kinase M lacks the phospholipid binding domain, it should show different localization and consequently have access to a different spectrum of substrates. The formation of protein kinase M is enhanced by phorbol ester binding both in vitro and in

vivo, and appreciable amounts accumulate in some cell systems. Nothing is known, however, about the contribution of protein kinase M to the biological responses observed for the phorbol esters. In previous studies, we have demonstrated that phorbol-ester activation of human neutrophil NADPH oxidase, the enzyme responsible for the oxidative burst, can be reconstituted using protein kinase C, phorbol esters, a neutrophil plasma membrane fraction, and appropriate cofactors. We now find that protein kinase M can replace the requirements for phorbol ester and protein kinase C in this reconstituted system. In intact Swiss 3T3 cells, phorbol ester treatment induces rapid morphological change. In collaboration with M. C. Willingham, we have demonstrated that microinjection of purified protein kinase M causes a similar but not identical change. Microinjected carrier protein had no effect. Our studies provide direct evidence, for the first time, that formation of protein kinase M may be of physiological significance.

Resiniferatoxin is a structural analog of the phorbol esters which is several orders of magnitude more inflammatory but which is unable to induce many of the other typical phorbol ester responses. Structural examination suggested homology to capsaicin, the pungent component in red pepper. We have now shown that resiniferatoxin, like capsaicin, causes pain, hypothermia, and neurogenic inflammation. These acute effects are followed by desensitization, as previously found for capsaicin, with cross-tolerance between resiniferatoxin and capsaicin. The resiniferatoxin-desensitized animals show an altered irritant response to typical phorbol esters. Early redding and edema are completely abolished; edema at late times is reduced in a strain-specific manner. The promotion-hypersensitive SENCAR strain retains most of the late-term edema response upon resiniferatoxin pretreatment; CD-1 mice retain only a limited response. Assessment of the relation of the two phases of the inflammatory response to hyperplasia and tumor promotion is in progress. Radiolabeled resiniferatoxin has been prepared for receptor analysis.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05445-04 CCTP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Regulation of Epidermal-Specific Differentiation Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. R. Roop	Microbiologist	LCCTP	NCI
Others:	S. H. Yuspa	Chief	LCCTP	NCI
	H. Nakazawa, T. Mehrel	Visiting Fellow	LCCTP	NCI
	D. Rosenthal	Biotechnology Fellow	LCCTP	NCI
	L. De Luca	Research Chemist	LCCTP	NCI
	P. Steinert	Senior Investigator	DB	NCI
	D. Hohl	Visiting Fellow	DB	NCI
	J. Stanley	Senior Investigator	DB	NCI

COOPERATING UNITS (if any) U. of Munich (T. Krieg); Cent. for Drugs and Biologics, Bethesda MD (J. Ridge); Baylor Coll. of Med., Houston, TX (J. Clark); U. of Washington, Seattle, WA (C. Fisher); U. of Oslo, Oslo, Norway (H. Huitfeldt); USUHS, Bethesda MD (E. Chang and S. Chung).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

cDNA clones corresponding to the major proteins expressed in mouse epidermis have been isolated and characterized. Using a combination of in situ hybridization with RNA probes (which are specific for individual mRNAs) and indirect immunofluorescence with monospecific antisera (which were elicited with synthetic peptides corresponding to unique sequences within each protein), it is possible to show that these genes belong to at least four subsets: those expressed predominantly in the proliferating basal layer of the epidermis; those expressed predominantly in the differentiated suprabasal spinous layers and, to a lesser extent, in the granular layer; those only expressed in the granular layer; and those only expressed under hyperproliferative conditions. Genes representing each subset have been isolated and sequenced. Various strategies have been employed to identify sequences regulating the expression of these genes, including vector constructs using different regions of the genomic clones to drive expression of the chloramphenicol acetyltransferase gene and the production of transgenic mice which express a human differentiation-associated keratin gene in a tissue- and developmental-specific pattern. A gene encoding a cysteine-rich protein, which is a major component of the cornified envelope, has been isolated and shown by in situ hybridization experiments to be expressed in the granular layer of the epidermis. A monospecific antiserum has been used to demonstrate that the C-terminal portion of this protein is only detectable on the inner surface of mature envelopes. Monospecific antisera that have been produced against mouse and human keratins and other epidermal-specific differentiation products have been used to study various stages of carcinogenesis, gene expression in mutant mice exhibiting developmental defects in epidermal differentiation, the induction of terminal differentiation in malignant cell lines by pharmacological agents, the in vivo kinetics of expression of the differentiation-associated keratins with respect to cell division, and requirements for the induction of terminal differentiation products in vitro.

PHS 6040 (Rev. 1/84)

GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged in this Project:

Dennis R. Roop	Microbiologist	LCCTP	NCI
S. H. Yuspa	Chief	LCCTP	NCI
H. Nakazawa	Visiting Fellow	LCCTP	NCI
T. Mehrel	Visiting Fellow	LCCTP	NCI
D. Rosenthal	Biotechnology Fellow	LCCTP	NCI
L. De Luca	Research Chemist	LCCTP	NCI
P. Steinert	Senior Investigator	DB	NCI
D. Hohl	Visiting Scientist	DB	NCI
J. Stanley	Senior Investigator	DB	NCI
G. Smith	Senior Investigator	DCBD	NCI

Objective:

To isolate and characterize the genes coding for the major differentiation products of epidermal cells. To study the expression of these genes during normal differentiation and during various stages of carcinogenesis and to define the mechanism regulating their expression.

Methods Employed:

The isolation of cDNA clones is accomplished by the purification of epidermal mRNA, reverse transcription and cloning of double-stranded cDNA in the plasmid pBR322 or gt11 expression vectors. The cDNA clones are characterized by hybridization-selection assays and by direct DNA sequence analysis. Genes are isolated by screening genomic libraries with nick-translated cDNAs and characterized by restriction endonuclease digestion and direct DNA sequence analysis. The expression of specific genes is monitored by RNA blot analysis and quantitated by slot-blot analysis. Transcripts for individual genes are detected in histological sections of skin by *in situ* hybridization with 35S-labeled RNA probes. Monospecific antisera are produced with synthetic peptides corresponding to unique sequences within each protein. The antisera are used to monitor normal and abnormal expression of these polypeptides by immunofluorescent staining, immunoblotting, and immunoprecipitation.

Major Findings:

The production of very specific antibodies with synthetic peptides that permit the detection of individual gene products and the development of an *in situ* hybridization technique that allows the localization of specific gene transcripts within cells in different layers of the epidermis have revealed the high degree of regulation that exists for various genes expressed at different differentiation states in the epidermis. One subset of keratin genes referred to as proliferation-associated, is only expressed in the basal layer. After cells commit to terminal differentiation and migrate away from the basement membrane, expression of these keratin genes is suppressed. In addition, there appears to

be regulation at the level of RNA stability since few transcripts are detected in the differentiated layers. Another set of keratin genes (differentiation-associated) is expressed concomitantly with the decision to terminally differentiate and this appears to occur in some cells prior to migration away from the basement membrane since transcripts and translation products can be detected in some cells still in contact with the basement membrane. As cells continue in their differentiation program and migrate through the spinous layer into the granular layer, expression of the differentiation-associated keratin genes is restricted at both the level of transcription and RNA stability. Concomitant with the suppression of these genes in the granular layer, another set of genes is activated. These genes encode a cysteine-rich component of the cornified envelope and filaggrin, a histidine-rich protein responsible for aggregating keratin filaments into the dense filament-matrix complex of the stratum corneum (studied in collaboration with Dr. Peter Steinert, Dermatology Branch (DB), NCI. A fourth subset of genes consists of keratin genes that are not expressed in normal epidermis but are expressed under hyperproliferative conditions such as in primary epidermal cell culture, benign and malignant epidermal tumors, epidermal hyperplasia induced by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), and squamous metaplasia.

The analysis of DNA sequence information has revealed unique regions within several additional proteins which have been utilized for the production of monospecific antisera with synthetic peptides. Antisera have been elicited against additional mouse keratins, human keratins, filaggrin, the cysteine-rich envelope component and a low molecular weight thiol proteinase inhibitor. These antisera have been used in the following studies:

1) To monitor stages of carcinogenesis. Changes in the proliferation status and/or differentiation state most likely occur as early events in epithelial carcinogenesis. The ability to detect these early events might permit diagnosis at a preclinical stage. The induction of new programs of keratin gene expression has been correlated with these changes. We have used specific keratin antisera to monitor these events. Initial experiments followed the induction of squamous metaplasia in tracheal epithelium of vitamin A-deficient hamsters or after exposure to chemical carcinogens (see Project Z01CP04798-18 CCTP). More recently these studies have been expanded to include esophageal and bladder epithelium of vitamin A-deficient hamsters. On the basis of promising results from these studies, we have begun to follow the induction of bladder cancer by chemicals in mice (studied in collaboration with Dr. Daniel Hohl, DB, NCI). The early induction of squamous metaplasia has also been detected in the uterus of rats exposed neonatally to diethylstilbestiol (studied in collaboration with Dr. Jim Clark, Baylor College of Medicine). Keratin changes associated with the early appearance of preneoplastic lesions and malignant conversion have also been observed during mouse mammary carcinogenesis (studied in collaboration with Dr. Gilbert Smith, Division of Cancer Biology and Diagnosis (DCBD), NCI). We have previously shown that loss of expression of the differentiation-associated keratins is a negative marker for malignant conversion in mouse skin carcinogenesis. Recent experiments have shown that expression of keratin K13 is a positive marker for malignant conversion (studied in collaboration with Drs. Roswitha Nischt and Jurgen Schweizer, German Cancer Center, Heidelberg, FRG).

2) To study keratin gene expression during abnormal development. Abnormal expression and processing of keratins has been demonstrated in pf/pf and Er/Er mutant mice which exhibit developmental defects in epidermal differentiation (studied in collaboration with Dr. Chris Fisher, Univ. of Washington.)

3) To follow the assembly of the cysteine-rich envelope precursors. Antibodies elicited against C-terminal sequences deduced from a cDNA clone suspected of encoding an envelope precursor protein were essential for confirming the identity of this clone. In addition, these antibodies have been used to follow the assembly of the precursor into the envelope and have revealed that the C-terminal sequences are only detectable on the inside of the envelope. This epitope has been shown to be conserved in the human protein and this has facilitated the isolation of the human cDNA clone (performed in collaboration with Drs. Daniel Hohl and Peter Steinert, DB, NCI).

4) To monitor the induction of terminal differentiation in malignant cell lines by pharmacological agents. Antisera elicited against the human differentiation-associated keratins have been used to demonstrate that τ-interferon can induce the terminal differentiation of A431 cells, a human epidermoid carcinoma cell line, (studied in collaboration with Dr. E. Chang, Uniformed Services University of the Health Sciences (USUHS), and Dr. J. Ridge, Center for Drugs and Biologics (CDB).

5) To follow the induction of differentiation in vivo. Antibodies against differentiation-associated keratins have been used in double-label experiments with antibodies against bromodeoxyuridine to determine that expression of these keratins occurs postmitotically. Furthermore, this study revealed that expression of these genes was sequential in that the Type II (67 kd) keratin appeared approximately 18 hours after DNA replication, followed two hours later by the Type I (59 kd) keratin (studied in collaboration with Dr. Henrik Huitfeldt, Univ. of Oslo).

6) To monitor the induction of terminal differentiation in vitro. Antisera against the differentiation-associated keratins have been used to show that expression of these proteins occurs in a low percentage of primary epidermal cells induced to differentiate by Ca^{2+}. This demonstrates that the in vitro system is permissive for the expression of these differentiation products. The availability of antisera against the differentiation-associated keratins as well as filaggrin and cysteine-rich envelope precursor, made it possible to modify the in vitro system so that a majority of the cells synthesize these products when they are induced to differentiate. The ability to induce differentiation in the in vitro system, that so closely mimics that occurring in vivo, should greatly facilitate attempts to define the mechanism regulating the expression of these genes as well as the differentiation process itself.

In order to elucidate the mechanism regulating the expression of these genes, members of each subset have been isolated and sequenced. In initial attempts to define sequences which regulate the expression of these genes, synthetic oligonucleotides corresponding to a 5' flanking region of the human differentiation-associated keratin gene (K1) have been inserted into a vector containing the

chloramphenicol acetyltransferase gene. These studies have revealed a keratinocyte-specific enhancer; however, additional enhancement of this activity is not achieved by inducing differentiation. This may be due to the absence of a differentiation-specific enhancer in these sequences or to difficulties in inducing terminal differentiation in the cell cultures employed. In an attempt to overcome these problems, transgenic mice have been produced in collaboration with Dr. Su Chung (USUHS). These mice contain the differentiation-associated human keratin gene, K1, which is flanked by 2 kilobases upstream and 3 kilobases downstream. This DNA fragment contains sufficient sequence information for tissue- and developmental-specific expression. Current experiments involving transgenic mice are aimed at defining the minimal sequences required for appropriate expression of this gene.

Additional information regarding sequences which function in maintaining the strict pattern of gene expression observed in the epidermis, may be obtained by comparing sequence data for several genes that are expressed at the same state of differentiation. Therefore, in collaboration with Dr. John Stanley DB, NCI, a project was initiated to clone the gene encoding the bullous pemphigoid antigen. This gene was of interest since it's expression is regulated like the proliferation-associated keratins. In addition, it is of interest clinically since the production of autoantibodies against this protein results in a subepidermal blistering disease. Sera from bullous pemphigoid patients were used to screen an expression library prepared against mRNA isolated from primary human epidermal cells. Cloned DNAs resulting from this screen are being characterized by DNA sequence analysis and will be used to isolate genomic clones.

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CONTRACT IN SUPPORT OF ALL LABORATORY PROJECTS

IMMUQUEST LABORATORIES, INC. (N1-CP-71092)

Title: Laboratory Rodent and Rabbit Facility for the Laboratory of Cellular Carcinogenesis and Tumor Promotion

Current Annual Level: $349,986.

Man Years: 5.50

Objectives: To provide in vivo support for several research projects within LCCTP: The contractor (1) carries out skin carcinogenesis experiments; (2) feeds vitamin-deficient or vitamin-supplemented diets; (3) removes, freezes and stores tumors or other tissues; (4) grafts epidermal cells or cell lines onto athymic mice; (5) prepares antisera from rabbits; and (6) provides for short-term holding of animals and delivery to LCCTP on request.

Major Contributions: The contract is to provide support for LCCTP in-house research; the major contributions of the contract can be found in the individual project descriptions.

Proposed Course: The contract term is March 1, 1987 through February 28, 1991.

ANNUAL REPORT OF

LABORATORY OF CHEMOPREVENTION
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The problem of the isolation, characterization, and biological role of transforming growth factors (TGFs) continues to be the major focus of 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 peptides. New methods to achieve purification have been developed in our laboratory, and we have completed the total purification of TGF-beta from three non-neoplastic tissues. The tissues are human placenta, human blood platelets and bovine kidney. The experimental use of TGF-beta in wound healing has been a finding of major importance and has provided a great deal of encouragement to proceed further with the entire problem of the molecular biology and molecular genetics of these growth factors. Finally, we are now involved in a major attempt to integrate studies of retinoids into our current program of studies on peptide growth factors.

The present activities of the laboratory are devoted almost exclusively to studying the chemistry and biology of type beta transforming growth factors. These studies include the interactions of these peptide growth factors with the genetic apparatus of the cell, particularly oncogenes, as well as their interactions with low molecular weight regulatory agents, such as retinoids and steroids. The laboratory is involved with the total spectrum of studies that can be done with growth factors, ranging all the way from mutating their chemical structure with the most advanced techniques of recombinant DNA research to evaluating their potential therapeutic usefulness as clinical agents in patients with defective wound healing.

We have characterized and purified to homogeneity a new peptide growth factor called transforming growth factor-beta (TGF-beta). Although this peptide was named for its ability to cooperate with members of the epidermal growth factor family to induce phenotypic transformation and anchorage-independent growth of non-neoplastic fibroblasts, it has been shown that TGF-beta can inhibit the anchorage-independent growth of certain tumor cells. This bifunctional character of TGF-beta is best shown in experiments using fibroblasts transfected with the *myc* gene; in these cells, TGF-beta can either stimulate or inhibit the anchorage-independent growth of the cells, depending on the complete set of other growth factors operant on the cells.

TGF-beta stimulates matrix production by mesenchymal cells. Many cell types including cells of the immune system, fibroblasts, and osteoblasts are known to secrete TGF-beta, suggesting that both fibrosis that accompanies chronic

inflammation and matrix formation by bone-forming cells may be dependent on TGF-beta. It has been postulated that excessive levels of TGF-beta might contribute to fibrotic diseases. We have shown, in a clinical setting, that excessive levels of TGF-beta are found in vitreous aspirates of eyes of patients with a disease called proliferative vitreoretinopathy and that levels of the protein increase with the severity of the fibrosis. Importantly, it has also been shown that the retinal pigment epithelial cell, previously postulated to play a central role in the disease process, can serve as a source of TGF-beta. We hope to extend these studies to include other fibrotic diseases.

Immunohistochemical studies of TGF-beta 1 expression in developing mouse embryos demonstrated that the expression of the peptide often correlated with morphogenesis and organogenesis. We have now shown TGF-beta 2 is involved in very early embryological development in the differentiation of mesoderm from ectoderm. In addition, medium conditioned by Xenopus tadpole cells, which is a powerful inducer of mesodermal markers, has been shown to contain TGF-beta and antibodies to TGF-beta interfere with the ability of this medium to act as an inducer. Present efforts are focused on purification of TGF-beta from this medium with the hopes of characterizing new forms of the peptide in the frog and of gaining insight into the action of growth factors in establishment of germ layers.

As part of our interest in the roles of TGF-beta in embryology, we have attempted to clone genes related to mammalian TGF-betas from cDNA libraries made from embryonic chicken and frog tissues. This has resulted in the identification of two novel forms of TGF-beta in the chicken and one in the frog. Comparison of the deduced amino acid sequences of these new TGF-betas with those of TGF-betas 1 and 2 shows areas of conservation which suggest specific functions. We are examining the expression patterns of these new genes in both embryonic and adult tissues and attempting to determine whether they might have specialized developmental roles. Antibodies are also being generated to these new TGF-betas to detect the protein products of these genes.

Control of TGF-beta expression is fundamental to the tissue-specific and time-dependent production of the protein found in embryonic development. Two aspects of this control currently under investigation are the production of alternately spliced TGF-beta 1 mRNAs and the identification of specific control elements in the promoter region of the TGF-beta 1 gene. An alternately spliced TGF-beta mRNA has been characterized which is predicted to encode a novel gene product which may target to the nucleus. Investigations aimed at identifying this protein are in progress. In a different approach, the promoter region for the TGF-beta 1 gene has been isolated from a human genomic library. Preliminary investigations have shown that this region contains both positive and negative control elements. We are currently attempting to identify the precise sequences in these regions responsive to inducers and repressors of TGF-beta 1 gene activity.

In vitro studies indicate that most cell types, whether derived from embryonic or adult tissue, produce and respond to transforming growth factor-beta (TGF-beta) under cell culture conditions and virtually all cells possess receptors for this peptide. Yet little is known regarding either the identity of the cells responsible for TGF-beta synthesis in vivo or the peptide's cellular targets and physiological roles. To address these issues we have raised polyclonal antibodies in rabbits to synthetic peptides corresponding to regions of mature TGF-beta and its precursor. Antibodies have been characterized by their

abilities to recognize TGF-beta in radioimmunoassays, enzyme-linked immunosorbent assays, Western blots, and for immunohistochemical systems. Two antibodies which are particularly useful for immunohistochemical localization of TGF-beta, recognize different epitopes of the protein and give different localizations; one antibody gives primarily intracellular staining and seems to recognize TGF-beta at sites of synthesis, while the other antibody gives extracellular staining and may recognize TGF-beta at sites of action or storage. Initial immunohistochemical studies on the role of TGF-beta in the development of the mouse have shown it to be associated with mesenchyme and localized in areas of active morphogenesis. By use of additional peptide antibodies we are expanding these studies to investigate the role of TGF-beta in development of the chick. In addition, we have used these antibodies to localize TGF-beta in neonatal and adult mouse and have seen staining on chondrocytes, renal distal tubules, placental chorion cells, adrenal cortex, cardiac myocytes, and bone marrow. Studies in the normal mouse are being used as a baseline for comparative studies of pathological conditions such as heart ischemia.

Extensive analysis of the distribution and modulation of the cellular receptor for TGF-beta has shown that binding of TGF-beta to its receptor is not a major control point in TGF-beta action. However, normal and transformed cells have been shown to secrete TGF-beta in a biologically inactive form that is unable to bind to the receptor, and it's anticipated that activation of this latent form will be a critical regulatory step in TGF-beta action. Using immunohistochemical techniques, the latent form of TGF-beta secreted by human platelets has been shown to be a high molecular weight complex in which mature TGF-beta is non-covalently associated with precursor sequences and a further unidentified component; this probably represents a delivery complex. A second latent form of TGF-beta, found in serum, has been identified as TGF-beta bound to alpha-2-macroglobulin; this probably represents a clearance complex. In collaboration with Genentech, Inc., the latent form of TGF-beta made by recombinant constructs has been purified to homogeneity and this material will be used to study the biological activities of latent TGF-beta, the in vivo activation mechanism and the in vivo pharmacokinetics of the latent form. It is anticipated that this will be the clinically useful form of the molecule.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05051-10 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Biology and Molecular Biology of Transforming Growth Factor-beta

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Lab	Institute
PI:	Anita B. Roberts	Staff Scientist	LC	NCI
OTHERS:	Sonia B. Jakowlew	Sr. Staff Fellow	LC	NCI
	Seong Jin Kim	Visiting Fellow	LC	NCI
	David Danielpour	Guest Researcher	LC	NCI
	Adam Glick	NRSA Fellow	LC	NCI
	Paturu Kondaiah	Visiting Fellow	LC	NCI
	Nannette B. Roche	Biologist	LC	NCI
	Pamela J. Dillard	Chemist	LC	NCI

COOPERATING UNITS (if any)

Wilmer Opthalmological Institute, Johns Hopkins University School of Medicine, Baltimore, MD, Bert Glaser

Laboratory of Molecular Genetics, NICHHD, Igor Dawid

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6.0	4.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The purpose of the project is twofold: to study the biology of transforming growth factor-beta (TGF-beta), particularly in terms of its effects on cell function, and to investigate the molecular biology of TGF-beta with emphasis on both identifying possible additional members of the TGF-beta family and evaluating the degree of conservation between species of both the precursor and processed TGF-beta peptides. With respect to the biology of TGF-beta, one of the principal effects of the peptide on cells of mesenchymal origin is to control synthesis of matrix proteins. Thus, aberrant expression of TGF-beta might be expected to contribute to the excessive fibrosis characteristic of several disease processes. Indeed, it has been possible to correlate levels of TGF-beta in vitreous aspirates of human eyes with the degree of severity of a fibrotic disease, proliferative vitreoretinopathy. Other lines of investigation have shown that the known ability of TGF-beta to regulate cellular proliferation and cellular differentiation might be important in the establishment of the germ layers of developing amphibian embryo. Use of specific antibodies to TGF-beta, as well as assays to detect and quantitate the peptide, suggest that TGF-beta might be essential in induction of mesoderm from ectoderm. These interests in embryology have also resulted in the cloning and sequencing of three new genes for proteins related to TGF-betas in cDNA libraries of chicken and frog embryos, as well as in identification of mechanisms of control of TGF-beta expression such as alternate splicing of mRNAs and specific positive and negative regulatory control regions of the TGF-beta 1 promoter. Understanding the mechanisms which control TGF-beta expression will be important not only to developmental processes, but to disease processes as well.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

Anita B. Roberts	Staff Scientist	LC	NCI
Sonia B. Jakowlew	Sr. Stafff Fellow	LC	NCI
David Danielpour	Guest Researcher	LC	NCI
Adam Glick	NRSA Fellow	LC	NCI
Paturu Kondaiah	Visiting Fellow	LC	NCI
Seong Jin Kim	Visiting Fellow	LC	NCI
Nannette B. Roche	Biologist	LC	NCI
Pamela J. Dillard	Chemist	LC	NCI

Objectives:

This project is directed towards three goals: 1) understanding the mechanisms whereby TGF-beta controls synthesis of matrix proteins in mesenchymal cells; 2) identifying new members of the TGF-beta family and investigating whether they might have specific patterns of expression or specific functions, and 3) examining mechanisms of control of TGF-beta expression and synthesis. With respect to control of matrix synthesis, investigations will be aimed at identifying the mechanisms of control by TGF-beta of matrix protein mRNA expression. Along other lines, cloning of genes related to TGF-beta in chicken and frog embryos will increase our understanding both of the function of specific conserved regions of the protein as well as of possible unique expression patterns or biological activities of the different TGF-betas.

Methods Employed:

Standard methods are utilized based on use of specific reagents such as iodinated TGF-beta, antibodies to TGF-beta, and cDNA probes for both growth factors and matrix proteins. In addition, constructs of the promoter for TGF-beta 1 linked to the gene for chloramphenicol acetlytransferase have been employed to assay for control elements in the TGF-beta promoter.

Major Findings:

TGF-beta stimulates matrix production by mesenchymal cells. Many cell types including cells of the immune system, fibroblasts, and osteoblasts are known to secrete TGF-beta, suggesting that both fibrosis that accompanies chronic inflammation and matrix formation by bone-forming cells may be dependent on TGF-beta. It has been postulated that excessive levels of TGF-beta might contribute to fibrotic diseases. We have shown, in a clinical setting, that excessive levels of TGF-beta are found in vitreous aspirates of eyes of patients with a disease called proliferative vitreoretinopathy and that levels of the protein

increase with the severity of the fibrosis. Importantly, it has also been shown that the retinal pigment epithelial cell, previously postulated to play a central role in this disease process, can serve as a source of TGF-beta. We hope to extend these studies to include other fibrotic diseases.

Immunohistochemical studies of TGF-beta 1 expression in developing mouse embryos demonstrated that the expression of the peptide often correlated with morphogenesis and organogenesis. We have now shown that TGF-beta 2 is involved in very early embryological development in the differentiation of mesoderm from ectoderm. In addition, medium conditioned by Xenopus tadpole cells, which is a powerful inducer of mesodermal markers, has been shown to contain TGF-beta and antibodies to TGF-beta which interfere with the ability of this medium to act as an inducer. Present efforts are focused on purification of TGF-beta from this medium with the hopes of characterizing new forms of the peptide in the frog and of gaining insight into the action of growth factors in establishment of the germ layers.

As part of our interest in the roles of TGF-beta in embryology, we have attempted to clone genes related to mammalian TGF-betas from cDNA libraries made from embryonic chicken and frog tissues. This has resulted in the identification of two novel forms of TGF-beta in the chicken and one in the frog. Comparison of the deduced amino acid sequences of these new TGF-betas with those of TGF-betas 1 and 2 shows areas of conservation which suggest specific functions. We are examining the expression patterns of these new genes in both embryonic and adult tissues and attempting to determine whether they might have specialized developmental roles. Antibodies are also being generated to these new TGF-betas to detect the protein products of these genes.

Control of TGF-beta expression is fundamental to the tissue-specific and time-dependent production of the protein found in embryonic development. Two aspects of this control currently under investigation are the production of alternately spliced TGF-beta 1 mRNAs and the identification of specific control elements in the promoter region of the TGF-beta 1 gene. An alternately spliced TGF-beta mRNA has been characterized which is predicted to encode a novel gene product which may target to the nucleus. Investigations aimed at identifying this protein are in progress. In a different approach, the promoter region for the TGF-beta 1 gene has been isolated from a human genomic library. Preliminary investigations have shown that this region contains both positive and negative control elements. We are currently attempting to identify the precise sequences in these regions responsive to inducers and repressors of TGF-beta 1 gene activity.

Publications:

Cromack DT, Sporn MB, Roberts AB, Merino MJ, Dart LL, Norton JA. Transforming growth factor-beta levels in rat wound chambers. J Surg Res 1987;42:622-8.

Falanga V, Tiegs SL, Alstadt SP, Roberts AB, Sporn MB. Transforming growth factor-beta: selective increase in glycosaminoglycan synthesis by cultures of fibroblasts from patients with progressive systemic sclerosis. J Invest Derm 1987;89:100-4.

Flanders KC, Roberts AB, Ling N, Fleurdelys, BE, Sporn MB. Antibodies to peptide determinants in transforming growth factor-beta and their applications. Biochemistry 1988;27:739-46.

Gerwin BI, Lechner JF, Reddel R, Roberts AB, Robbins K, Gabrielson EW, Harris CC. A comparison of production of transforming growth factor-beta and platelet-derived growth factor-like growth factors by normal human mesothelial cells and mesothelioma cell lines. Cancer Res 1987;47:6180-4.

Heine UI, Munoz EF, Flanders KC, Ellingsworth LR, Lam H-YP, Thompson NL, Roberts AB, Sporn MB. The role of transforming growth factor-beta in the development of the mouse embryo. J Cell Biol 1987;105:2861-76.

Jakowlew SB, Kondaiah P, Flanders KC, Thompson NL, Dillard PJ, Sporn MB, Roberts AB. Increased coordinate expression of growth factor mRNAs accompanies viral transformation of rodent cells. Oncogene Res 1988;2:135-48.

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Roberts AB, Sporn MB. Transforming growth factor-beta: stimulator or inhibitor of angiogenesis? In: Rifkin DB, Klagsbrun M, (eds.) Angiogenesis: mechanisms and pathobiology. Current communications in molecular biology, Cold Spring Harbor Laboratory. 1987;69-71.

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Segarini PR, Roberts AB, Rosen DM, Seyedin SM. Membrane characteristics of two forms of transforming growth factor-beta. J Biol Chem 1987;262:14655-62.

Sporn MB, Roberts AB. Peptide growth factors are multifunctional. Nature 1988;332:217-9.

Sporn MB, Roberts AB. Suppression of carcinogenesis by retinoids: interactions with peptide growth factors and their receptors as a key mechanism. In Hyashi: Y, et al. (eds) Diet, nutrition and cancer. Utrecht, Japan Sci. Soc. Press 1986;149-58.

Sporn MB, Roberts AB. Transforming growth factor-beta: new chemical forms and new biological roles. Biofactors 1988;1:89-93.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05396-05 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Development of Methods to Study the Functions of TGF-beta

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Shinichi Watanabe	Sr. Staff Fellow	LC	NCI
OTHERS:	Andrew Geiser	IRTA Fellow	LC	NCI
	J. Michael Thomas	Biologist	LC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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 (Use standard unreduced type. Do not exceed the space provided.)

In order to study the function of transforming growth factor-beta (TGF-beta) types 1 and 2, we are constructing several modified small nuclear RNA sites to block splicing of precursor RNA for TGF-beta in cells. Modified genes were inserted into the retrovirus vectors and recombinant virus was used to infect cells. The other approach to block the expression of TGF-beta genes is to disrupt the genes themselves by homologous recombination by using modified retrovirus vectors. This approach is still too preliminary to discuss in detail. The second project is to dissect the genes involved in signal transduction of TGF-beta after binding to their receptors. We are using insertional mutagenesis to construct mutant cells which do not respond to TGF-beta. Insertional mutagenesis will allow the isolation of genes which cause resistance to TGF-beta action.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Shinichi Watanabe	Senior Staff Fellow	LC	NCI
Andrew Geiser	IRTA Fellow	LC	NCI
Michael Thomas	Biologist	LC	NCI

Objectives:

There are two major objectives to develop methodology to study the function of TGF-beta: 1) to make cells or animal models which are not expressing the TGF-beta gene and 2) to dissect the action of TGF-beta after binding to its receptors. The first objective is directed toward understanding the importance of TGF-beta by making mutant cells (or animal models) by disrupting the expression. The second objective is to understand what kinds of events are involved in signal transduction of TGF-beta.

Methods Employed:

We are using standard molecular biological techniques and extensive use of retrovirus vectors. Retrovirus vectors are a much more versatile means of introducing genes to a wide variety of cells.

Major Findings:

We have introduced the modified small nuclear RNA U1 gene by retrovirus mediated gene transfer into A549 cells. The modified gene was expressed well in A549, but the copy number of the modified U1 RNA was lower than wild type RNA. This made it difficult to obtain good inhibition of TGF-beta expression in A549 cells. This observation led to infection of A549 cells at high multiplicicty of infection by co-culture of A549 and producer cell lines for the retrovirus-carrying modified U1 gene.

The cell line producing recombinant retrovirus for insertional mutagenesis was obtained from another researcher, but this cell line was contaminated with helper viruses. This forced us to construct a cell line which produces only recominant virus. We have this cell line now and are using it to carry out insertional mutagenesis.

Publications:

Lazar E, Watanabe S, Dalton S, Sporn MB. Transforming growth factor alpha: mutation of aspartic acid 47 and leucine 48 results in different biological activities. Mol Cell Biol 1988;8:1247-52.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05398-05 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Characterization of Latent Forms of Transforming Growth Factor-beta

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Lalage M. Wakefield	Visiting Associate	LC	NCI
OTHER:	Diane M. Smith	Biologist	LC	NCI

COOPERATING UNITS (if any)

Genentech, Inc., San Francisco, Dr. Arthur Levinson;
Biotechnology Research Institute, Montreal, Dr. Maureen O'Conner-McCourt

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.6	1.0	.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Transforming growth factor-beta (TGF-beta) is a multi-functional peptide that regulates growth and differentiation of a wide variety of cell types. The purpose of this project is to determine the role that the endogenously-produced TGF-beta may play in the control of growth of normal and transformed cells, and to study the regulation of TGF-beta action in this context. To this end, polyclonal antisera have been raised against TGF-beta and synthetic peptides corresponding to regions of the putative precursor, and these are being used as tools in the immunochemical characterization of the latent forms of TGF-beta. Extensive analysis of the distribution and modulation of the cellular receptor for TGF-beta has shown that binding of TGF-beta to its receptor is not a major control point in TGF-beta action. However, normal and transformed cells have been shown to secrete TGF-beta in biologically inactive form that is unable to bind to the receptor, and it is anticipated that activation of this latent form will be a critical regulatory step in TGF-beta action. Using immunochemical techniques, the latent form of TGF-beta secreted by human platelets has been shown to be a high molecular weight complex in which mature TGF-beta is non-covalently associated with precursor sequences and a further unidentified component; this probably represents a delivery complex. A second latent form of TGF-beta, found in serum, has been identified as TGF-beta bound to alpha-2-macroglobulin; this probably represents a clearance complex. In collaboration with Genentech, Inc., the latent form of TGF-beta made by recombinant constructs has been purified to homogeneity and this material will be used to study the biological activities of latent TGF-beta, the in vivo activation mechanism and the in vivo pharmacokinetics of the latent form. It is anticipated that this will be the clinically useful form of the molecule.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Lalage M. Wakefield	Visiting Associate	LC	NCI
Diane M. Smith	Biologist	LC	NCI

Objectives:

The purpose of this project is to examine the role of transforming growth factor beta (TGF-beta) in the control of normal cell growth and in the process of malignant transformation. The mechanism of action of this growth factor is being studied at a biochemical level with particular emphasis on the activation of the latent form of TGF-beta secreted by cells in culture and on the initial interaction of the active TGF-beta with the cell surface receptor. With greater understanding of the mechanism of action of TGf-beta in normal growth control, analogs and potential inhibitors may be synthesized and tested with a view to producing effective chemotherapeutic agents.

Methods Employed:

A method has been developed for the radioiodination of TGF-beta to high specific activity for use in specific radioreceptor assays to characterize TGF-beta binding to a variety of cell lines in culture, and to allow accurate quantitation of unknown amounts of TGF-beta in biological samples. TGF-beta activity secreted by cells is characterized biochemically by immunoprecipitation and Western blot techniques using specific anti-peptide antisera. The latent forms of TGF-beta are being purified by fast liquid chromatography, employing a variety of separation techniques including gel filtration, ion exchange, hydrophobic interaction and affinity chromatography. The ability of cells to grow in an anchorage-independent manner in soft agar is used as an assay for the transformed phenotype, and the number and size of cell colonies obtained are measured by using an Omnicon image analyzer. Anchorage-dependent growth is quantitated by determining changes in cell number for cells grown in monolayer.

Major Findings:

Characterization of the TGf-beta Receptor

Binding of TGF-beta to the cell membrane initiates the chain of events leading to cell transformation, so TGF-beta-receptor interaction and its modulation are of particular interest in any mechanistic studies. A method was developed for the iodination of TGF-beta without loss of biological activity for use in a radioreceptor assay. High affinity TGF-beta receptors have been found on all of over 60 cell types assayed to date, including cells of mesenchymal, epithelial and hematopoietic origin and many tumor-derived cells. There is a strong inverse relationship between the affinity of the receptor for TGFs and the number of receptors expressed per cell, indicating a striking degree of

conservation of the level of ligand binding. None of the agents that modulate TGF-beta action effect the binding of TGF-beta to target cells, suggesting modulation of TGF-beta binding is not a major control point in TGF-beta action. Unlike the platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) receptors, the receptor for TGF-beta does not show extensive ligand-induced down-regulation and in some cell types down-regulation does not occur at all. In addition, there is never more than a twofold change in TGF-beta receptor number on cell transformation, again in contrast to observations with other growth factor receptors. Thus, the TGF-beta receptor appears to be unique in the degree to which the level of TGF-beta binding is conserved in different cell types under different conditions, probably reflecting a very critical role for TGF-beta in the regulation of growth and differentiation in many cell types.

Identification and Characterization of Latent Forms of TGF-beta

Human platelets, when induced to degranulate by thrombin, secrete transforming growth factor beta (TGF-beta) in a biologically latent form. In this form, TGF-beta cannot bind to its cellular receptor, nor can it be immunoprecipitated by polyclonal antisera to TGF-beta, suggesting that the receptor-binding site and other TGF-beta epitopes may be masked. Western blot analysis of the platelet secretions indicates that the latent form of TGF-beta is a 220-235 kDa complex, in which mature TGF-beta (25 kDa) is non-covalently associated with sequences from the remainder of the precursor (75 kDa), and a third unidentified entity (~135 kDa). The third component is immunologically unrelated to other growth factor binding proteins. The complex is glycosylated, and gel filtration analysis suggests it may exist in solution as higher molecular weights aggregates. Further chromatographic analysis indicates that in its latent form, the platelet TGF-beta cannot bind to alpha$_2$-macroglobulin (alpha$_2$M), but that if the platelet latent TGF-beta is activated by transient acidification, the released active TGF-beta will bind to an alpha$_2$M. We have identified the latent form of TGF-beta found in serum as an alpha$_2$M-TGF-beta complex and we propose that the latent TGF-beta secreted by platelets may be a cellular delivery complex, whereas the latent form found in serum may represent a clearance complex. Thus, alpha$_2$M may scavenge excess TGF-beta that is released when the platelet latent form is activated, possibly by the clotting process. Finally we have shown that the latent form of TGF-beta secreted by a variety of cell types in culture is similar, if not identical, to that secreted by platelets.

The human lung carcinoma line A549 appears to have lost the ability to activate the latent endogenous form of TGF-beta that it secretes and can therefore no longer limit its own growth in an autocrine fashion, whereas the parent normal cell can respond to the latent TGF-beta that it secretes. Thus, activation of latent secreted TGF-beta may be a step that is disrupted on transformation. In collaboration with Genentech, Inc., we have purified to homogeneity a latent form of TGF-beta synthesized by recombinant constructs. This has many of the same properties as the natural form and will be used in in vitro studies to analyze the cellular mechanism for activation of the latent form, and in in vivo studies to determine whether the pharmacokinetics of the latent form will make it the form of choice for clinical therapeutic use.

Publications:

O'Conner-McCourt MD, Wakefield LM. Latent transforming growth factor-beta in serum: a specific complex with alpha-2-macreglobulin. J Biol Chem 1987;262:14090-9.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05550-01 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Immunohistochemical Localization of Transforming Growth Factor-beta

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Kathleen C. Flanders	Sr. Staff Fellow	LC	NCI
Others:	Nancy L. Thompson	Biotechnology Fellow	LC	NCI
	David S. Cissel	Biologist	LC	NCI
	Joseph M. Smith	Biologist	LC	NCI
	Ursula I Heine	Staff Scientist	LCC	NCI
	Larry Mullen	Animal Technician	LC	NCI
	Peter Brown	Sr. Staff Fellow	LC	NCI

COOPERATING UNITS (if any)

Fernando Bazoberry and Ward Casscells, NHLBI

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

In vitro studies indicate that most cell types, whether derived from embryonic or adult tissue, produce and respond to transforming growth factor-beta (TGF-beta) under cell culture conditions. Virtually all cells possess receptors for this peptide; yet little is known regarding either the identity of cells responsible for TGF-beta synthesis in vivo or the peptides' cellular targets and physiological roles. To address these issues we have raised polyclonal antibodies in rabbits to synthetic peptides corresponding to regions of mature TGF-beta and its precursor. Antibodies have been characterized by their abilities to recognize TGF-beta in radioimmunoassays, enzyme-linked immunoabsorbent assays, Western blots and immunohistochemical systems. Two antibodies which are particularly useful for immunohistochemical localization of TGF-beta, recognize different epitopes of the protein and give different localizations; one antibody gives primarily intracellular staining and seems to recognize TGF-beta at sites of synthesis, while the other antibody gives extracellular staining and may recognize TGF-beta at sites of action or storage. Initial immunohistochemical studies on the role of TGF-beta in the development of the mouse have shown it to be associated with mesenchyme and localized in areas of active morphogenesis. By use of additional peptide antibodies we are expanding these studies to investigate the role of TGF-beta in development of the chick. In addition, we have used these antibodies to localized TGF-beta in neonatal and adult mouse and have seen staining in chondrocytes, renal distal tubules, placental chorion cells, adrenal cortex, cardiac monocytes and bone marrow. Studies in the normal mouse are being used as a baseline for comparitive studies of pathological conditions such as heart ischemia.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Kathleen Flanders	Sr. Staff Fellow	LC	NCI
Nancy L. Thompson	Guest Researcher	LC	NCI
Peter Brown	Senior Staff Fellow	LC	NCI
David Cissel	Biologist	LC	NCI
Joseph M. Smith	Biologist	LC	NCI
Ursula I. Heine	Staff Scientist	LCC	NCI
Larry Mullen	Animal Technician	LC	NCI

Objectives:

The purpose of this project is to examine the role of transforming growth factor-beta (TGF-beta) in embryonic development, the maintenance of normal growth homeostasis, and the pathogenesis of a variety of malignant and non-malignant diseases. To accomplish this, we have raised antibodies to peptides corresponding to sequences of mature TGF-beta and its precursor and have characterized the abilities of antibodies to recognize TGF-beta in immunohistochemical localization studies. Localization of TGF-beta in mouse and chick embryos at various gestational ages provides evidence that TGF-beta is an important developmental factor. Localization of TGF-beta in adult mouse tissues suggests that it has a role in normal growth and provides a baseline for comparative studies of pathological disease states, as well as suggesting additional *in vivo* roles for TGF-beta.

Methods Employed:

Polyclonal antisera were raised in rabbits to peptides corresponding to various regions of TGF-beta. Each antiserum was characterized by enzyme-linked immunosorbent assay, radioimmunoassay, Western blot and ability to inhibit binding of iodinated TGF-beta to its receptor. Immunohistochemical staining was done on paraffin-embedded tissue using the sensitive avidin-biotin peroxidase technique. *In situ* hybridization techniques are being developed to correlate tissue mRNA and protein levels.

Major Findings:

Characterization of TGF-beta Antibodies

Region-specific antisera have become useful reagents to detect TGF-beta in Western blot and immunoprecipitation assays. Furthermore, based on the ability of the antisera to block binding of iodinated TGF-beta to its receptor, it appears that the carboxyl terminal region of TGF-beta plays a significant role in binding of the native ligand to its receptor. A number of antisera also are able to detect immunoreactive TGF-beta in tissue sections from several species, making them useful immunohistochemical reagents. Differential immunohistochemical localization of TGF-beta

(intracellular vs. extracellular) with the antibodies suggests a conformational change in TGF-beta upon secretion from the cell. This change may play a role in generating the active peptide.

Role of TGF-beta in Development

Initial studies of the immunohistochemical localization of TGF-beta in mouse embryos showed that it is often associated with mesenchyme M areas of active remodeling and morphogenesis of areas of epithelial-mesenchymal interaction. Studies with newly developed antibodies indicate which cells may be synthesizing TGF-beta. Similar staining patterns in mouse and chick embryos support the role of TGF-beta in morphogenesis and pattern development and as an important modulator of the development of several organs, such as heart and kidney.

Role of TGF-beta in Normal and Disease States

Immunohistochemical localization of TGF-beta in the adult mouse revealed cell type specific expression of the protein in a variety of cells, including costal chondrocytes, renal distal tubules, placental chorion cells, adrenal cortical cells, cardiac myocytes, bone marrow and ovarian interstitial cells. These findings suggest a possible role of TGF-beta in normal growth homeostasis or as a modulator of processes, steroidogenesis. We plan to use the study of normal mouse as a baseline for comparative studies of pathological conditions. For example, using a rat model of heart ischemia we have found that following infection there is a loss of immunoreactive TGF-beta from cardiac myocytes and an increase of immunoreactive TGF-beta in arterial smooth muscle and viable border zone myocytes. This suggests a role for TGF-beta in fibrinolysis, wound healing or angiogenesis in the heart.

Publications:

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Heine, UI, Munoz, EF, Flanders, KC, Ellingsworth, LR, Lam, PH-Y, Thompson, NL, Roberts, AB, Sporn, MB. Role of transforming growth factor-beta in the development of the mouse embryo. J Cell Biol 1987;105:2861-76.

ANNUAL REPORT OF

THE LABORATORY OF COMPARATIVE CARCINOGENESIS
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The Laboratory of Comparative Carcinogenesis (LCC) 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 specificities in carcinogenesis; (3) investigates the roles of nutrition, metabolism, the perinatal age period and pregnancy in modifying susceptibility to chemical carcinogens; and (4) conducts biological and morphologic studies on the pathogenesis of naturally occurring and induced tumors in experimental animals.

Organizational Changes: At the beginning of this fiscal year, the Inorganic Carcinogenesis Section was established, incorporating and expanding the program and personnel of the Inorganic Carcinogenesis Working Group that previously existed in the Office of the Chief. During this year, the Ultrastructural Studies Section was abolished, and that aspect of its program dealing with gap junctional cell-cell communication and its interruption by tumor promoting agents was transferred to the Tumor Pathology and Pathogenesis Section.

Summary Report: The Laboratory of Comparative Carcinogenesis provides a major focus within the Chemical and Physical Carcinogenesis Program for studies on the mechanisms of experimental carcinogenesis that involve primary neoplasia in animals as experimental end points, with a primary goal of establishing a rigorous experimental basis for extrapolation of mechanistic concepts in chemical carcinogenesis from experimental species to human beings. Two major categories of chemical agents are the subject of special research emphasis in this Laboratory. The first are the inorganic carcinogens, especially the cations of the metallic elements nickel and cadmium. A second category of agents of major importance, because of their wide use in human medicine, consists of drugs that are sedative, anticonvulsant, analgesic, or anxiolytic, and that may be prescribed and taken in large doses for long periods by substantial numbers of individuals. Many of these agents increase the incidence of neoplasia, especially hepatocellular tumors, in experimental species even though the compounds are generally nonmutagenic. This has served to identify many of these drugs, including long-acting barbiturates and hydantoin sedatives and anticonvulsants, various aniline derivatives such as acetaminophen, and a number of the benzodiazepines as nongenotoxic chemical carcinogens. The mechanisms of action of these nongenotoxic carcinogens are urgently in need of study to determine whether their prolonged use is potentially as deleterious in humans as prolonged use of phenacetin, an effective analgesic but a potent renal carcinogen.

Identification of specific transforming genes (oncogenes) in both human and experimental tumors has stimulated great interest and intensive efforts in many laboratories to clarify the roles of those genes in the pathogenesis of cancer. As many of the known oncogenes derive from normal elements of the mammalian

genome, there is a real possibility that the biochemical mechanisms of neoplastic transformation may be definable by thorough analysis of the properties of the oncogenes and their cellular homologs. Of special interest in chemical carcinogenesis are oncogenes that behave as dominant genetic elements and that are activated to this behavior either by single-base mutations, such as the ras family of genes. This mechanism of activation can be caused, in principle, by genotoxic chemical carcinogens, which can be provisionally identified as such by their mutagenic or clastogenic effects. The mechanisms of action of such agents, long considered to involve damage to DNA, may eventually be reconciled with molecular virology, and the crucial events in cellular transformation, by both chemical and biological agents, defined through analysis of the activation and behavior of oncogenes. Detection and critical evaluation of the roles of dominant oncogenes, especially mutant genes of the ras and erb families, have become a major unifying theme in the research activities of several Sections within this Laboratory.

Research on the carcinogenicity of synthetic fecapentaene-12 (FP-12) has produced no evidence that this highly mutagenic natural product is capable of inducing neoplasms in either rats or mice, in the colon or in other tissues. Studies continue using highly stabilized preparations containing α-tocopherol to retard oxidative degradation, and the more highly liquid soluble diacetyl derivative.

Major research programs on metabolic determinants of transplacental carcinogenesis in rodents and nonhuman primates and on the chemistry and biochemistry of nitrosamines also continue and are described in detail both in the following summary reports of each Section and in the individual project reports.

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.

The Primate Research Working Group has continued its observations on patas and cynomolgus monkeys that are at risk for tumor development following prenatal exposure to organic nitroso compounds or to aflatoxin B_1, or that were subjected to initiation of the carcinogenic process by exposure to N-nitrosodiethylamine during adulthood followed by prolonged oral administration of a barbiturate as a putative promoting agent.

Nonhuman primates of the species Erythrocebus patas (patas) and Macaca fascicularis (cynomolgus) are subjected to direct-acting or metabolism-dependent chemical carcinogens by transplacental or direct exposure. In some cases the carcinogen-treated animals are subsequently exposed to chemicals that promote the development of neoplasms in rodents. Mechanisms of organ and species differences in the effects of chemical carcinogens and tumor promoters among rodent and nonhuman primate species are investigated. Induced tumors are evaluated by light microscopy using standard staining procedures, histochemical techniques and electron microscopy and are assayed for in vitro cultivability and transplantability to rodents. Selected tumors are subjected to DNA extraction and attempts are made to transfect NIH 3T3 cells with their DNA. These studies have shown that intrinsic susceptibility to transplacental carcinogenesis is greatest in nonhuman primates early in gestation and have provided the only

animal model of chemically inducible gestational choriocarcinoma. The association of chronic ulcerative colitis and multifocal colonic carcinoma in the cotton-top tamarin (Saguinus oedipus) is being investigated in collaboration with Oak Ridge Associated Universities, with primary attention being given to a search for indirect evidence for a fecal mutagen/carcinogen in this species, since direct search for a fecal mutagen has been unsuccessful.

The Molecular Biology Working Group has focused its studies on signal transduction and the control of developmental gene expression on a simple model system, the cellular slime mold Dictyostelium discoideum. This model system is being used to study mechanisms which control developmental gene activation during normal differentiation. Postaggregation Dictyostelium cells transcribe an additional 26% of their genome which is not expressed in earlier pre-aggregation stage cells. Cell-cell interaction is a necessary prerequisite for the synthesis and stability of these new differentiation-specific messenger RNAs. Additionally, the transciption rate and stability of these messenger RNAs are further regulated by a cyclic AMP-mediated process. We have demonstrated that 1) lyzosomatrophic agents such as $(NH_4)_2SO_4$ can replace the need for cell-cell interaction for postaggregation gene expression; 2) cAMP acts to regulate post aggregation gene expression through the cell surface cAMP receptor; 3) accumulation of mRNA for differentiation-specific genes expressed in prestalk cells is regulated through a different kinetic form of the cell surface receptor than those expressed in prespore cells; 4) activation of the cAMP receptor-associated adenylate cyclase does not play a role in the second messenger signal transduction system utilized for activation of expression of either the prespore or the prestalk genes; 5) prespore genes but not prestalk genes utilize a Ca^{++}/Calmodulin-dependent second messenger signal transduction system for their activation; 6) pathways that induce the expression of differentiation-specific genes in prespore cells suppress the expression of genes transcribed during growth; finally 7) analysis of one of the cAMP regulated prespore genes reveals that it is highly homologous to the neural adhesion protein N-CAM and that both its synthesis and cell surface appearance alternate in strict sequence with another cell surface adhesion molecule at key morphogenetic stages.

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 roles of morphogenetic differentiation in controlling the phenotypic expression of neoplastic transformation, the degree of malignancy of tumors, and the susceptibility of developing organs to carcinogenesis are studied using organ culture and tissue transplantation techniques, with current emphasis on the kidney. A serum-free culture system has now been devised that allows for both growth and branching of ureteric bud and proliferation of metanephric mesenchyme. Tubulogenesis has been observed in mesenchyme maintained under these conditions in the absence of an inducing tissue.

The ability of transplacentally administered carcinogens to induce genotoxic damage in cells of embryos or fetuses exposed at different stages of gestation was determined for rat, mouse, and Syrian hamster. Cells were isolated from

exposed embryos and gene mutation at three loci (resistance to 6-thioguanine (6-TG) and ouabain and to diphtheria toxin (DT) in the hamster) were assayed in vitro with simultaneous determination of survival ability. Induced DT and TG mutant frequency (mutants per surviving cell) was greatest when treatment was day 9 of gestation for mesenchymal cells of the Syrian hamster fetus. Cells from the brain also had a large increase in frequency of mutants when fetuses were exposed to NEU at day 9 of gestation and also appeared to have another peak of sensitivity at day 6-7 of gestation. In addition, an almost completed study has as its objective to determine the number of mutants induced in individual fetuses which have been treated with N-nitrosoethylurea (NEU) at different days of gestation. Another study is to determine the spontaneous mutation frequencies in the Syrian hamster using litters of day 13 fetuses. This was 2×10^{-7} DT mutants per viable cell and 2×10^{-7} TG resistant mutants per viable cell. Ninety-five and ninety-nine percent confidence limits were calulated for these mean spontaneous frequencies in order to compare with cells from litters which had been transplacentally treated with a mutagen. In addition the number of 6-TG^r mutant cells was determined in a large number of individual day 13 fetuses. Mutants could not be detected in half of these fetuses, despite testing an average of 53 plates per fetus.

The expression of activated cellular oncogenes in chemically induced rat tumors and in comparable human neoplasms and the relationship of oncogene expression to progression from the normal to the neoplastic phenotype are studied using 3T3 transfection and hybridization techniques and monoclonal antibodies directed against the specific oncogene products. The consistent activation of K-ras in rat renal mesenchymal tumors has been shown to occur by a G → A transition mutation in the second position of codon 12, which has been demonstrated in 3T3 transformants and in DNA from primary tumors whether the DNA transformed 3T3 cells or not. Further studies on the neu oncogene in the pathogenesis of schwannomas, in a different strain of rat than was previously used, confirm the consistent presence in an additional 30 such tumors (100% of those tested) of neu activated by a T → A transversion mutation in that segment of the neu locus encoding the putative transmembrane region of the protein encoded by the gene, a growth factor receptor-tyrosine kinase-type molecule. Further studies are in progress to determine whether neu is comparably activated by point mutation in chemically induced schwannomas of other species of rodents, and in human tumors including schwannomas.

Significant progress has been made with a murine model for the pharmacogenetics of transplacental carcinogenesis by a polycyclic aromatic hydrocarbon (PAH), 3-methylcholanthrene (MC). Genetics allowing induction of metabolism of this chemical predisposed mouse fetuses to susceptibility to tumor initiation in lung and liver, whereas inducibility in the mother allowed protection of the fetuses. Pretreatment with a noncarcinogenic enzyme inducer β-naphthoflavone (BNF) enhanced fetal protection if the mother was inducible, but enhanced tumorigenesis in fetal organs if she was not. Transplacental induction of cytochrome P-450-dependent PAH activating enzyme activity and of gene expression for this P-450 were followed for MC and BNF. Dramatic induction occurred and MC and BNF were noted to have different time- and organ-specific induction patterns. Monoclonal antibody and PAH-inducible P-450s and cDNA probes for the genes specifying these enzymes were used to delineate these phenomena. Transplacental induction of the phase II detoxicating activity, uridine diphosphoglucuronic acid transferase, was found to be inducible by BNF but not MC. Transplacental MC, but not BNF, had a permanent imprinting effect on MC metabolism of the livers of the adult offspring,

and this effect was dependent on genetically-controlled induction of metabolism by either the fetus or the mother. Gene methylation differences are being explored as a possible mechanism for this effect. In other perinatal experiments in progress or recently completed, N-nitrosodimethylamine has been found to be a transplacental carcinogen in the mouse, causing liver tumors and, interesting, sarcomas. Liver tumors initiated by N-nitrosoethylurea in this study have been analyzed for activated oncogenes and found to contain H-*ras* in some cases. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been administered to several strains of mice transplacentally and neonatally with limited effect.

An interdisciplinary approach to analysis of the effect of the common environmental carcinogen N-nitrosodimethylamine (NDMA) has been fruitful. Striking effects of ethanol, which is being increasingly implicated in the etiology of human cancers, have been demonstrated. Co-administration of ethanol in the drinking water with NDMA has been found to cause a consistent and significant increase in numbers of lung tumors and in promutagenic DNA adducts in the lung. Tumor promotion by ethanol was ruled out as a contributing factor. Competitive inhibition of the liver enzyme acting on NDMA, a demethylase (cytochrome P-450j), is the postulated mechanism. This enzyme has been studied biochemically and pharmacokinetically in mouse, monkey, and several other species, with inhibition by a specific monoclonal antibody to confirm the nature of the P-450. The activity appears to be highly conserved across species, so that results from the mouse have particular relevance to the human. Utilization of this system is ongoing. Interesting results have also been obtained with N-nitrosocimetidine (NCM), derivative of a commonly-used pharmaceutical. Though not a complete carcinogen, this alkylating agent both enhanced progression of preformed skin papillomas to malignancy, and initiated tumors which could be caused to develop by subsequent application of a tumor promoter. These NCM-initiated tumors were all found to contain DNA that transformed 3T3 cells and were mutated in the second position of codon 61 of the H-*ras* oncogene. Other experiments that are ongoing in this project include immunohistochemical studies of localization of cytochromes P450 and of DNA adducts using monoclonal antibody reagents, and investigation of the protective effect *in vivo* of pretreatment of mice with inducers of enzymes metabolizing carcinogens.

Previous studies have shown that treatment of a rat hepatoma cell line with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) caused concurrent decreases of the steroid-inducible levels of both tyrosine aminotransferase (TAT) enzyme activity and TAT-specific RNA. These studies have been extended and it has now been shown that MNNG treatment had no effect on the RNA levels of the constitutive housekeeping gene α-tubulin nor on the steroid- and metal-inducible metallothionein (MT) gene. Although attempts were made to measure the levels of the glucocorticoid receptor by both biochemical and molecular methods, receptor levels were too low (< 100 fmol/mg protein) to quantitate accurately. However, the lack of effect of MNNG on MT RNA levels suggests that the inhibitory effect of the carcinogen is not mediated through alterations in receptor level or affinity. The steroid hormone system has also been employed to examine the effects of an oncogene on expression of a cytoskeletal protein. A polyoma virus middle T gene has been recombined with a steroid-inducible promoter, rendering the oncogene sensitive to induction by steroids. It has been shown in two separately transfected clones that increases in the levels of the middle T gene cause increases in α-tubulin gene expression. Also being studied is the role of drug metabolism in determining susceptibility to chemical carcinogens. The induction kinetics of cytochromes P_1- and P_3-450 have been determined in fetal mice after exposure to various

inducers. The P-450s exhibited both tissue- and inducer-dependent specificity. It was also demonstrated that P_3-450 is induced during the fetal period. Finally, a long-term carcinogenicity study has been initiated to determine if alterations in expression of the genes regulating drug metabolic enzymes affect the toxicological and carcinogenic consequences of DES administration. The biochemical studies as well as the chemical treatment of all the animals for the 2 year carcinogenicity study have been completed.

The Inorganic Carcinogenesis Section (1) investigates mechanisms of carcinogenesis by inorganic compounds, with emphasis on nickel and cadmium; (2) isolates and characterizes metal-binding proteins and determines their roles in modifying toxicity of carcinogenic metal cations; and (3) studies inhibition of carcinogenesis by essential trace elements.

Investigations of the effects of essential divalent metals, Mg, Ca, Zn, and Fe, on the carcinogenicity of Ni have been continued in bioassay and biochemical studies aimed at elucidation of the mechanisms of nickel carcinogenesis. The results indicate that Mg, Ca, and possibly Fe, affect transmembrane transport of Ni and greatly modify local inflammatory/immune response to nickel carcinogens, especially by macrophages, whereas Zn, a much weaker antagonist, has no effect on Ni transport and no detectable influence on the local immune response, including macrophage activation. Ni/Ca interactions have been studied with calmodulin. Ni binds to calmodulin in the presence of Ca; two binding sites are involved. Thus Ni may interfere with regulatory functions of the Ca-calmodulin complex. The disruption of cell-cell communication in 3T3 cells, shown last year, indicated some tumor-promotional activity of this metal. Subsequently, a bioassay to test promotional activity of Ni toward renal tumors initiated with methyl-(methoxymethyl)nitrosoamine is underway. Following the observations of local inflammatory/immune responses to Ni and the other metals, a study on possible involvement of active oxygen species and related enzymatic systems in nickel carcinogenesis has been undertaken. Three important effects of nickel subsulfide (Ni_3S_2), the strongest nickel carcinogen, have been discovered: (1) Ni_3S_2 and Ni(II) derived by oxidative dissolution of Ni_3S_2, inhibit enzymatic activity of catalase and glutathione peroxidase; (2) Ni_3S_2 in the presence of oxygen causes polymerization of histones in calf thymus nucleohistone in vitro; this reaction is enhanced by small peptides and/or hydrogen peroxide; (3) Ni_3S_2 in the presence of oxygen causes oxidation of 2'-deoxyguanosine to potentially mutagenic 8-hydroxy-2'deoxyguanosine in vitro. Effects (2) and (3) suggest that Ni_3S_2, a supposedly nongenotoxic carcinogen, is in fact capable of damaging cellular genetic material. Effect (1) is contributory to the other two. Thus, for the first time a close resemblance was found between the action of Ni_3S_2 and that of classical carcinogens.

The mechanisms of cadmium carcinogenesis are under active investigation. Repeated subcutaneous injections of cadmium resulted in a rapid onset and high incidence of highly malignant tumors at the injection site and had an apparent association with malignant testicular tumors. The Syrian hamster was shown to be susceptible to cadmium-induced testicular carcinogenesis, while mice strains resistant to the acute effects of cadmium in the testes proved resistant also to its carcinogenic effects. Chronic oral exposure to cadmium in rats was associated with prostatic tumors, providing further evidence for a link between cadmium and prostatic carcinogenesis. Zinc was shown to inhibit cadmium carcinogenesis in a route- and site-dependent manner. Rare testicular tumors were also observed in rats in association with cadmium treatment including sertoli cell tumors, rete testis

adenocarcinoma and seminomas indicating a general susceptibility within the cells of the testes. A marked enhancement of cadmium cellular efflux, a reduction in levels of nuclear cadmium and a reduction in DNA cadmium content were observed in cells isolated from the testes of rats made resistant to cadmium carcinogenesis by zinc treatment. The DNA hypomethylating agent, deoxyazacytidine caused both a marked increase in the synthesis of metallothionein, the protein most frequently associated with cadmium tolerance, and cellular tolerance to cadmium. Analysis of restriction enzyme digests of deoxyazacytidine-treated DNA indicated that this tolerance coincided with a hypomethylation of the MT gene, a condition clearly associated with enhanced expressibility. It was shown that, like the rat, mouse and monkey testes, the hamster ovaries are deficient in metallothionein. Hamster ovaries undergo an acute phase response to cadmium similar to that of the testes. An absence of metallothionein in the rat prostate was also established. Thus, a consistent absence of metallothionein in targets of cadmium toxicity and carcinogenesis is observed and the inability to express the metallotheionein gene is probably a very important factor in susceptibility of a given tissue to cadmium carcinogenesis.

The Chemistry Section (1) plans and conducts laboratory research on the chemistry of organic and inorganic carcinogens; (2) investigates mechanisms of carcinogen formation, with the aim of understanding and ultimately preventing formation of such compounds; (3) studies chemical reactivity of carcinogens to identify reaction paths and products causally related to tumor formation as well as alternative pathways that may destroy carcinogens or otherwise interrupt carcinogenic reaction sequences; and (4) conducts comparative investigations of molecular interactions between chemical carcinogens and the cells of different organs and species to identify factors that contribute to organ specificity and species differences in chemical carcinogenesis.

Mechanisms potentially responsible for formation, activation, and detoxication of carcinogenic N-nitroso compounds in the human body are under intense investigation. Further evidence supporting the involvement of the alpha-nitrosamino radical as the critical intermediate in both activation and inactivation of the potent carcinogen, N-nitrosodimethylamine (NDMA), has been obtained by designing a non-enzymatic model for the latter process. The Fenton degradation of NDMA produced formaldehyde, methylamine, and nitrate, each equimolar to nitrosamine consumed, suggesting that the presumably inactivating metabolic pathway of similar course (denitrosation) could be elevated from its normally minor role (~ 15%) in the overall metabolism to become the major or even exclusive fate of NDMA if a means can be found to protect the initial radical from further oxidation by cytochrome P-450 and other scavenging reactions. A non-enzymatic reaction that parallels the macrophage-induced oxidative activation of reduced nitrogen species to ultimate nitrosating agents in several key respects has also been devised; oxidation of coordinated ammonia in the presence of secondary amines produced novel complexes of the carcinogenic N-nitroso derivatives. The dissociation constant for N-protonated NDMA in aqueous acid has been estimated and a novel complex of an O-protonated nitrosamine has been characterized in which each acidic proton is strongly, symmetrically, and collinearly hydrogen-bonded with two nitrosamine molecules rather than one in a structure reminiscent of the bifluoride ($F-H-F^-$) and bihydroxide ($HO-H-OH^-$) ions. The clearance of N-nitrosomethyl(2-hydroxyethyl)amine proved faster in female rats than in males, suggesting a pharmacokinetic origin for the greater susceptibility of females to its hepatocarcinogenic action.

The Tumor Pathology and Pathogenesis Section (TPPS) (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 other Sections of the Laboratory.

The potential contributions of oncogenes and chronic hyperplasia to hepatocarcinogenesis of the mouse liver was studied. The involvement of ras oncogenes in mouse liver carcinogenesis was investigated in B6C3F1 and C3H mice. N-nitrosodiethylamine (DEN) was found to induce tumors in B6C3F1 mice which were characterized by 1 of 3 types of mutations in Codon 61 of the H-ras oncogene. The incidence and distribution of the mutations were identical to those found in liver tumors of control mice. Since the incidence of H-ras activation was significant in the hepatocellular adenomas, it is speculated that H-ras activation may be important for tumor initiation rather than tumor promotion or progression. In contrast, spontaneous and induced liver tumors in C3H/HeNCr mice had a much lower level of H-ras activation, suggesting the activation of H-ras in this mouse was not an important event in multistage carcinogenesis.

Acute and chronic increases in levels of DNA synthesis by hepatocytes and renal tubular cells were studied by autoradiography, Brdu immunohistochemistry and thymidine kinase biochemistry in B6C3F1 mouse liver to determine the role of target-cell hyperplasia in tumor promotion and carcinogenesis by nongenotoxic carcinogens. The liver toxins, acetaminophen and di(2-ethylhexyl)phthalate (DEHP), were found to cause persistent increases in levels of DNA synthesis, increases which were unrelated to any tumor promoting or carcinogenic activity. DEHP, a mouse renal toxin, produced a dramatic persistent elevation of levels of DNA synthesis in kidney tubules, despite its lack of carcinogenic or tumor promoting activity.

The cellular origin and antigenic components of bronchiolar-alveolar pulmonary tumors of mice (induced by N-nitrosoethylurea) and hamsters (induced by N-nitrosodiethylamine) were studied. The mouse tumors of the papillary type have often been described in the literature as being of Clara cell origin. Using antisera to surfactant apoproteins, alveolar cell fetal antigens, Clara cell antigens, or rat lysozyme, the mouse tumors were found to arise from alveolar type II cells. The less-differentiated papillary tumors arose from fetal alveolar cells, while the better-differentiated solid and alveolar tumors arose from more differentiated alveolar type II cells. In contrast, hamster tumors often arose from bronchiolar Clara cells. As they progressively grew in size, they usually became less differentiated. Our studies of mouse and hamster lung tumors provide important histogenetic information for proper terminology and classification of these tumors.

Structure-tumor promoting activity relationships of various barbiturates, hydantoins, oxazolidinediones and benzodiazepine tranquilizers were found. Long-acting compounds were usually active as tumor promoters and enzyme inducers in target organs. A close relationship was found to exist between induction of specific cytochrome P-450 species and tumor promoting abilities of barbiturates and hydantoins. Although phenobarbital induced hepatic enzymes in rats, mice and patas monkeys, species susceptible to phenobarbital liver tumor promotion, it

failed to have similar effects in the resistant species, hamsters and cynomolgus monkeys. A liver tumor-promoting index based on these findings was developed.

The H-ras oncogene protein product p21 was characterized as to its cellular and tissue localization and possible role in carcinogenesis in rodents and humans. We used 10 different antibody preparations, commercially available or prepared at NIH by intramural investigators. Western blotting with antibodies to H-ras p21 from E. coli expression vectors, revealed 9 of 10 antibodies immunoreacted with H-ras p21. The pattern of specific immunohistochemical staining was always characteristically on the cell membrane of Harvey virus induced sarcomas. From 20-50% of the tumor cells were immunoreactive. In contrast, less than 1% of cells transformed by chemicals with an activated H-ras oncogene were immunoreactive on the cell membrane. Some antibodies reacted with cytoplasmic components in normal cells but never on the cell membrane. Our findings provide valuable information on the true localization of p21 immunoreactive cellular antigens. Much of our results are in contrast to those previously published. Much of the previously published studies appear to have reported nonspecific staining rather than actual localization of H-ras p21.

Contributions to the pathogenesis of AIDS in monkeys and humans have been made by immunohistochemical localization of lentiviral antigens in cells and tissues of the affected species. We have found that human immunodeficiency virus (HIV) gag proteins have cross-reactive epitopes with the cytomegalovirus (CMV) major capsid protein (153 KD). The potential implications of this include the roles of viral activation, latency and disease progression since a high proportion of AIDS patients are infected with CMV. HIV and Simian immunodeficiency virus (SIV) antigens, usually gag proteins were found in endothelial cells of brain, lymph nodes and bone marrow and bone marrow stem cells for the first time. Chimpanzees with experimental HIV infection had abundant viral antigens in blood and bone marrow lymphocytes but little or none in lymph node lymphoid cells. Our refinement of the ABC immunohistochemical technique has allowed for direct comparison with in situ hybridization studies for lentiviral RNA currently underway.

Fecapentaene-12 and diacetylfecapentaene-12, potential human gastrointestinal carcinogens, were found to be potent bacterial mutagens, but weaker mammalian mutagens. Despite the mutagenic activity of these chemicals, no biological activity has been found for skin tumor initiation, promotion or complete carcinogenesis in mice, colon carcinogenesis (by intrarectal administration), or carcinogenesis by subcutaneous administration in rats or mice. The total doses administered have been limited by solubility, stability and cost.

The Ultrastructural Studies Section plans and conducts research to investigate the differentiation of potentially neoplastic epithelial cells and its relation to phenotypic expression of the neoplastic genotype with special emphasis on various cell interactions during the transformation process. The Section's special goals are to evaluate 1) cellular mechanisms of tumor promotion in initiated epithelial cells, 2) the regulatory role of growth factors in differentiation and cell proliferation; and 3) the interaction of tumor cells with their environment. The work is organized into two projects, both of which terminate this year.

It has been proposed that the loss of contact inhibition and gap-junctional communication has a significant role in tumor promotion providing initiated cells with the means to escape from the controlling influence of their neighbors. In

our continuing study of tumor promotion, using the epidermis-derived JB6 cell system as a model, we have now employed the radioisotope transfer technique and microinjection of fluorescent dye to examine gap-junctional intercellular communication during promotion by 12-O-tetradecanoylphorbol-13-acetate (TPA). The results obtained using the radioisotope transfer technique provide evidence for the capability of nonpromotable, promotable as well as tumorigenic transformed cells to communicate in homologous pairings in the presence of TPA, albeit with reduced activity; however, transformed cells were most sensitive to the effects of TPA with respect to a partial loss of gap-junctional communication. In heterologous pairings, the combination of transformed cells with nontransformed cells was most sensitive to TPA. A complete disruption of intercellular communication, however, was especially noteworthy when focus-forming, promotable cells were exposured to TPA. By using microinjection, we could show that only cells of foci completely lacked the capability to communicate with their neighbors which were situated in adjacent flat monolayers. The study indicates that the interruption of cell-cell communication is confined to focus-forming cells at a time when the H-ras gene is expressed in these cells. The latter has been shown previously by us. The results give evidence that there exists a link between the expression of the H-ras gene and the loss of gap-junctional intercellular communication during promoter-dependent focus formation. A comparison of the state of gap-junction-dependent intercellular communication to the capability to form colonies on irradiated feeder cell layers gave most interesting results. We obtained evidence that, in the model system used, the interruption of cell-to-cell communication may not be of primary importance for the establishment of colonies. An initial low intercellular communication does not necessarily result in good colony formation and, contrarily, an initial good communication is not indicative for a reduced colony formation at a later time.

In a second study, we have employed the probing of gap-junctional cell communication as a means to uncover tumor-promoting activity of chemical agents such as Ni(II). NIH 3T3 cells were used, as it is well known that they are sensitive to tumor promoters. Ni(II) salts at various concentrations were tested for their capability of interrupting cell-cell communication. We found a concentration-dependent reduction of such communication indicating that Ni(II) salts resemble classical tumor promoters such as TPA. It is suggested that the role of nickel in carcinogenesis, therefore, should be reinterpreted as that of a tumor promoter.

Our collaborative study with the Laboratory of Chemoprevention, NCI, in which we are investigating the regulatory role of the transforming growth factor-beta (TGF-β) in embryonal development has provided exciting results. Probing the distribution of TGF-β with antibodies raised against synthetic peptides corresponding to various regions of the TGF-β monomer has shown a high concentration of the growth factor in mouse embryos of 13 to 15 days gestation, when organogenesis is most intense. TGF-β was localized in tissues of ectodermal and mesenchymal origin. The presence of TGF-β in the latter is of special importance as it is well known that TGF-β has the capability of stimulating mesenchyme to produce extracellular matrix, which, in turn, is necessary for the expression of cell-specific proteins in the adjacent tissue. Thus, the action of the growth factor may represent one of the first steps in a cascade of events leading to cytodifferentiation in the developing embryo.

The involvement of TGF-β in the formation of extracellular matrix components is now examined in the developing embryo by relating the growth factor to matrix components such as fibronectin, laminin, and collagen.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04542-16 LCC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Chemistry of Nitroso Compounds & Other Substances of Interest in Cancer Research

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
Others:	Y.-H. Heur	Visiting Fellow	LCC	NCI
	M. Stershic	Staff Fellow	LCC	NCI
	A. J. Streeter	Visiting Associate	LCC	NCI
	R. Nims	Chemist	LCC	NCI

COOPERATING UNITS (if any) PRI, Frederick, MD (W. Andrews, G. Lunn, B. Hilton, J. Hrabie, L. Ohannesian, E. Sansone); U. of N. Carolina (T. Meyer, B. Sullivan); U. of Zürich (P. Kleihues, I. Schmerold, E. von Hofe); SRI International (E. Reist); Naval Research Lab. (J. Flippen-Anderson, C. George); Varian Assoc. (D. Wilbur)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	4	1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

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

Mechanisms potentially responsible for formation, activation, and detoxication of carcinogenic N-nitroso compounds in the human body are under intense investigation. Further evidence supporting the involvement of the alpha-nitrosamino radical as the critical intermediate in both activation and inactivation of the potent carcinogen, N-nitrosodimethylamine (NDMA), has been obtained by designing a non-enzymatic model for the latter process. The Fenton degradation of NDMA produced formaldehyde, methylamine, and nitrate, each equimolar to nitrosamine consumed, suggesting that the presumably inactivating metabolic pathway of similar course (denitrosation) could be elevated from its normally minor role (~ 15%) in the overall metabolism to become the major or even exclusive fate of NDMA if a means can be found to protect the initial radical from further oxidation by cytochrome P-450 and other scavenging reactions. A non-enzymatic reaction that parallels the macrophage-induced oxidative activation of reduced nitrogen species to ultimate nitrosating agents in several key respects has also been devised; oxidation of coordinated ammonia in the presence of secondary amines produced novel complexes of the carcinogenic N-nitroso derivatives. The dissociation constant for N-protonated NDMA in aqueous acid has been estimated and a novel complex of an O-protonated nitrosamine has been characterized in which each acidic proton is strongly, symmetrically, and collinearly hydrogen-bonded with two nitrosamine molecules rather than one in a structure reminiscent of the bifluoride ($F-H-F^-$) and bihydroxide ($HO-H-OH^-$) ions. The clearance of N-nitrosomethyl(2-hydroxyethyl)amine proved faster in female rats than in males, suggesting a pharmacokinetic origin for the greater susceptibility of females to its hepatocarcinogenic action.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. K. Keefer	Chief, Chemistry Section	LCC	NCI
Y.-H. Heur	Visiting Fellow	LCC	NCI
M. Stershic	Staff Fellow	LCC	NCI
A. J. Streeter	Visiting Associate	LCC	NCI
R. Nims	Chemist	LCC	NCI

Objectives:

Generally, to apply the methods and concepts of chemistry toward the solution of important problems in cancer research, especially by elucidating new mechanisms of formation, destruction, metabolism, and biological action of nitrosamines and related carcinogens. Specifically, (1) to establish 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 may be devised for intercepting these carcinogens before human exposure can occur; (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 and other substances of interest in cancer research as a means of contributing to the general fund of knowledge about such materials.

Major Findings:

In our studies of nitrosamine formation mechanisms, we have discovered a possible model of the biological reaction in which reduced nitrogen species (-3 oxidation state) serve as a stoichiometric source of nitrosating agent (+3 nitrogen) in the synthesis of carcinogenic N-nitroso compounds in vivo or in macrophage culture. Ammonia (-3 nitrogen) has been both oxidized and activated toward nitrosamine-forming attack on secondary amines by coordination to a suitably electrophilic metal center (collaboration with Drs. Meyer and Sullivan of the University of North Carolina).

As to the destruction of carcinogenic N-nitroso compounds, a reliable procedure for chemically detoxicating hazardous wastes generated in cancer research laboratory manipulations of N-nitrosated ureas, urethanes, guanidines, and sulfonamides has been developed, validated by interlaboratory comparisons, and published (collaboration with Drs. Lunn, Sansone, and Andrews of Program Resources, Inc.). In addition, the use of aluminum-nickel alloy as a reducing agent in organic chemistry has been reviewed, with emphasis on the reagent's utility in destruction of toxic wastes (collaboration with Dr. Lunn).

In our biological chemistry investigations, the pharmacokinetic parameters of N-nitrosomethyl(2-hydroxyethyl)amine (NMHA) and N-nitrosomethylethylamine (NMEA) have been measured in Fischer rats. NMHA is cleared somewhat more slowly than N-nitrosodimethylamine (NDMA), indicating that the hydroxyl group slows metabolism

rather than accelerating it (as might have been expected if conjugation and dehydrogenation reactions were both rapid and extensive). Clearance of NMHA is significantly greater in the female, suggesting a pharmacokinetic origin for the sex difference noted by others in responsiveness to this hepatocarcinogen (collaboration with Dr. Hrabie, Program Resources, Inc.). By contrast, NMEA is cleared approximately as rapidly as NDMA, but deuteration at the β-carbon atom has no effect on any of the pharmacokinetic parameters. This is surprising, because β-deuteration shifts both the extent of DNA alkylation (collaboration with Drs. Kleihues, Schmerold, and von Hofe of the University of Zürich and Dr. Reist of SRI International) and the organotropic carcinogenicity toward increasing activity in the esophagus, leading us to predict, originally, that the bioavailability would be larger for the deuterated material. Further work will be aimed at elucidating the origins of this organotropic shift. Finally, further evidence supporting the involvement of the α-nitrosamino radical as the critical first intermediate in both the activating dealkylation of NDMA and the competing enzymatic denitrosation route has been obtained by generating this radical through exposure of NDMA to the Fenton reagent. By producing the radical in the absence of further oxidation by cytochrome P-450 or other scavenging reactions, the predicted products of pure denitrosation were formed on an equimolar basis with nitrosamine consumed, proving that the degradation of NDMA can be shifted from predominant (85% in the microsomes) dealkylation to 100% denitrosation if the radical is allowed to decompose unimolecularly.

Contributions to knowledge concerning the fundamental physicochemical properties of the nitrosamines include: the first characterization of a nitrosamine coordinated to an electrophilic center via its central (nitroso) nitrogen atom (collaboration with Drs. Meyer and Sullivan); evidence that the amino nitrogen of NDMA is significantly protonated at equilibrium in aqueous acid and calculation of a dissociation constant for the process (collaboration with Drs. Hilton and Hrabie of Program Resources, Inc., and Dr. Wilbur of Varian Assoc.); the first X-ray crystallographic confirmation that the preferred site of nitrosamine protonation is at oxygen, with the acidic proton being coordinated equally strongly to two nitrosamine molecules rather than one in an unexpectedly strong O-H-O hydrogen bond (collaboration with Drs. Flippen-Anderson and George of the Naval Research Laboratory and Drs. Hrabie and Ohannesian of Program Resources, Inc.); the demonstration that the nature of the coordinating electrophile is a powerful determinant of reaction course in the nucleophile-induced decomposition of O-coordinated nitrosamines, displacement of N- vs. O-substituents being observable in limiting cases (collaboration with Dr. Ohannesian).

Publications:

Fanning JC, Keefer LK. Rapid formation of a potent nitrosating agent by solvolysis of ionic nitrite in dichloromethane. J Chem Soc, Chem Commun, 1987;955-6.

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Keefer LK, Wang S-M, Anjo T, Fanning JC, Day CS. Preparation of a thallium(I) diazotate. Structure, physicochemical characterization, and conversion to novel N-nitroso compounds. J Am Chem Soc (In Press).

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Lijinsky W, Kovatch RM, Keefer LK, Saavedra JE, Hansen TJ, Miller AJ, Fiddler W. Carcinogenesis in rats by cyclic N-nitrosamines containing sulphur. Fd Chem Toxic 1988;26:3-7.

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Resce JL, Fanning JC, Day CS, Uhm S-J, Croisy AF, Keefer LK. Structure of the Fe(salen)ONO_2 dimer, a ferric complex with a unidentate nitrate ligand. Acta Cryst, Section C, 1987;43:2100-4.

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Yang CS, Wade D, Anjo T, Keefer LK. Kinetic isotope effect on the demethylation and denitrosation of N-nitrosodimethylamine in vitro. IARC Sci Publ 1987;84:124-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP04582-13 LCC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mechanisms of Inorganic Carcinogenesis: Nickel

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	K. S. Kasprzak	Visiting Scientist	LCC	NCI
Others:	M. P. Waalkes	Pharmacologist	LCC	NCI
	J. M. Ward	Chief, Tumor Pathol. & Pathogen. Section	LCC	NCI
	N. Konishi	Visiting Fellow	LCC	NCI
	R. Rodriguez	Intramural Research Training Associate	LCC	NCI
	N. Raos	Visiting Fellow	LCC	NCI

COOPERATING UNITS (if any)
Program Resources, Inc., Frederick, MD (B. Diwan, H. Issaq, C. Riggs, O. Weislow, B. Kiser); Pathology Associates (R.M. Kovatch); Bionetics Research, Inc., Basic Research Program, Frederick, MD (L. Hernandez)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Inorganic Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

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

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)
Investigations of the effects of essential divalent metals, Mg, Ca, Zn, and Fe, on the carcinogenicity of Ni have been continued in bioassay and biochemical studies aimed at elucidation of the mechanisms of nickel carcinogenesis. The results indicate that Mg, Ca, and possibly Fe, affect transmembrane transport of Ni and greatly modify local inflammatory/immune response to nickel carcinogens, especially by macrophages, whereas Zn, a much weaker antagonist, has no effect on Ni transport and no detectable influence on the local immune response, including macrophage activation. Ni/Ca interactions have been studied with calmodulin. Ni binds to calmodulin in the presence of Ca; two binding sites are involved. Thus Ni may interfere with regulatory functions of the Ca-calmodulin complex. The disruption of cell-cell communication in 3T3 cells, shown last year, indicated some tumor-promotional activity of this metal. Subsequently, a bioassay to test promotional activity of Ni toward renal tumors initiated with methyl-(methoxymethyl)nitrosoamine is underway. Following the observations of local inflammatory/immune responses to Ni and the other metals, a study on possible involvement of active oxygen species and related enzymatic systems in nickel carcinogenesis has been undertaken. Three important effects of nickel subsulfide (Ni_3S_2), the strongest nickel carcinogen, have been discovered: (1) Ni_3S_2 and Ni(II) derived by oxidative dissolution of Ni_3S_2, inhibit enzymatic activity of catalase and glutathione peroxidase; (2) Ni_3S_2 in the presence of oxygen causes polymerization of histones in calf thymus nucleohistone _in vitro_; this reaction is enhanced by small peptides and/or hydrogen peroxide; (3) Ni_3S_2 in the presence of oxygen causes oxidation of 2'-deoxyguanosine to potentially mutagenic 8-hydroxy-2'deoxyguanosine _in vitro_. Effects (2) and (3) suggest that Ni_3S_2, a supposedly nongenotoxic carcinogen, is, in fact, capable of damaging cellular genetic material. Effect (1) is contributory to the other two. Thus, for the first time a close resemblance was found between the action of Ni_3S_2 and that of classical carcinogens.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Enagaged in this Project:

K. S. Kasprzak	Visiting Scientist	LCC	NCI
M. P. Waalkes	Pharmacologist	LCC	NCI
J. M. Ward	Chief, Tumor Pathol. & Pathogen Section	LCC	NCI
N. Konishi	Visiting Fellow	LCC	NCI
R. Rodriguez	Intramural Research Training Associate	LCC	NCI
N. Raos	Visiting Fellow	LCC	NCI

Objectives:

To study the mechanisms of nickel carcinogenesis by investigating a hypothesis that nickel and possibly other metal carcinogens act, at least in part, through interactions with the essential divalent metals, calcium, magnesium, zinc, iron, and others. An alternative hypothesis assuming that nickel may damage cellular genetic material through initiation of radical chain reactions with the participation of oxygen, some polypeptides and proteins, causing DNA-protein and protein-protein cross-linking, and oxidation of DNA bases, is also investigated. Uncoding the molecular nature of these interactions would provide some new insights into the mechanisms of nickel-induced carcinogenesis and eventually open up prospects of practical application of the essential metals in cancer prevention and treatment.

Major Findings:

Iron appears to be the strongest inhibitor of nickel carcinogenesis of all metals tested. The ranking in rat's muscle is the following: Fe > Mg >>Zn. Ca has no effect. Inhibition of nickel carcinogenesis by iron is mediated by stimulatory action of the latter upon the macrophage activity otherwise suppressed by nickel at the site of its i.m. injection. As shown for the first time by reaction kinetic mesurements, nickel suppresses the activity of catalase and glutathione peroxidase, allowing hydrogen peroxide to contribute to the toxic and carcinogenic action of nickel. Iron has no effect on that inhibition. It may, however, substitute for these two enzymes in destroying H_2O_2, and thus counteract nickel. The immunostimulatory effects of iron resemble those by magnesium described last year, and manganese, reported elsewhere, which also resulted in suppression of nickel carcinogenesis. Studying the effects of magnesium and iron, as well as other inflammation stimulants and suppressants, on nickel carcinogenesis revealed the importance of the cellular immune responses to the process of tumor induction by nickel. This, in turn, focused our attention upon possible roles of active oxygen species and related enzymatic control systems in nickel carcinogenesis.

As a result, a new original hypothesis has been formulated and tested. It assumes that nickel may damage the cellular genetic material not by direct binding to it, but rather indirectly, by catalyzing oxidation and decarboxylation of some polypeptides and/or proteins resulting in generation of reactive radicals which can attack DNA and histones and cause modification of DNA bases, DNA-histone and/or histone-histone cross-linking. Relevant experiments initiated last year have been continued with the use of gel chromatography, high performance liquid chromatography (HPLC), and special analysis. They showed for the first time that (1) solid nickel carcinogen, Ni_3S_2, in the presence of oxygen causes

polymerization of histones in calf thymus nucleohistone or individual isolated histones; (2) Ni_3S_2 in the presence of ambient oxygen causes oxidation of 2'-deoxyguanosine to mutagenic 8-hydroxy-2'-deoxyguanosine and at least one more product whose identification is underway; (3) these effects of Ni_3S_2 are modified by hydrogen peroxide and tetraglycine. Thus, at least two new effects were found by which Ni_3S_2, a supposedly non-genotoxic carcinogen, can, in fact, cause damage to genetic material--polymerization of histones and oxidative modification of DNA bases.

In continuation of nickel/calcium interactions studies, experiments were conducted both in vivo and in vitro on nickel effects on calmodulin, a calcium-activated regulatory protein. As found by radioimmune assay, enzyme kinetic analysis, and radiolabeled methods, treatment of rats with nickel causes more rat renal calmodulin to become bound to insoluble cellular fractions and inhibits enzyme-activating ability of calmodulin. These effects are most likely caused by nickel binding to calmodulin, even in the presence of calcium; two binding sites are involved. Nickel may thus interfere with the messenger/regulatory role of calcium in cell proliferation and differentiation.

Publications:

Kasprzak KS. Nickel. In: Fishbein L, Furst A, Mehlman MA, eds. Advances in modern environmental toxicology. XI. Genotoxic and carcinogenic metals - environmental and occupational occurrence and exposure. Princeton: Princeton Sci Publ Co, 1987;145-83.

Kasprzak KS, Kiser RF, Weislow OS. Magnesium counteracts nickel-induced suppression of T-lymphocyte response to Concanavalin A. Magnesium. Exp and Clin Res (In Press).

Kasprzak KS, Kovatch RM, Poirier LA. Inhibitory effect of zinc on nickel subsulfide carcinogenesis in Fischer rats. Toxicology. (In Press)

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Kasprzak KS, Ward JM, Poirier LA, Reichardt DA, Denn AC III, Reynolds CW. Nickel-magnesium interactions in carcinogenesis: dose effects and involvement of natural killer cells. Carcinogenesis 1987;8:1005-11.

Miki H, Kasprzak KS, Kenney S, Heine UI. Inhibition of intercellular communication by nickel(II): antagonistic effect of magnesium. Carcinogenesis 1987;8:1757-60.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP04812-20 LCC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cell Interactions During Transformation of Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
Others:	I. Yamadori	Visiting Fellow	LCC	NCI
	K. S. Kasprzak	Visiting Scientist	LCC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

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

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

To define the role of gap-junctional communication in tumor promotion, nonpromotable, promotable, and tumorigenic transformed epidermis-derived cells of line JB6 cells were subjected to the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA). Cell-cell communication was measured either by the radioisotope transfer technique or by microinjection of fluorescent dye. The capability of TPA-treated cells to escape from the controlling influence of non-promotable neighboring cells was monitored by the evaluation of colony formation on irradiated feeder cell layers. Our results give evidence for a blocked cell-cell communication during the initial exposure to TPA. However, the number of colonies evolving from promotable cell clones in the presence of TPA does not correlate to the magnitude of the initial interruption of intercellular communication. Thus, it has to be concluded that, in the model system used, the suppressive effect of TPA on intercellular communication may not primarily be related to the promoting effect on cell growth. Also, reduced intercellular communication is not a decisive factor in maintaining malignancy. Interruption of gap-junctional intercellular communication was used as indicator in a short-term test model to uncover tumor-promoting properties in chemical agents, such as Ni-(II)-salts.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
I. Yamadori	Visiting Fellow	LCC	NCI
K. S. Kasprzak	Visiting Scientist	LCC	NCI

Objectives:

1) To evaluate inhibition of intercellular communication by tumor promoters as a mechanism for tumor promotion, using, as a model system, tm.xpromotable epidermis-derived cells of line JB6 together with appropriate controls.

2) To establish a short-term test to uncover tumor-promoting properties in chemical agents using interruption of gap-junctional intercellular communication as indicator.

Major Findings:

It has been proposed that the loss of gap-junctional communication plays a significant role in tumor promotion, providing initiated cells with further means to escape from the controlling influence of their surrounding normal neighbors. We have studied the effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on intercellular communication in nonpromotable, promotable and tumorigenic transformed mouse epidermal cells of line JB6. Using the radioisotope transfer technique that monitors the behavior of cells during short-time exposures to the agent under examination, we established that in homologous pairings of promotable, nonpromotable and transformed clones, it is the transformed cell that is most sensitive to the effect of TPA on intercellular communication. In heterologous pairings, the combination of transformed with nontransformed cells is more sensitive to TPA than the pairings of nontransformed cells. Furthermore, microinjection experiments established a remarkable lack of communication between focus-forming cells and surrounding flat cells. Extension of this study to include a comparison of the state of intercellular communication at day 1 to the capacity to form colonies on irradiated feeder cells (10 days) gave the surprising result that colony formation may be independent of the initial state of intercellular communication. Thus, low intercellular communication between promotable and feeder cells at day 1 may not necessarily be followed by good colony formation and, contrarily, good intercellular communication at day 1 may yield many colonies. It is only later during actual colony formation that intercellular communication is definitely reduced between colony forming and surrounding nonpromotable cells. Our results indicate that the effect of the tumor promoter TPA on communication may not be of primary importance for cells to start independent growth in the model system used.

To uncover the tumor-promoting activity of a chemical agent, we used the interruption of gap-junctional communication between NIH 3T3 cells as a model system and employed divalent nickel salts as test agent (see project Z01CP04582-13). Using the radioisotope transfer technique, nickel(II) sulfate was found to disrupt cell-cell communication in a dose-related manner from 98% of the control values to 2% at 75 mM Ni. At these concentrations viability of the cells was not

affected. The results indicate that nickel(II) sulfate is capable of inhibiting cell-cell communication at concentrations that do not cause cytotoxic effects on NIH 3T3 cells; thus, nickel(II) resembles classical tumor promoters such as TPA. The role of nickel in carcinogenesis, therefore, may include tumor promotion effects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05092-10 LCC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Transplacental Carcinogenesis and Tumor Promotion in Nonhuman Primates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Rice	Chief	LCC	NCI
Others:	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
	S. Rehm	Visiting Scientist	LCC	NCI
	L. M. Anderson	Expert	LCC	NCI
	P. J. Donovan	Chemist	LCC	NCI
	A. O. Perantoni	Staff Fellow	LCC	NCI

COOPERATING UNITS (if any)

SEMA, Inc., Rockville, MD (J. Phillips); Baylor College of Medicine, Houston, TX (L. J. Lu); Oak Ridge Associated Universities, Oak Ridge, TN (N. Clapp)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Office of the Chief, Primate Research Working Group

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	2.0	1.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Nonhuman primates of the species Erythrocebus patas (patas) and Macaca fascicularis (cynomolgus) are subjected to direct-acting or metabolism-dependent chemical carcinogens by transplacental or direct exposure. In some cases the carcinogen-treated animals are subsequently exposed to chemicals that promote the development of neoplasms in rodents. Mechanisms of organ and species differences in the effects of chemical carcinogens and tumor promoters among rodent and nonhuman primate species are investigated. Induced tumors are evaluated by light microscopy using standard staining procedures, histochemical techniques and electron microscopy and are assayed for in vitro cultivability and transplantability to rodents. Selected tumors are subjected to DNA extraction and attempts are made to transfect NIH 3T3 cells with their DNA. These studies have shown that intrinsic susceptibility to transplacental carcinogenesis is greatest in nonhuman primates early in gestation and have provided the only animal model of chemically inducible gestational choriocarcinoma. The association of chronic ulcerative colitis and multifocal colonic carcinoma in the cotton-top tamarin (Saguinus oedipus) is being investigated in collaboration with Oak Ridge Associated Universities, with primary attention being given to a search for indirect evidence for a fecal mutagen/carcinogen in this species, since direct search for a fecal mutagen has been unsuccessful.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Rice	Chief	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
S. Rehm	Visiting Scientist	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
P. J. Donovan	Chemist	LCC	NCI
A. O. Perantoni	Staff Fellow	LCC	NCI

Objectives:

To study and characterize the variable sensitivities of different organ systems in nonhuman primates to carcinogens which act directly or require *in vivo* metabolism for carcinogenic activity during the prenatal and postnatal periods. To attempt to demonstrate the phenomenon of tumor promotion in nonhuman primates.

Major Findings:

Studies on carcinogenesis by ethylnitrosourea (ENU) and diethylnitrosamine (DEN) in *Erythrocebus patas*, an Old World monkey, continue and have been expanded to include aflatoxin B_1 (AFB_1). Additional cases of mesenchymal and epithelial tumors were observed in the offspring of monkeys that received ENU intravenously during pregnancy, especially when exposure occurred during the first trimester of gestation. These additional findings support the tentative conclusions drawn previously that, like rodents, this species of nonhuman primate 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 that received a similar dose directly.

A survey of a variety of neoplasms induced by ENU in patas monkeys for transfection activity in NIH 3T3 cells has been positive in only 1 instance. It is not clear whether this reflects a significant divergence from rodents, in which demonstration of activated oncogenes in chemically induced tumors is often facile, or whether the specific kinds of patas tumors available for survey represent entities in which activation of known oncogenes is rare.

The finding that chronic ulcerative colitis in the cotton-top tamarin, *Saguinus oedipus*, is associated with a high incidence of multifocal colonic carcinoma presents a unique opportunity to study the etiology of colonic carcinoma in a system that strikingly mimics the comparable association of ulcerative colitis and colonic carcinoma in humans. In collaboration with Dr. Neal Clapp of Oak Ridge Associated Universities, we have undertaken several studies to explore the etiology of tamarin chronic carcinoma.

Direct search for fecal mutagens in cotton-top tamarins has not been successful. Ames assays on fecal samples have been inconsistent and generally negative, and this approach is being abandoned. P-32 postlabeling assays for atypical DNA adducts in colonic mucosa are in progress.

Publications:

Rice JM, Rehm S, Donovan PJ, Perantoni AO. Comparative transplacental carcinogenesis by direct acting and metabolism dependent alkylating agents in rodents and non-human primates. IARC Sci Publ (In Press).

CONTRACT IN SUPPORT OF THIS PROJECT

SEMA, Inc., N01-CP-71079

Title: Resources for Transplacental Carcinogenesis and Tumor Promotion in Old World Monkeys

Current Annual Level: $465,450

Man Years: 4.7

Objectives:

This contract provides animal care and technical support for colonies of 185 patas and 100 cynomolgus monkeys. The project is designed to demonstrate and characterize transplacental carcinogenesis and tumor promotion in the Old World monkey species patas (*Erythrocebus patas*) and cynomolgus (*Macaca fascicularis*). In addition, related phenomena are studied, including the increased risk of adult female patas exposed to chemicals during pregnancy, tumor promotion in both patas and cynomolgus monkeys, mechanisms of metabolism, cell and organ specificities and species differences in the effects of both chemical carcinogens and tumor promoters.

Major Contributions:

Models for chemically inducible gestational choriocarcinoma and for pre-eclamptic toxemia of pregnancy have resulted from studies performed under this contract. Ethylnitrosourea (ENU) has been shown to be a potent carcinogen in the patas and the rhesus (*Macaca mulatta*) monkeys. In both species, the fetus is more susceptible than is the adult, and this susceptibility is more pronounced during the first and early second trimesters of pregnancy. However, the kinds of tumors seen in the two species differ in their characteristics and distribution.

Diethylnitrosamine (DEN) given to pregnant patas monkeys during gestation did not cause tumors in the offspring or mothers after 4 years of observation. However, after 24 to 30 months of subsequent daily doses of phenobarbital comparable to therapeutic anticonvulsant levels in man, both offspring and mothers developed hepatocellular adenomas and carcinomas. Phenobarbital clearly can promote hepatocarcinogenesis in this species as it does in rats. However, it appears to have no promoting effect on similarly induced tumors in the cynomolgus monkey.

Except for the association between in utero exposure to diethylstilbestrol and the increased risk of vaginal adenocarcinoma during early adulthood, there is little known concerning the effects of carcinogens on the human fetus. Transplacental chemical carcinogenesis studies have been limited to rodent species which differ greatly from man. Most significant is the more rapid rate of fetal and neonatal growth and maturation in rodents. Nonhuman primates also have shorter gestations and mature more rapidly than do humans, but they are more similar to man in fetal growth, placentation and early development than are rodents. Some tumors induced to date in rhesus and patas monkeys by transplacental exposure to carcinogens resemble some congenital tumors or tumors of infancy and childhood seen in man, suggesting that prenatal exposure of humans to chemicals may be a

factor in human pediatric cancer causation. The demonstration of tumor promotion in nonhuman primates provides significant evidence of the importance of this phenomenon to man.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05093-10 LCC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

In Vitro Studies on Organ Specificity in Transplacental Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Rice	Chief	LCC NCI
Others:	P. Donovan	Chemist	LCC NCI
	A. Perantoni	Staff Fellow	LCC NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (B. Diwan)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section, Developmental Biology Working Group

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The roles of morphogenetic differentiation in controlling the phenotypic expression of neoplastic transformation, the degree of malignancy of tumors, and the susceptibility of developing organs to carcinogenesis are studied using organ culture and tissue transplantation techniques, with current emphasis on the kidney. A serum-free culture system has now been devised that allows for both growth and branching of ureteric bud and proliferation of metanephric mesenchyme. Tubulogenesis has been observed in mesenchyme maintained under these conditions in the absence of an inducing tissue. The ability of transplacentally administered carcinogens to induce genotoxic damage in cells of embryos or fetuses exposed at different stages of gestation was determined for rat, mouse, and Syrian hamster. Cells were isolated from exposed embryos and gene mutation at three loci (resistance to 6-thioguanine (6-TG) and ouabain and to diphtheria toxin (DT) in the hamster) were assayed in vitro with simultaneous determination of survival ability. Induced DT and TG mutant frequency (mutants per surviving cell) was greatest when treatment was day 9 of gestation for mesenchymal cells of the Syrian hamster fetus. Cells from the brain also had a large increase in frequency of mutants when fetuses were exposed to NEU at day 9 of gestation and also appeared to have another peak of sensitivity at day 6-7 of gestation. In addition, an almost completed study has as its objective to determine the number of mutants induced in individual fetuses which have been treated with NEU at different days of gestation. Another study aims determined the spontaneous mutation frequencies Syrian hamster using litters of day 13 fetuses. This was 2 X 10-7 DT mutants per per viable cell and 2 X 10-7 TG resistant mutants per viable cell. 95 and 99% confidence limits were calulated for these mean spontaneous frequencies in order to compare with cells from litters which have been transplacentally treated with a mutagen. In addition, the number of 6-TGr mutant cells was determined in a large number of individual day 13 fetuses. Mutants could not be detected in half of these fetuses, despite testing an average of 53 plates per fetus.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Rice	Chief	LCC NCI
P. J. Donovan	Chemist	LCC NCI
A. O. Perantoni	Staff Fellow	LCC NCI

Objectives:

To identify and characterize those aspects of morphogenetic differentiation that modify the consequences of prenatal exposure to chemical carcinogens, especially in the nervous and genitourinary systems. The ultimate objective is to elucidate the control of expression of the neoplastic phenotype in transformed cells. To devise and apply improved quantitative selective mutation systems to embryonal and fetal primary cells in culture from donors previously treated in utero with chemical carcinogens. To determine the time course of maximum sensitivity to induced gene mutation of cells from embryos or fetuses transplacentally exposed to carcinogens at different stages of gestation. To determine quantitative dose curves for transplacentally induced gene mutation by selected carcinogens. To determine the sensitivity of various species to metabolism-independent transplacental chemical carcinogens and to determine inter- and intra-litter variations in response to chemical carcinogens. To determine the organ specificity in various species of gene mutations transplacentally induced by nitrosoethylurea. To apply in vitro transformation assays to cells isolated from embryos of different species treated transplacentally with chemical carcinogens. To correlate the above quantitatively determined in vitro parameters with transplacental tumorigenesis data.

Major Findings:

Renal differentiation. Efforts to make a comparative study of mouse and rat renal differentiation have been hampered in the past by our inability to establish culture medium conditions favorable to both proliferation and differentiation of ureteric bud and renal mesenchyme. We now have devised a serum-free culture milieu which not only allows for the independent growth and branching of the ureteric bud but also proliferation and apparent tubulogenesis in metanephrogenic mesenchyme that, by Grobstein's and Saxen's criteria, should not have been "induced" to differentiate. The studies by Saxen et al. suggested that close apposition or direct contact of epithelium and mesenchyme was necessary for tubulogenesis in mesenchyme. From our studies it appears that this phenomenon can be reproduced with a complex culture medium composed primarily of soluble maintenance and growth factors. While we have yet to establish the presence of similar growth requirements and conditions in vivo, our studies indicate that these fetal elements do have the potential for responsiveness. Having identified putative growth and differentiation factors for renal tissues, we can apply this information in our studies of embryonal renal tumors to ascertain if an addition to K-*ras* activation as previously described, alterations in growth factor or receptor molecules or their production are also associated with tumorigenesis.

Transplacental Mutagenesis We have previously reported quantitative transplacental dose curves for various carcinogens. Gravid animals are injected with different doses of carcinogen at precise stages of gestation. Primaries are

made from cells derived from either individual fetuses or entire litters of day 13 fetuses which are then stored over liquid nitrogen, after slow freezing. Cells are thawed and cultures are initiated which are carried in conventional media for an expression time of 5 days. They are then seeded for either determination of survival or selection with 6-thioguanine or diphtheria toxin. The spontaneous mutation frequencies of somatic cells isolated from a pool of 100 day 13 fetuses was established after 25 repeated experiments that totaled 600 plates. A study comparing 26 litters for DT resistance and 15 litters for TG resistance gave essentially the same answer. The frequency for DT resistance was 2.6×10^{-7} and that for TG resistance was 3.1×10^{-7}. Confidence limits of these values were calculated to enable comparison with mutagen-treated litters. In addition the spontaneous mutation values for 6 thioguanine resistance was determined for a series of individual day 13 hamsters. At least 50 dishes were assayed for each fetus. The results indicated that in at least half the fetuses no mutants could be detected. The mean mutant frequency was similar to that determined previously (5.3×10^{-7}). A statistical study of this distribution is in progress to compare it to theoretical distribution. A shorter study was undertaken with individual fetuses to determine the distribution of frequencies of resistance to DT. These were found to have a mean of 2.9×10^{-7}. Compared to mutation frequencies of cell lines grown in vitro as V-79 or CHO, these frequencies are extraordinarily low (about two orders of magnitude), which might reflect an especially low mutation rate in vivo. It might be possible to make an estimate of this mutation rate whether spontaneous or induced, if some idea of the number of developmental compartments involved is known and and which are being measured in vitro using this mutation system. Some idea of this can be obtained by the extent of mutation in individual fetuses after transplacental N-nitrosoethylurea (NEU) at different ages of gestation. This had been previously determined by us using pools of fetal cells from different litters of hamsters which had been treated with NEU at different days of gestation. Using these pools of cells, it was found that the induction of mutants was greatest when treatment was at day 9 of gestation. Current studies are determining the extent of mutation induction in individual fetuses after treatment with NEU at different ages of gestation from day 6 to day 12. The number of fetuses that have to be used for proper statistics is greatly increased for earlier time treatments as most are negative. Positive fetuses resulting from earlier treatments have a corresponding greater mutant frequency. Studies are still in progress. Cells from other tissues that have been treated with either different carcinogens or NEU at different times of gestation also can be cultured in special media and subjected to the above selection with either 6-TG or DT.

Publications:

Rice JM, Diwan BA, Donovan PJ, Perantoni AO. Mechanisms of transplacental carcinogenesis: mutation, oncogene activation, and tumor promotion. Banbury Report, New York: Cold Spring Harbor Laboratory 1987;26:137-53.

Rice JM. Editorial -- Fetal susceptibility to viral and chemical carcinogens. Lab Invest 1988;58:1-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05288-07 LCC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Signal Transduction and the Control of Developmental Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. D. Blumberg	Expert	LCC	NCI
Others:	R. Das	Visiting Fellow	LCC	NCI
	K. Higinbotham	Biologist	LCC	NCI

COOPERATING UNITS (if any)
Macquarie University, Sydney, Australia (L.H. Browne, K.L. Williams); St. Louis University Medical School, St. Louis, Mo. (H. Sadeghi, C. Klein)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Office of the Chief, Molecular Biology Working Group

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.25	1.25	1.0

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

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)
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. Postaggregation Dictyostelium cells transcribe an additional 26% of their genome which is not expressed in earlier pre-aggregation stage cells. Cell-cell interaction is a necessary prerequisite for the synthesis and stability of these new differentiation-specific messenger RNAs. Additionally, the transciption rate and stability of these messenger RNAs are further regulated by a cyclic AMP-mediated process. We have demonstrated that 1) lyzosomatrophic agents such as $(NH_4)_2SO_4$ can replace the need for cell-cell interaction for postaggregation gene expression; 2) cAMP acts to regulate post aggregation gene expression through the cell surface cAMP receptor; 3) accumulation of mRNA for differentiation-specific genes expressed in prestalk cells is regulated through a different kinetic form of the cell surface receptor than those expressed in prespore cells; 4) activation of the cAMP receptor-associated adenylate cyclase does not play a role in the second messenger signal transduction system utilized for activation of expression of either the prespore or the prestalk genes; 5) prespore genes but not prestalk genes utilize a Ca++/Calmodulin-dependent second messenger signal transduction system for their activation; 6) pathways that induce the expression of differentiation-specific genes in prespore cells suppress the expression of genes transcribed during growth; finally, 7) analysis of one of the cAMP regulated prespore genes reveals that it is highly homologous to the neural adhesion protein N-CAM and that both its synthesis and cell surface appearance alternate in strict sequence with another cell surface adhesion molecule at key morphogenetic stages.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. D. Blumberg	Expert	LCC	NCI
R. Das	Visiting Fellow	LCC	NCI
K. Higinbotham	Biologist	LCC	NCI

Objectives:

The aim of these studies is to understand how cells respond to signals in their external environment, transduce them intracellularly, and induce the expression of genes specific to one pathway of differentiation as opposed to another. We are utilizing, as a model system, a lower eukaryotic microorganism, the cellular slime mold, *Dictyostelium discoideum*. This is one of the simplest organisms to undergo true multicellular differentiation. The predominant feature of the development cycle of *Dictyostelium* is the aggregation of unicellular, free-living amoebae into a multicellular organism. Differentiation of amoebae within the newly formed aggregates generates the two distinct cell types found in the mature fruiting body: spore cells and stalk cells. *Dictyostelium* exhibits many features of development seen in more complex eukaryotic organisms. Specific cell-cell contacts are found, a homogeneous population differentiates into discrete cell types, and there is specific cell migration and pattern formation. These morphogenetic changes are accompanied by major changes in the pattern of gene expression. In particular, postaggregation *Dictyostelium* cells contain 2000-3000 new messenger RNA species that are absent from earlier preaggregation-stage cells. These new aggregation-dependent sequences compose 30% of the mass of the messenger RNA in the late cells and, together with their heteronuclear RNA precursors, represent the transcription products of an additional 26% of the single copy portion of the genome.

The initiation of transcription on this portion of the genome is dependent upon cell-cell interaction. Additionally, both the rate of transcription and subsequent stability of the mRNAs transcribed off this portion of the genome are further regulated by a cAMP-mediated process.

The major objective of our work is to elucidate the molecular mechanisms by which these genes are coordinately activated and to understand the controls which regulate the transcription rate and stability of these mRNAs in response to cAMP and cell-cell interaction.

Major Findings:

We have extended our previous findings that NH_4^+ ion can relieve the requirement for multicellularity (cell-cell interaction), cAMP regulates gene expression through interactions at the cell surface receptor not by activation of protein kinase A, and a Ca^{++}/calmodulin-mediated signal transduction system is utilized for prespore, but not prestalk, gene expression.

New findings this year are that the mRNA level and cell surface appearance of a cAMP regulated prespore protein alternates at key morphogenetic stages with another cell surface adhesion molecule.

Another aspect of the study of these cAMP regulated postaggregation genes deals with the identification of the protein products that they encode. Recently the D19 gene, one of a group of three of our cAMP co-regulated prespore genes that is under the control of the Ca^{++} dependent signal transduction system described above, has been sequenced by Jeff Williams' lab at the Imperial Cancer Research Fund Laboratories. The D19 gene has been shown to encode the PsA cell surface glycoprotein defined by the MUD-1 antibody which has recently been sequenced by Keith Williams' group in Australia. Interestingly, these studies have shown that the D19 gene contains sequences homologous to a major *Dictyostelium* cell adhesion molecule as well as to the chicken neural cell adhesion protein N-CAM. We have shown that both the accumulation of the D19 mRNA and the cell surface appearance of the protein alternate in strict sequence with another cell surface glycoprotein thought to be involved in cell adhesion or some aspect of cell recognition. This switching of cell surface glycoproteins with major changes in the program of morphogenesis is similar to that observed during embryogenesis in higher systems.

Publications:

Blumberg DD. Guide to molecular cloning techniques: The creation of an RNase-free environment. In: Berger S, Kimmel A, eds. Methods in enzymology. New York: Academic Press, 1987;21-4.

Blumberg DD. Guide to molecular cloning techniques: Equipping the laboratory. In: Berger S, Kimmel A, eds. Methods in enzymology. New York: Academic Press, 1987;3-20.

Blumberg DD, Comer JF, Higinbotham KG. A Ca^{++} dependent signal transduction system participates in coupling expression of some cAMP dependent pre-spore genes to the cell surface receptor. Dev Genetics (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05299-07 LCC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Interspecies Differences in Transplacental Carcinogenesis and Tumor Promotion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. A. Lubet	Expert	LCC	NCI
Others:	J. M. Rice	Chief	LCC	NCI
	L. M. Anderson	Expert	LCC	NCI
	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Sect.	LCC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (B. Diwan); Univ. of Ohio Medical School, Toledo, OH (J. Klaunig)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.6	0.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Tumor promotion phenomena in two-stage carcinogenesis were systematically explored in various rodent species in conjunction with transplacental carcinogenesis. Structure-promoting activity relationships of various barbiturates, hydantoins, oxazolidinediones, and benzodiazepine tranquilizers were investigated by sequential administration to animals of a transient, low level exposure to a genotoxic carcinogen followed by the test agent under study. A close relationship was found to exist between the induction of certain cytochrome P-450 species and tumor-promoting abilities of barbiturates and hydantoins. Two oxazolidinediones, trimethadione and dimethadione, non-inducers of cytochrome P-450, failed to promote liver carcinogenesis in rats. Unlike diazepam and oxazepam, a benzodiazepine tranquilizer, clonazepam did not possess liver tumor-promoting activity in mice. Phenobarbital increased liver weight and enhanced hepatic alkoxyresorufin O-dealkylase and aminopyrine N-demethylase activities in rats, mice and patas monkeys susceptible to liver tumors but failed to induce any of these parameters to a significant extent in species resistant to liver tumor promotion, hamsters and cynomolgus monkeys.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. A. Lubet	Expert	LCC	NCI
J. M. Rice	Chief	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI

Objectives:

1) To investigate the relationship between molecular structures and promoting activity of various anticonvulsant, sedative, hypnotic, and anxiolytic drugs available commercially for human therapeutics, i.e., barbiturates, hydantoins, oxazolidine-2,4-diones, acetylureas and benzodiazepines; 2) to determine the cell- and organ-specific tumor promoting effects of anticonvulsant drugs in various rodent species and to explore the genetic and biochemical mechanisms underlying such activities; 3) to charcterize and systematically define the limits of organ, strain, and species specificities of promotion by these compounds; and 4) to utilize promotable JB6 mouse epidermal cells, primary rat hepatocytes or bladder or renal tubular epithelial cells to investigate the mechanisms of the promoting action of these agents.

Major Findings:

To test predictions that barbiturates that are (1) long-acting sedatives and (2) potent inducers of cytochrome $P\text{-}450_b$-mediated monooxygenase activities would be effective promoters of hepatocarcinogenesis, a series of clinically useful barbiturates was selected on the basis of literature values for duration of action as a sedative. These compounds were tested for ability to promote hepatocellular carcinogenesis in male F344 rats initiated with N-nitrosodiethylamine and for activity as inducers of cytochrome $P\text{-}450_b$. The liver tumor promoter phenobarbital showed the greatest ability to induce cytochrome $P\text{-}450_b$ and had the greatest duration of action. The longer-acting sedatives allobarbital and aprobarbital were predicted and confirmed to promote, but were found to be only moderate cytochrome $P\text{-}450_b$ inducers. The nonsedatives, 5-phenyl- and 5-ethylbarbituric acid, were predicted to be inactive as tumor promoters; both were found to be inactive or at most very weakly promoting and were poor cytochrome $P\text{-}450_b$ inducers. In the case of the shorter acting sedatives, Nembutal (pentobarbital) was found to promote, and was exceptionally potent as a cytochrome $P\text{-}450_b$ inducer, while secobarbital showed little or no promoting activity and was only a moderate inducer of cytochrome $P\text{-}450_b$. Pentobarbital thus proved an important exception to the hypothesis that only long-acting sedative barbiturates would promote hepatocarcinogenesis. When the degree of cytochrome $P\text{-}450_b$ induction was plotted against a quantitative value for liver tumor-promoting activity (relative promotion index), a strong correlation ($P < 0.01$) was obtained between these parameters. An empirical parameter constructed for each barbiturate by multiplying duration of sedative action by a measure of cytochrome $P\text{-}450_b$ induction could readily distinguish those compounds with little or no promoting activity for hepatocarcinogenesis from those compounds with moderate or strong activity.

Tumor promoting activities of trimethadione and dimethadione were investigated in rat liver. Male F344 rats were given a single initiating dose of N-nitrosodiethylamine (DEN) and beginning 2 weeks later were placed on either normal diet or diet containing 500 ppm of phenobarbital (PB) or equimolar doses of trimethadione or dimethadione for the remaining experimental period. At 52 weeks, focal hepatocellular proliferative lesions (FHPL)/cm^2 were numerous in rats given PB following DEN initiation. No such increase in the number of FHPL/cm^2 was observed in rats that received DEN followed by either trimethadione or dimethadione. Unlike PB, both trimethadione and dimethadione failed to increase P-450-mediated alkoxyresorufin O-dealkylase and aminopyrine N-demethylase activities in rat liver.

Ethylphenylacetylurea (EPAU) and diethylacetylurea (EEAU), the ring hydrolysis products and urinary metabolities of the multi-tissue tumor promoting barbiturates, PB and barbital (BB), respectively, were fed to F344/NCr male rats previously given a single initiating injection of N-nitrosodiethylamine. EPAU was a weak promoter of hepatocellular adenomas, whereas EEAU had minimal effects. The acetylureas were also weak to moderate inducers of P-450_b-mediated benzyloxyresorufin O-dealkylase activity, with EPAU causing ~ 10x induction, while EEAU increased dealkylation <3x. Neither EPAU nor EEAU had any effect on tumor development in the thyroid, unlike both PB and BB. In contrast EEAU, but not EPAU, strongly promoted development of renal cortical epithelial tumors, and may be at least partly responsible for the promoting effects of BB on the renal cortex. Although an intact heterocyclic ring system is thus not absolutely necessary for promotion of either renal or hepatocellular neoplasms by barbiturates or structurally related compounds, the ring-opened derivatives are considerably weaker as liver tumor promoters. In contrast, ring opening is accompanied by loss of promoting activity for thyroid follicular epithelium. Results with the acetylureas, barbiturates, and hydantoins would seem to imply that only those compounds which are strong inducers of the alkoxyresorufin O-dealkylase activities are thyroid tumor promoters.

The benzodiazepine tranquilizer clonazepam, unlike diazepam and oxazepam (Carcinogenesis 1987;7:789-794), failed to promote liver carcinogenesis in male B6C3F1 mice. Clonazepam was also ineffective in inhibiting intercellular communication in male B6C3F1 mouse hepatocytes in primary culture.

The reciprocal F_1 progeny between the susceptible DBA/2N and the resistant C57BL/6N strains of mice (Carcinogenesis 1987;7:215-220) were also susceptible to liver tumor promotion by PB. In addition to hepatocellular carcinogenesis, the male F_1 progeny derived from DBA/2 females crossed with C57BL/6N males (D2B6F1) were susceptible to the development of hepatoblastoma when they were exposed to a regimen of DEN initiation and PB promotion. PB consistently enhanced development of malignant hepatoblastomas, as well as hepatocarcinogenesis, in D2B6F1 males, but not in intact D2B6F1 females or in B6D2F1 males that were genetically identical to D2B6F1 males except for the reverse origin of their x and y chromosomes.

In view of the major efforts in rats which have tended to link induction of alkoxyresorufin O-dealkylase activities with liver tumor promoting activity in the barbiturate and hydantoins, we have attempted to expand this relationship to other species. We found that phenobarbital was a liver tumor promoter in DEN-initiated mice and rats but not in initiated hamsters. In parallel with these results, phenobarbital induced alkoxyresorufin O-dealkylase activities in mice and rats but not in hamsters. We have expanded these findings to two species of

nonhuman primates (Erythrocebus patas and Macacca fascularis). Phenobarbital was both an enzyme inducer and liver tumor promoter in Erythrocebus patas. In contrast, phenobarbital was both a weak inducer and promoter in Macacca fascularis. The mechanistic basis for this association is currently being investigated.

Publications:

Diwan BA, Rice JM, Nims RW, Lubet RA, Hu H, Ward JM. P-450 enzyme induction of 5-ethyl-5-phenylhydantoin and 5,5-diethylhydantoin analogs of barbiturate tumor promoters phenobarbital and barbital, and promotion of liver and thyroid carcinogenesis initiated by N-nitrosodiethylamine in rats. Cancer Res 1988;48:2492-7.

Lubet RA, Nims RW, Ward JM, Rice JM, Diwan BA. Induction of cytochrome P-450b and its relationship to liver tumor promotion. J Amer Coll Toxicol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05301-07 LCC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Biology and Pathology of Natural and Experimentally Induced Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
Others:	S. Rehm	Visiting Scientist	LCC	NCI
	R. Benveniste	Medical Officer	LVC	NCI
	P. Nara	Expert	OD	NCI
	A. Perantoni	Staff Fellow	LCC	NCI

COOPERATING UNITS (if any) Natl. Inst. of Allergy and Infectious Diseases, Bethesda, MD (E. Santos); VA Hosp., Pittsburgh, PA (G. Singh); Univ. of Leiden, The Netherlands (A. Ten Have-Opbroek); Natl. Inst. of Hygienic Sciences, Tokyo (K. Takahashi); Johns Hopkins University, Baltimore, MD (W. Gibson)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Application of immunohistochemical, ultrastructural and morphologic techniques revealed conclusive evidence for the origin of chemically-induced papillary lung tumors of the mouse from alveolar cells rather than from bronchiolar Clara cells, as reported by some investigators. These controversial tumors were found to be of alveolar origin based on specific antigen localization and unique ultrastructural features found only in alveolar Type II cells. In contrast, chemically induced bronchiolar tumors of the Syrian hamster were found to arise from bronchiolar Clara cells by the use of similar techniques, although progressive evidence of de-differentiation was also found. A sensitive ABC immunohistochemical technique was developed to detect lentiviral antigens in autopsy and biopsy specimens of humans with human immunodeficiency virus (HIV) infection and monkeys with simian immunodeficiency virus (SIV) infection. Monoclonal and polyclonal antisera to HIV and SIV proteins detected viral gag proteins in inflammatory macrophages and giant cells, tumor cells, endothelial cells and bone marrow cells. Thus, our technique was very sensitive for detecting infected cells for diagnosis and studies of the pathogenesis of the associated diseases and tumors. Immunohistochemical studies of human tissues with HIV and cytomegalovirus (CMV) infection led to detailed immunoblotting studies which revealed that the major gag protein of HIV and major capsid protein of CMV cross-react. Antisera to H-ras p21 detected the antigen in fixed tissue sections of Harvey sarcomas on the cell membrane in T24 cells, and in the cytoplasm of some normal tissues and in liver tumors of mice that contain an activated H-ras oncogene. Immunoblotting, used to detect p21 in comparison with levels detected by immunohistochemistry, revealed that one monoclonal antibody claimed to react with p21 did not react to p21 or with rodent tumors with an activated H-ras oncogene. Interpretation of published studies involving p21 immunohistochemistry require caution and additional study.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
S. Rehm	Visiting Scientist	LCC	NCI
R. Benveniste	Medical Officer	LVC	NCI
P. Nara	Expert	OD	NCI
A. Perantoni	Staff Fellow	LCC	NCI

Objectives:

To study and characterize the biology, pathology and pathogenesis of naturally occurring and experimentally-induced tumors of laboratory animals. To develop animal models for the study of human tumors and associated diseases. To develop methods utilizing experimental animals to better understand the nature, causes and prevention of human cancer, especially methods involving immunohistochemistry.

Major Findings:

Lentiviral (HIV, SIV) antigens were localized to cells and tissues in various stages of the diseases caused by lentiviruses through the application of avidin-biotin immunohistochemistry in order to help elucidate the pathogenesis of these infections. Polyclonal and monoclonal antibodies and a refined ABC technique were used on fixed tissue sections from biopsy and autopsy specimens of human and nonhuman primates. HIV antigens, especially the major gag proteins p24, p17, and Pr55, were found in several cell types including multinucleated giant cells, macrophages and endothelial cells of the brain, dendritic reticular cells, rare immunoblasts in hyperplastic lymph node follicles, endothelial cells in high-endothelial venules (HEV) of lymph nodes, rare small lymphocytes in lymph nodes, sinus histocytes/macrophages in nodes, pulmonary macrophages, and lymphoma cells of one case. The CD4 lymphocyte antigen (HIV, receptor) was found in endothelial cells in HEV (1 case) and brain (1 case); similar cells immunoreacted with antibodies to HIV p24. HEV has been implicated in lymphocyte scrubbing, recirculation and pathogenesis of viral diseases and associated neoplasms.

Immunohistochemistry of experimental SIV infection in macaques revealed SIV antigens in some unusual sites including bone marrow endothelium and pancreatic islet cells. Viral gag proteins were not, however, found in lymphocytes, the lymphoma of origin of the viral isolate nor in megakaryocytes. Chimpanzees, experimentally infected with HIV, were found to reveal HIV infection of bone marrow lymphocytes, immature myeloid cells and megakaryocytes but not in peripheral lymph node cells. From in situ hybridization studies, HIV RNA was found within more cells in lymphoid tissues than by immunohistochemical detection of HIV antigens, but no such difference was observed in bone marrow.

An incidental finding of immunoreactivity of HIV antisera to cytomegalovirus (CMV) intranuclear inclusion bodies provided incentive for a detailed molecular virology study of possible cross-reactivity of HIV gag proteins and CMV proteins. After extensive immunoblotting analysis, it was found that HIV gag proteins

showed cross-reaction to the CMV major capsid protein (MCP, 153KD). The implications of this finding include a possible mechanism responsible for HIV and CMV pathogenesis after co-infection or initial infection by one agent. Serum antibody responses to one virus may cause false positive diagnoses in serological tests to the other virus.

Different morphologic, ultrastructural, and immunohistochemical antigenic localization methods were used to identify the cell of origin of the controversial mouse papillary lung tumors and pulmonary bronchiolar neoplasms of hamsters. The tumors in the mouse were induced transplacentally with N-nitrosoethylurea and in the adult hamster with several s.c. injections of N-nitrosodiethylamine. Most convincing evidence was seen for alveolar type II cell origin of the mouse lung tumors and for Clara cell origin of the hamster lung tumors with different immunocytochemical techniques and the use of specific antibodies identifying cell-specific antigens in these pulmonary cells. Two different antibodies were raised in rabbits against mouse surfactant apoproteins specific for alveolar type II cells. The antigens were obtained from either lung lavage fluid (anti-SAP-M) or lung homogenate (SAALS). Anti-SAP-M recognizes a M_r 35,000 surfactant-associated protein and SAALS was characterized as being specific for surfactant apoproteins of molecular weights of 26,000, 32,000 and 38,000 daltons and a M_r 12,000 protein. The majority of the papillary mouse lung tumors were immunoreactive for specific mouse surfactant apoproteins (intracytoplasmic or intranuclear) irrespective of the method or antibody used. Anti rat-Clara cell antigen (CCA) is cross-reactive with normal mouse Clara cells. CCA is a Clara cell protein C that has M_r about 10,000, is present in lung lavage fluid and consists of three isotypes. In the mouse not a single lung tumor was immunoreactive for CCA. In the hamster, however, pulmonary preneoplastic and neoplastic lesions were immunoreactive for a specific hamster CCA. The lung tumors of the hamster also develop squamous metaplasia losing the immunoreactivity for CCA and becoming reactive for cytokeratin. These studies provide convincing evidence for the common alveolar origin of mouse lung tumors and Clara cell origin of hamster lung tumors.

The oncogene product of the H-ras oncogene, p21, was localized to selected normal cells, tissues and tumors after avidin-biotin immunohistochemistry in order to determine the usefulness of this technique for detecting oncogene activation and possible contributing mechanisms of carcinogenesis. Ten antibodies were used including polyclonal and monoclonal antibodies to normal or transforming p21 produced from bacterial expression vectors or p21 peptides. Western blotting of the preparations provided important verifying information on their immunoreactivity with expression vector-generated p21. One antibody, RAP-5, a mouse monoclonal to a peptide composed of amino acids 10-17 with valine at Codon 12, consistently did not react in Western blots nor in fixed tissue sections with tumors with a characterized activated H-ras oncogene even in T24 tumors with the valine mutation at codon 12. The nine other antibodies to p21 always immunoreacted with cell membranes of Harvey virus-induced sarcoma cells. One antibody preparation to a p21 peptide reacted with cell membranes of a low proportion of rat bladder hyperplasias and tumors induced by a nitrosamine. Subsequently, a low incidence of H-ras activation was found by tumor DNA transfection of NIH 3T3 cells. Several antibodies reacted with cytoplasm of components from some normal cells or tissues. One preparation, mouse monoclonal #51, reacted with cytoplasmic granules (probably mitochondria) in numerous tissues. Western blotting of kidney preparations revealed the reactive antigen with a molecular weight significantly greater than 21KD. The significance of this finding is under study. It could represent a

cross-reacting epitope of a mitochondrial antigen, a precursor to p21, p21 in another form for a new function, or the same epitope on another antigen. Our studies on detection of H-ras p21 in autopsy specimens provide important information and techniques for practical application of this method in detecting oncogene activation. Our work also emphasizes a need for caution in interpretation of published reports using these techniques.

Publications:

Anderson AO, Ward JM. Endocytic stripping of ligands from migrant lymphocytes in high endothelial venules (HEV): implications for immunomodulation vs. viral pathogenesis. Adv Exp Med Biol (In Press).

Benveniste, RE, Morton WR, Clark EA, Tsai CC, Ochs HD, Ward JM, Kuller L, Knott WB, Hill RW, Gale MJ, Thouless ME. Inoculation of baboons and macaques with SIV/Mne, a primate lentivirus closely related to HIV-2. J Virol (In Press).

Frith CH, Ward JM. A color atlas of neoplastic and nonneoplastic lesions in aging mice. Amsterdam: Elsevier, 1988;154.

Nonoyama T, Reznik G, Bucci T, Ward, JM. Mouse hepatoblastomas: a histologic, ultrastructural and immunohistochemical study. Vet Pathol (In Press).

Rehm S, Ward, JM, Ten Have-Opbroek AAW, Anderson, LM, Singh G, Katyal SL, Rice, JM. Mouse papillary lung tumors transplacentally induced by N-nitrosoethylurea: evidence for alveolar type II cell origin by comparative light microscopic, ultrastructural, and immunohistochemical studies. Cancer Res. 1988;48:148-60.

Rhodes RH, Ward JM. Immunohistochemical localization of human immunodeficiency viral antigens in formalin-fixed spinal cords with AIDS myelopathy provides evidences of histopathogenesis. Clin Neuropathol (In Press).

Rhodes R, Ward JM. Immunohistochemistry of human immunodeficiency virus in the central nervous system and a hypothesis concerning the pathogenesis of AIDS meningoencephalitis. In: Sommer S, Racz P, Meyer PR, eds. Progress in AIDS pathology. New York: Field & Wood (In Press).

Ward JM, Hagiwara A, Tsuda H, Tatematsu M, Ito N. H-ras p21 and peanut lectin immunoreactivity of hyperplastic, preneoplastic and neoplastic urinary bladder lesions in rats. Jpn J Cancer Res 1988;79:152-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05303-07 LCC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Pathogenesis and Promotion of Natural and Induced Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
Others:	T. Enomoto	Visiting Fellow	LCC	NCI
	A. Perantoni	Staff Fellow	LCC	NCI
	L. Anderson	Expert	LCC	NCI
	N. Konishi	Visiting Fellow	LCC	NCI
	P. Donovan	Chemist	LCC	NCI
	D. Devor	Biologist	LCC	NCI
	G. Smith	Biological Laboratory Technician	LCC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (B. Diwan, J. Henneman); Pathology Institute, Holback, Denmark (K. Ostergaard)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The role of oncogenes, chronic toxicity and aging on carcinogenesis in mouse liver was studied. Activation of the H-ras oncogene by point mutation was found in a proportion of hepatocellular adenomas and carcinomas induced in B6C3F1 mice by N-nitrosodiethylamine or occurring spontaneously in C3H mice. The three types of mutations found at Codon 61 of H-ras was similar in type and incidence to those occurring spontaneously in B6C3F1 mice. The incidence of H-ras activation in liver tumors of C3H mice, a strain with a high spontaneous rate, was significantly lower than for B6C3F1 mice. These findings provide evidence for the role of H-ras in tumor initiation rather than promotion and suggest other factors play a major role in the multistage process of mouse hepatocarcinogenesis. Aging mouse hepatocytes were shown to be significantly more sensitive to phenobarbital carcinogenesis than those of young mice. Age-related changes in metabolism of phenobarbital and oncogene activation are possible mechanisms of this interesting phenomenon. We found similar findings for rats. Chronic toxicity in mouse liver and kidney was induced by acetaminophen or di(2-ethylhexyl)phthalate as measured by tritiated thymidine or Brdu uptake and histopathology. These studies provided evidence that chronic hyperplasia does not always lead to organ-specific carcinogenesis or tumor promotion.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
T. Enomoto	Visiting Fellow	LCC	NCI
A. Perantoni	Staff Fellow	LCC	NCI
L. Anderson	Expert	LCC	NCI
N. Konishi	Visiting Fellow	LCC	NCI
P. Donovan	Chemist	LCC	NCI
D. Devor	Biologist	LCC	NCI
G. Smith	Biological Laboratory Technician	LCC	NCI

Objectives:

To characterize the sequential morphologic and biologic steps in the development of organ-specific cancers and tumor promotion and to study the nature of cellular and organ-specific activities of nongenotoxic carcinogens and tumor promoters.

Major Findings:

In an effort to determine the mechanisms responsible for mouse liver carcinogenesis, especially by chemicals without genotoxic activity, we studied the role of oncogenes, aging and chronic toxicity on induction of liver tumors in mice. Several exciting findings, reported for the first time, reveal important aspects of the mechanisms responsible for mouse hepatocarcinogenesis.

Oncogenes, especially those of the ras family, have been found to play a role in rodent and human carcinogenesis. In collaboration with other groups within LCC (see project Z01CP05399-05), we have used transfection of NIH 3T3 cells and oligonucleotide hybridization as screening methods for detection of H-ras activation and point mutation. In spontaneous hepatocellular adenomas and carcinomas of C3H/HeNCr mice, we found a very low rate of focus formation in the 3T3 transfection assay (1/39 and 1/6, respectively). This result was confirmed by lack of hybridization with the use of mutant probes to H-ras at Codon 61 and positive hybridization with probes for the wild type oncogene. In contrast, B6C3F1 mice, derived from a parental C3H mouse, revealed spontaneous and induced (DEN) liver tumors with high rates (30-70%) of H-ras activation, especially at Codon 61. The relatively high incidence of H-ras activation even in benign tumors (adenomas) of B6C3F1 mice suggests that this potential mechanism of carcinogenesis may not be so important for the development of carcinomas but rather plays an important role in the initiation process. On the other hand in C3H mice, this role was not found. Even in tumors induced or promoted by phenobarbital in C3H mice, H-ras activation was an uncommon event. We found also that aging mouse hepatocytes are highly susceptible to phenobarbital "carcinogenesis", suggesting that aging hepatocytes have activated oncogenes which may have initiated carcinogenesis or that metabolism, pharmacokinetics and bioresponses to phenobarbital are significantly modified in these aging hepatocytes and in aging mice.

Nongenotoxic tumor promoters are usually so-called "weak" carcinogens. Often, they must be administered at doses which cause chronic organ-specific toxic

lesions. These lesions and their association with chronic increases in levels of DNA synthesis in target cells for tumor promotion and carcinogenesis are not well studied or correlated. We studied the chronic hepatotoxic effects of barbital sodium, acetaminophen, phenobarbital and di(2-ethylhexyl)phthalate (DEHP) on mouse liver and kidney. B6C3F1 mice were fed diets containing one of these chemicals for periods of up to 18 months. Levels of thymidine kinase and tritiated thymidine or Brdu uptake, liver weights and histopathology were evaluated. We found that relative increases of any parameter of chronic toxicity or hyperplasia (increased level of DNA synthesis) was totally independent of the biological activity of the chemical studied. For example, phenobarbital, a potent liver tumor promoter, did not induce chronic increases of hepatic levels of DNA synthesis, while DEHP, a much weaker liver tumor promoter, could induce a chronic persistent increase in these levels in hepatocytes. Acetaminophen, a nonhepatocarcinogen in our B6C3F1 mice, also induced chronic increases in DNA synthesis at dietary levels without evidence of carcinogenesis or tumor promotion at these exposures. In the kidney of B6C3F1 mice, DEHP produced marked time-related cumulative nephropathy with severe focal chronic degenerative and hyperplastic renal tubular lesions. Levels of DNA synthesis in renal tubular epithelium was 20-100 times normal without evidence of carcinogenesis (in 2-year studies) or tumor promotion (after transplacental N-nitrosoethylurea [ENU] initiation). This finding was extremely significant in light of recent findings with some renal toxins which induce hyperplasia and renal tumors. In an effort to explain the possible mechanism of renal carcinogenesis by nongenotoxic chemicals, it was suggested previously, by others, that an agent which induces this chronic toxicity and hyperplasia was carcinogenic as a consequence of this induced chronic hyperplasia (Short et al., Lab Invest 1987;57:564-577). Our findings, at least with DEHP in mouse kidney and DEHP and acetaminophen in mouse liver, would question this mechanism as the driving force for carcinogenesis and tumor promotion by nongenotoxic carcinogens.

In an effort to define mechanisms of renal tumor promotion in vivo and in vitro in rats and rat cells and in vitro in human cells, we developed an in vitro renal cell assay system, initially for rat renal tubular epithelial cells and for studies in progress on human renal tubular epithelial cells. Primary and early passage rat renal epithelial cells were cultivated in vitro and a focus-forming assay was used to evaluate the effects of rat renal tumor promoters and carcinogens on these cells. Chemicals studied in vivo in rats and subsequently in vitro included barbital sodium, phenobarbital and nitrilotriacetic acid (NTA). Early findings showed that colony size could be increased by tumor promoters but numbers of colonies were not changed. Biochemical parameters will be measured. A similar rat urinary bladder assay has proved more difficult to develop but shows some promise as well.

Publications:

Stowers SJ, Wiseman RW, Ward JM, Miller EC, Miller JA, Anderson MW, Eva A. Detection of activated proto-oncogenes in N-nitrosodiethylamine-induced liver tumors: a comparison between B6C3F1 mice and Fischer 344 rats. Carcinogenesis 1988;9:271-6.

Ward JM, Lynch P, Riggs C. Rapid development of hepatocellular neoplasms in aging male C3H/HeNCr mice given phenobarbital. Cancer Lett 1988;39:9-18.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05352-06 LCC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Metabolic and Pharmacological Determinants in Perinatal Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. M. Anderson	Expert	LCC	NCI
Others:	J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
	M. S. Miller	Senior Staff Fellow	LCC	NCI
	D. P. Chauhan	Visiting Fellow	LCC	NCI
	A. Perantoni	Staff Fellow	LCC	NCI
	T. Enomoto	Visiting Fellow	LCC	NCI
	S. Park	Expert	LMC	NCI
	H. V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)
Program Resources, Inc., Frederick, MD (H. Issaq); Pathology Associates (R. Kovatch); American Health Foundation, Valhalla, NY (S. Hecht); and University of Texas (L.J. Lu)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.25	1.5	0.75

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

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

This project consists of whole-animal, biochemical, and molecular approaches to determination of the factors that determine and modulate susceptibility to carcinogenesis during the perinatal period. Significant progress has been made with a murine model for the pharmacogenetics of transplacental carcinogenesis by a polycyclic aromatic hydrocarbon (PAH), 3-methylcholanthrene (MC). Genetics allowing induction of metabolism of this chemical predisposed mouse fetuses to susceptibility to tumor initiation in lung and liver, whereas inducibility in the mother allowed protection of the fetuses. Pretreatment with a noncarcinogenic enzyme inducer β-naphthoflavone (BNF) enhanced fetal protection if the mother was inducible, but enhanced tumorigenesis in fetal organs if she was not. Transplacental induction of cytochrome P-450-dependent PAH activating enzyme activity and of gene expression for this P-450 were followed for MC and BNF. Dramatic induction occurred and MC and BNF were noted to have different time- and organ-specific induction patterns. Monoclonal antibody and PAH-inducible P-450s and cDNA probes for the genes specifying these enzymes were used to delineate these phenomena. Transplacental induction of the phase II detoxicating activity, uridine diphosphoglucuronic acid transferase, was found to be inducible by BNF but not MC. Transplacental MC but not BNF had a permanent imprinting effect on MC metabolism by the livers of the adult offspring, and this effect was dependent on genetically-controlled induction of metabolism by either the fetus or the mother. Gene methylation differences are being explored as a possible mechanism for this effect. In other perinatal experiments in progress or recently completed, N-nitrosodimethylamine has been found to be a transplacental carcinogen in the mouse, causing liver tumors and, interestingly, sarcomas. Liver tumors initiated by N-nitrosoethylurea in this study have been analyzed for activated oncogenes and found to contain H-ras in some cases. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been administered to several strains of mice transplacentally and neonatally with limited effect.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. M. Anderson	Expert	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
M. S. Miller	Senior Staff Fellow	LCC	NCI
D. P. Chauhan	Visiting Fellow	LCC	NCI
A. Perantoni	Staff Fellow	LCC	NCI
T. Enomoto	Visiting Fellow	LCC	NCI
S. Park	Expert	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

To identify and characterize the organismal, biochemical, and molecular factors that determine susceptibility to carcinogens during the perinatal period, and that modulate the development of perinatally-initiated tumors; to discover the perinatal effects of important toxicants to which humans are exposed; and to develop animal models for study of the etiology of common human childhood cancers.

Major Findings:

Previous work in this section demonstrated, with a transplacental pharmacogenetic murine model, that the genetically-regulated capacity of both fetuses and mothers to respond to inducers of polycyclic aromatic hydrocarbon metabolism has an important determining role in susceptibility to tumorigenesis in lung and liver by 3-methylcholanthrene (MC). Inducibility in the fetus potentiates this process, but inducibility in the mother affords protection to the fetuses. We have now confirmed and extended these observations with regard to pretreatment with a noncarcinogenic enzyme inducer, β-naphthoflavone. When mothers are inducible, such pretreatment enhances tumorigenesis in inducible fetuses under certain conditions of dose and sex. These observations have important implications for both evaluating human risk and designing preventive measures. Transplacental induction of aryl hydrocarbon hydroxylase (AHH) by MC and BNF has been assayed as a measure of the cytochrome P-450-dependent phase I activation of PAHs, and found to increase dramatically (20 to 40-fold over basal for fetal liver and lung), with different specific time- and organ-dependent differences between MC and BNF. The nature of the P-450 isozyme was confirmed by inhibition (60-70%) of the enyzme activity by a monoclonal antibody to this P-450. Use of a cDNA probe specific for P_1450 in Northern and slot blots showed that the increased enzyme activity was reflective of greatly increased synthesis of mRNA. Transplacental induction of a phase II detoxicating enzyme for PAHs, uridine diphosphoglucuronic acid transferase, was also studied. BNF caused significant induction (two- to threefold) but MC did not, in contrast to the large increase in phase I activity--a differential of significance with regard to the observed tumorigenic effects. Another interesting consequence of transplacental exposure to MC was an imprinting effect, resulting in a significant increase in MC metabolism in the livers of the adults. This occurred only in situations where either the mother or the fetus was inducible, and therefore was dependent on induced metabolism of MC. BNF had no such effect. A cDNA probe from the 5 region of

the P-450 gene is being used to assess altered DNA methylation as a mechanism for this effect.

Other ongoing work with this model includes ^{32}P-postlabeling analysis of DNA adducts after fetal exposure, and continuation of whole-animal experiments with other carcinogens and modes of treatment, and with congenic mice. N-nitroso compounds, which have been suggested as human transplacental carcinogens on the basis of epidemiological studies, have been studied for perinatal effects in murine models. N-Nitrosodimethylamine (NDMA), a common environmental nitrosamine, was found for the first time to be a transplacental carcinogen in C3H mice. It caused liver tumors and, interestingly, sarcomas; tumors of mesodermal origin are among the more common cancers of childhood. It also resulted in one schwannoma and thus gave some support to the concept of implication of metabolism-dependent nitrosamines in causation of neurogenic tumors. Liver tumors initiated by N-nitrosoethylurea, the positive control of this study, were analyzed for activated oncogenes and found to contain activated H-*ras* in some cases. Also, an extensive test of the perinatal effects of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been carried out with mice. Final analysis of the data is nearly complete; NNK is a most weak transplacental carcinogen but may be more active in the neonate.

Other perinatal experiments that are in progress or planned include (1) analysis of the effects of polychlorinated biphenyls as enhancers of neonatally-initiated tumors; (2) investigation of diethylstilbestrol as a perinatal carcinogen in the rat and modulation of this process by metabolism inducers; (3) transplacental carcinogenic effects of nitrofen, a herbicide which is a highly specific teratogen.

Publications:

Anderson LM, Jones AB, Miller MS, Chauhan DP. Metabolism of transplacental carcinogens. IARC Sci Publ (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05353-06 LCC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Sensitivity Factors in Special Carcinogenesis Models

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. M. Anderson	Expert	LCC	NCI
Others:	J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
	A. O. Perantoni	Staff Fellow	LCC	NCI
	T. Enomoto	Visiting Fellow	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
	S. S. Park	Expert	LMC	NCI
	H. V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)
Temple University, Philadelphia, PA (G. Harrington, H. Pylypiw, and P. N. Magee); Smith Kline Laboratories, King of Prussia, PA (C. Gombar); University of South Florida, Tampa, FL (A. Giner-Sorolla)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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

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

The several experiments in this project relate to the common theme of discovery and delineation of the factors that determine and modify susceptibility to carcinogenesis. An interdisciplinary approach to analysis of the effect of the common environmental carcinogen N-nitrosodimethylamine (NDMA) has been fruitful. Striking effects of ethanol, which is being increasingly implicated in the etiology of human cancers, has been demonstrated. Co-administration of ethanol in the drinking water with NDMA has been found to cause a consistent and significant increase in numbers of lung tumors and in promutagenic DNA adducts in the lung. Tumor promotion by ethanol was ruled out as a contributing factor. Competitive inhibition of the liver enzyme acting on NDMA, a demethylase (cytochrome P-450j) is the postulated mechanism. This enzyme has been studied biochemically and pharmacokinetically in mouse, monkey, and several other species, with inhibition by a specific monoclonal antibody to confirm the nature of the P-450. The activity appears to be highly conserved across species, so that results from the mouse have particular relevance to the human. Utilization of this system is ongoing. Interesting results have also been obtained with N-nitrosocimetidine (NCM), derivative of a commonly-used pharmaceutical. Though not a complete carcinogen, this alkylating agent both enhanced progression of preformed skin papillomas to malignancy and initiated cells which could be caused to develop by subsequent application of a tumor promoter. These NCM-initiated tumors were all found to contain DNA that transformed 3T3 cells and were mutated in the second position of codon 61 of the H-*ras* oncogene. Other experiments that are ongoing in this project include immunohistochemical studies of localization of cytochromes P-450 and of DNA adducts using monoclonal antibody reagents, and investigation of the protective effect *in vivo* of pretreatment of mice with inducers of enzymes metabolizing carcinogens.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. M. Anderson	Expert	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
A. O. Perantoni	Staff Fellow	LCC	NCI
T. Enomoto	Visiting Fellow	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
S. S. Park	Expert	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

To ascertain mechanisms for qualitative or quantitative differences in effects of chemical carcinogens as a function of cellular events (metabolic activation/detoxication, DNA damage and repair) and of organismal phenomena (disposition, kinetics). This project utilizes agents of unique chemical or human exposure interest and special animal models where susceptibility characteristics present favorable situations for mechanistic analysis.

Major Findings:

N-nitrosodimethylamine (NDMA) is a common environmental contaminant and is found to circulate in human blood. Ethanol is, of course, consumed by humans and is being increasingly implicated in the etiology of human cancer by epidemiological studies. Earlier work in this section showed that chronic co-treatment of mice with NDMA and ethanol (a common mode of human exposure to these chemicals) resulted in higher blood and tissue levels of NDMA than when the carcinogen was given alone. We have now demonstrated that under these conditions co-treatment with ethanol causes a large, two- to fivefold increase in numbers of lung tumors initiated by NDMA at several doses. It was found that this effect was not due to tumor promotion by the ethanol. Mechanisms and characterization of this important effect and its relevance to human risk are being pursued with biochemical studies of NDMA metabolism, analysis of promutagenic adducts in liver and lung, and cross-species comparison of NDMA pharmacokinetics. NDMA demethylase activity in mouse liver has Michaelis-Menten kinetics similar to those in rat liver and is effectively inhibited by a monoclonal antibody to rat liver cytochrome P-450j (the ethanol-inducible form of P-450). Analysis of pharmacokinetic parameters for NDMA in mouse, rat, rabbit, dog, pig, and monkey, carried out by Dr. C. Gombar of Smith Kline Laboratories and including our data for mouse and patas monkey (see project Z01CP05092-10), show very similar clearance rates for all of these species. The data together indicate highly conserved pharmacology of NDMA and hence high relevance of animal studies to the human. Analysis of formation and repair of DNA adducts in lung and liver of mouse and monkey by NDMA, with and without ethanol, is in progress and thus far indicates that co-treatment with ethanol may greatly affect the numbers of promutagenic lesions in DNA of distal targets.

N-Nitrosocimetidine (NCM) is derived by nitrosation from a widely-prescribed drug. It is an alkylating genotoxicant, but not carcinogenic by itself. We have now found that NCM has effectiveness as an incomplete skin carcinogen on SENCAR mice. Skin papillomas that had been initiated with dimethylbenzanthracene

and promoted with 12-O-decanoylphorbol-13-acetate (TPA) were found to progress to malignancy more rapidly when they were further treated with NCM, compared with acetone controls. In addition, repeated treatment with NCM initiated skin tumors that could be promoted with TPA; 4/4 of these yielded DNA that transfected 3T3 cells. This is the highest frequency of transformation by skin tumors initiated by an alkylating agent thus far reported. Two of these were found to contain a mutation in the second position of the 61 codon of the H-*ras* oncogene (see project Z01CP05399-05); analysis of the other two is in progress. NCM thus has interesting potential as a model compound for dissecting the mechanisms involved in tumor initiation, promotion, and complete carcinogenesis. Reassessment as a potential human carcinogen may also be in order.

Other studies ongoing include immunohistochemical monoclonal antibody localization of isozymes of cytochromes P-450 and of promutagenic DNA adducts (O^6-methylguanine) as related to susceptibility to toxic/carcinogenic effects; and *in vivo* experiments on the role of metabolic activation and detoxication in susceptibility to tumorigenesis, including use of congenic mice.

Publications:

Anderson LM. Increased numbers of N-nitrosodimethylamine-initiated lung tumors in mice by chronic co-administration of ethanol. Carcinogenesis (In Press).

Anderson LM, Ward JM, Park SS, Jones AB, Gelboin HV, Rice JM. Immunohistochemical determination of inducibility phenotype with a monoclonal antibody to a methylcholanthrene-inducible isozyme of cytochrome P-450. Cancer Res 1987;47:6079-85.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05399-05 LCC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Oncogene Expression in Chemically Induced Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. M. Rice	Chief	LCC	NCI
Others:	A. O. Perantoni	Staff Fellow	LCC	NCI
	T. Enomoto	Visiting Fellow	LCC	NCI
	C. D. Reed	Senior Health Services Officer	LCC	NCI
	C. Majumdar	Expert	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
	L. M. Anderson	Expert	LCC	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section, Developmental Biology Working Group

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

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

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

The expression of activated cellular oncogenes in chemically induced rat tumors and in comparable human neoplasms and the relationship of oncogene expression to progression from the normal to the neoplastic phenotype are being studied using 3T3 transfection and hybridization techniques and monoclonal antibodies directed against the specific oncogene products. The consistent activation of K-*ras* in rat renal mesenchymal tumors has been shown to occur by a G → A transition mutation in the second position of codon 12, which has been demonstrated in 3T3 transformants and in DNA from primary tumors whether the DNA transformed 3T3 cells or not. Further studies on the *neu* oncogene in the pathogenesis of schwannomas, in a different strain of rat than was previously used, confirm the consistent presence in an additional 30 such tumors (100% of those tested) of *neu* activated by a T → A transversion mutation in that segment of the *neu* locus encoding the putative transmembrane region of the protein encoded by the gene, a growth factor receptor-tyrosine kinase-type molecule. Further studies are in progress to determine whether *neu* is comparably activated by point mutation in chemically induced schwannomas of other species of rodents and in human tumors including schwannomas.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Rice	Chief	LCC	NCI
A. O. Perantoni	Staff Fellow	LCC	NCI
T. Enomoto	Visiting Fellow	LCC	NCI
C. D. Reed	Senior Health Services Officer	LCC	NCI
C. Majumdar	Expert	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
L. M. Anderson	Expert	LCC	NCI

Objectives:

To identify activated oncogene sequences in specific types of chemically induced neoplasms in rats, mice, and other species in comparison with normal, nonneoplastic tissues in the same animals. To isolate and characterize the oncogene sequences found as mutant or wild-type alleles. To generate immunologic probes for available oncogene products and to apply these to detect expression of oncogenes at different stages of tumor development as well as during embryonic or fetal development.

Major Findings:

We have evaluated NIH 3T3 transformants derived from renal mesenchymal tumor DNAs for mutations in the activated K-*ras* oncogene using selective oligonucleotide hybridization and have found a consistent G → A transition mutation in the second position of codon 12. This mutation was identified in 12/18 transformants and in 4/4 primary tumor DNAs, indicating that the mutation did not result from the transfection process. We are currently attempting to eliminate technical considerations in our efforts to characterize the lesion(s) in the remaining tumors with transforming K-*ras* sequences. By amplification of genomic sequences using K-*ras*-specific primers, we should have adequate sensitivity to determine if a G → A transition is also responsible for activation of K-*ras* in these tumors. In the event that a similar mutation is not observed, amplification procedures also provide suitable material for sequencing specific regions in the K-*ras* oncogene. In addition, we have observed G → A transitions in at least 5 of the 23 transfection-negative tumor DNAs and expect to apply gene amplification procedures to evaluate the generality of mechanism since we have reported the inefficiency of transfection for the detection of large oncogenes.

Previously, we reported the selective activation of the *neu* oncogene in F344 rats by a specific T → A transversion in transplacentally N-nitrosoethylurea (ENU)-induced schwannomas, but not in gliomas. To determine the general association of this mutation with schwannomas, ENU-induced neurogenic tumors from Sprague-Dawley rats were evaluated for transversions. In these experiments, 30/30 schwannomas and 0/19 oligodendrogliomas contained the same T → A mutation in the region encoding the putative transmembrane domain as we described for the F344 rat. These studies, therefore, indicate the association of the T → A transversion in the *neu* oncogene with schwannomas is not unique to the F344 rat. We are currently analyzing similar tumors derived from different species or initiated with different chemical carcinogens. Since the wild-type oligonucleotide probe used in selective hybridization of rat tissues does not react with the other

species under study, gene amplification and sequencing will be necessary to determine the consistency of mechanism.

Publications:

Perantoni AO, Rice JM, Reed CD, Watatani M, Wenk ML. Activated neu oncogene sequences in primary tumors of the peripheral nervous system induced in rats by transplacental exposure to ethylnitrosourea. Proc Natl Acad Sci USA, 1987;84:6317-21.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05465-04 LCC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

The Regulatory Role of Retinoids and Growth Factors in Tissue Differentiation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
Others:	A. B. Roberts	Research Chemist	LC	NCI
	M. B. Sporn	Chief	LC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., FCRF, Frederick, MD (E. F. Munoz)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Ultrastructural Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The involvement of tumor growth factor-beta (TGF-β) in embryonal development of the mouse was investigated in embryos of 10 to 18 days of gestation, using antibodies raised against synthetic peptides of the TGF-β monomer to localize the growth factor. TGF-β was found in a variety of tissues of ectodermal and mesenchymal origin, predominantly around day 15 when organogenesis is most intense. The wide distribution of TGF-β indicates its involvement as a regulator in major events of cytodifferentiation. As TGF-β is known to be a modulator of the extracellular matrix, the relationship of the growth factor to the synthesis of matrix components (fibronectin, collagen I, laminin) was evaluated in the developing mouse embryo.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ursula I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
Anita B. Roberts	Research Chemist	LC	NCI
Michael B. Sporn	Chief	LC	NCI

Objectives:

To investigate the role of the transforming growth factor-beta (TGF-β) in regulating cell differentiation and proliferation during embryonal development.

Major Findings:

High concentrations of TGF-β were detected in embryos of 13-15 days gestation, at a time when organogenesis is most intense. TGF-β was predominantly localized in tissues of mesenchymal origin, such as notochord, somites, the ossification centers of ribs, vertebrae, and long bones, and the connective tissue network of various organs including muscular tissue and dermis. In the latter case, a close correlation of staining to areas of active vascularization was detected. TGF-β was also localized in tissues of neural crest origin, including ossification centers of bones in the cranial, facial or oral region, as well as the two innermost coverings of the brain (pia mater and arachnoid) and spinal cord. TGF-β was also closely associated with cushion tissue of the heart that is instrumental in the construction of the heart valves and septae. The results are indicative of a complex role of TGF-β in embryonal development with special involvement in bone formation and stimulation of mesenchymal tissue, which in turn, is known to activate cytodifferentiation. The appearance of extracellular matrix components, especially fibronectin and collagen I, could be correlated to the presence of TGF-β. This correlation was especially pronounced during organogenesis of lung and kidney.

Publications:

Heine UI, Munoz EF, Flanders KC, Ellingsworth LR, Lam H-YP, Thompson NL, Roberts AB, Sporn MB. The role of transforming growth factor-beta in the development of the mouse embryo. J Cell Biology 1987;105:2861-6.

Junker JL, Heine UI. The effect of adhesion factors fibronectin, laminin and type IV collagen on spreading and growth of transformed and control liver epithelial cells. Cancer Res 1987;47:3802-7.

Roberts AB, Flanders KC, Condaiag P, Thompson NL, Van Obberghan-Schiling E, Wakefield L, Rossi P, deCrombrugghe B, Heine U, Sporn MB. Transforming growth factor beta; biochemistry and roles in embryogenesis, tissue repair, remodeling, and carcinogenesis. Recent Prog Horm Res (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05487-03 LCC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Carcinogenesis and Mutagenesis by Fecapentaenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Sect.	LCC	NCI
Others:	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
	A. J. Streeter	Visiting Associate	LCC	NCI
	P. J. Donovan	Chemist	LCC	NCI
	J. M. Rice	Chief	LCC	NCI
	D. E. Devor	Biologist	LCC	NCI

COOPERATING UNITS (if any)
Stanford Research Institute, Palo Alto, CA (W. Bradford); Program Resources, Inc., Frederick, MD (L. Ohannesian, J. Henneman, W. Andrews)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.2	0.8

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

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

Fecapentaenes are direct-acting human fecal mutagens and are therefore potential candidates as human colon carcinogens. Several in vitro studies by other investigators have demonstrated potent mutagenic effects of fecapentaene-12 (FP-12) in bacterial cells, low cell transforming activity in vitro and mammalian mutagenic activity. The chemical purity and stability of FP-12 was determined to develop effective handling procedures during rodent exposure. Maintenance under argon in DMSO, and with added Vitamin E, proved useful. The diacetate of FP-12 was also synthesized. Rodent carcinogenesis experiments were carried out to determine potential carcinogenic activity. Skin painting studies in SENCAR mice showed lack of tumor-initiating activity, complete carcinogenesis or tumor-promoting activity in three separate experiments, some of which were repeated to conclusively demonstrate the negative findings. Intrarectal and subcutaneous administration to mice and rats have not provided convincing evidence of the carcinogenesis of FP-12. Total doses of up to 16 mg of FP-12 were used. The limitation of these in vivo assays involved the potentially low total doses utilized. FP-12 was mutagenic in vivo in rats by the subcutaneous granuloma pouch assay, but no tumors developed. Our studies provide no evidence for the carcinogenicity of FP-12, although a weak carcinogenic effect has not been eliminated.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
A. J. Streeter	Visiting Associate	LCC	NCI
P. J. Donovan	Chemist	LCC	NCI
J. M. Rice	Chief	LCC	NCI
D. E. Devor	Biologist	LCC	NCI

Objectives:

To establish the carcinogenicity of, and study the mechanisms of carcinogenesis by, fecapentaene-12 in rats and mice.

Major Findings:

Minimal decomposition was observed when special handling procedures were employed, including use of a controlled atmosphere (argon) to ensure minimal contact of the solutions with oxygen, especially important during manipulation of concentrated stock solutions. These procedures were developed after examining data from stability studies showing that decomposition was extremely rapid (half-life as short as 20 min.) when 6 mM fecapentaene-12 in 9:1 ethanol:dimethylsulfoxide was exposed to air. The fact that the half-life increased dramatically when this solution was diluted fivefold suggested that the decomposition was primarily a radical process initiated by autoxidation. This suggested, in turn, that fecapentaene solutions might be stabilized by addition of appropriate radical scavengers. Promising results in this direction have been obtained with α-tocopherol. It should be noted that the fecapentaene-12 synthesized for these studies is actually a mixture giving at least four distinct peaks by high performance liquid chromatography (HPLC). It has been suggested that the several components are stereoisomers, presumably differing little in biological activity. Efforts are currently underway to separate these by preparative HPLC so that their spectral, chromatographic, and especially biological (e.g., mutagenic) properties can be individually examined. Hopefully, the structures of all components can be assigned from these data in the near future.

Skin initiating activity could not be demonstrated after repeated 12-O-tetradecanoly-phorbol-13-acetate (TPA) exposure. SENCAR mice received an initiating dose of 0.56 mg FP-12 in ethanol, 3 mg FP-12 in dimethylsulfoxide (DMSO), or 1 mg (diacetate of FP-12) twice weekly for 5 weeks (total 10 mg). Total doses of 5.6 or 10 mg in ten weekly or biweekly exposures also did not induce tumors within 30 weeks. In contrast, 50 μg of 7,12-dimethylbenz[a]anthracene (DMBA) was highly effective as an initiator. Intrarectal administrations to mice and rats were carried out with FP-12 in ethanol or DMSO. Seven to ten weekly exposures to total doses of 0.49-1.8 mg in ethanol and 16 mg in DMSO were not significantly associated with colon or other tumors even up to 18 months. FP-12 given intrarectally (IR) to B6C3F1 mice (total dose 0.81 mg) was associated with one small intra-epithelial colonic carcinoma, and an additional three mice had foci of atypical colonic hyperplasia when killed at 51 weeks. Two of 25 rats

given a low total dose (1.8 mg) of FP-12 intrarectally developed colon polypoid adenomas. In another later experiment, however, in which FP-12 dissolved in DMSO, for a total dose of 16 mg, no colon tumors were seen after 18 months.

Mutagenicity studies in Salmonella indicator strains confirm that this compound is a potent bacterial mutagen. When tested with TA 100, it yielded a value of 5300 revertants/microgram or 1325 revertants/nanomole; when compared with some of the compounds in Ames' original survey of published mutation data, it can be seen to be among the most mutagenic of compounds. For example, the supermutagen, N-nitrosomethylnitroguanidine (MNNG), yielded 1375 revertants/nanomole. In contrast FP-12, transplacentally administered to Syrian hamster fetuses, was found to be marginally mutagenic (1-3 X control). The granuloma pouch assay is a direct mutagenicity test which measures the extent of mutation induced in the fibroblasts of induced granulomatous tissue. FP-12 administered in the granuloma pouch in male F344 rats at a dose of 0.25 mg or 1 micromole/pouch was weakly mutagenic causing a 31-fold increase over the spontaneous mutation rate for 6-thioguanine resistance, but no sarcomas developed in the pouch. Sarcomas did develop when comparably mutagenic doses of MNNG were given to positive control rats.

In summary, there is no convincing evidence from rodent experiments at this time for the postulated carcinogenic activity of the human fecal mutagen, FP-12. Negative findings may have occurred because of the low doses utilized and to a potentially weak carcinogenic activity of FP-12 (in relation to other direct-acting mutagens, such as nitrosoureas, and to DMBA) or to insufficient periods of observation for tumor appearance. Studies are still in progress, specifically toward reconciling the discrepancy between bacterial mutagenesis by FP-12 and its lower apparent potency in mammalian systems. We will attempt to demonstrate DNA damage in tissues (skin and colon) for which we failed to demonstrate carcinogenic, tumor-initiating or tumor-promoting activity.

Publications:

Ward JM, Anjo T, Ohannesian L, Keefer LK, Devor DE, Donovan PF, Smith GT, Henneman JR, Streeter AJ, Konishi N, Rehm S, Reist EJ, Bradford WW, Rice JM. Inactivity of fecapentaene-12 as a rodent carcinogen or tumor initiator and guidelines for its use in biological studies. Cancer Let (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05488-03 LCC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mechanisms of Inorganic Carcinogenesis: Cadmium

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. P. Waalkes	Pharmacologist	LCC	NCI
Others:	M. S. Miller	Senior Staff Fellow	LCC	NCI
	A. O. Perantoni	Staff Fellow	LCC	NCI
	K. S. Kasprzak	Visiting Scientist	LCC	NCI
	T. Koizumi	Visiting Fellow	LCC	NCI
	S. Rehm	Visiting Scientist	LCC	NCI
	S. Rhodes	Intramural Research Training Associate	LCC	NCI
	Z. Wahba	Intramural Research Training Associate	LCC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (C. Riggs, H. Issaq); Food and Drug Administration, Rockville, MD (P. Goering); School of Hygiene and Public Health, Johns Hopkins University, Baltimore, MD (L. Ewing)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Inorganic Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The mechanisms of cadmium carcinogenesis are under active investigation. Repeated subcutaneous injections of cadmium resulted in a rapid onset and high incidence of highly malignant tumors at the injection site and had an apparent association with malignant testicular tumors. The Syrian hamster was shown to be susceptible to cadmium-induced testicular carcinogenesis, while mice strains resistant to the acute effects of cadmium in the testes proved resistant also to its carcinogenic effects. Chronic oral exposure to cadmium in rats was associated with prostatic tumors, providing further evidence for a link between cadmium and prostatic carcinogenesis. Zinc was shown to inhibit cadmium carcinogenesis in a route- and site-dependent manner. Rare testicular tumors were also observed in rats in association with cadmium treatment including sertoli cell tumors, rete testis adenocarcinoma and seminomas, indicating a general susceptibility within the cells of the testes. A marked enhancement of cadmium cellular efflux, a reduction in levels of nuclear cadmium and a reduction in DNA cadmium content were observed in cells isolated from the testes of rats made resistant to cadmium carcinogenesis by zinc treatment. The DNA hypomethylating agent, deoxyazacytidine, caused both a marked increase in the synthesis of metallothionein, the protein most frequently associated with cadmium tolerance, and cellular tolerance to cadmium. Analysis of restriction enzyme digests of deoxyazacytidine-treated DNA indicated that this tolerance coincided with a hypomethylation of the MT gene, a condition clearly associated with enhanced expressibility. It was shown that, like the rat, mouse and monkey testes, the hamster ovaries are deficient in metallothionein. Hamster ovaries undergo an acute phase response to cadmium similar to that of the testes. An absence of metallothionein in the rat prostate was also established. Thus, a consistent absence of metallothionein in targets of cadmium toxicity and carcinogenesis is observed and the inability to express the metallotheionein gene is probably a very important factor in susceptibility of a given tissue to cadmium carcinogenesis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. P. Waalkes	Pharmacologist	LCC	NCI
M. S. Miller	Senior Staff Fellow	LCC	NCI
A. O. Perantoni	Staff Fellow	LCC	NCI
K. S. Kasprzak	Visiting Scientist	LCC	NCI
T. Koizumi	Visiting Fellow	LCC	NCI
S. Rehm	Visiting Scientist	LCC	NCI
S. Rhodes	Intramural Research Training Associate	LCC	NCI
Z. Wahba	Intramural Research Training Associate	LCC	NCI

Objectives:

To study the mechanisms of carcinogenesis by cadmium by investigating target site specificity, tissue susceptibility determinants, and the genetic basis for susceptibility to cadmium toxicity.

Major Findings:

The mechanisms of cadmium carcinogenesis are under active investigation at the organismal, cellular, and molecular levels. On the organismal level repeated subcutaneous injections of cadmium were found to result in a rapid onset of a high incidence of highly malignant injection site tumors, as reflected by distant metastasis and local invasiveness. Such repeated exposures also appeared to be associated with malignant testicular tumors. The number of species shown to be susceptible to cadmium-induced testicular carcinogenesis was extended to include the Syrian hamster, as cadmium proved highly effective (>90 incidence) in inducing interstitial cell tumors in these animals. Also in chronic studies, mice strains resistant to the acute effects of cadmium on the testes (hemorrhagic necrosis, tubular degeneration) proved resistant to its carcinogenic effects as well, indicating a genetic basis for the resistance. Chronic oral exposure to cadmium in rats was associated with an increased incidence of prostatic tumors, providing further evidence for a link between cadmium and prostatic carcinogenesis. Analysis of the inhibitory effects of zinc on cadmium carcinogenesis in rats indicated zinc works in both a route-dependent and site-dependent fashion, preventing testicular interstitial cell tumors and reducing injection site tumors. Prostatic tumors were observed in animals in which the testicular effects of cadmium were prevented by zinc, thus allowing appropriate androgen support to the sex accessory tissue. Unusual or rare testicular tumors were observed in the rat in association with cadmium treatment including sertoli cell tumors, rete testicular adenocarcinoma and seminomas, indicating a susceptibility to cadmium of various cell types within the testes.

On the cellular level a series of studies investigated the interactions between cadmium and zinc in isolated interstitial cells. In vivo zinc treatments, at doses able to prevent the testicular toxicity of cadmium, prior to isolation of cells, indicated several potential mechanisms for the inhibition of cadmium carcinogenesis by zinc in these cells. These included a marked enhancement of cadmium efflux, a reduction in levels of nuclear cadmium and a reduction in cadmium content of all subnuclear fractions including chromatin and DNA iteslf. Further investigations into the mechanisms of genetic resistance to cadmium

revealed that the DNA hypomethylating agent deoxyazacytidine caused a marked increase in the synthesis of metallothionein in response to cadmium exposure in cultured cells accompanied by tolerance to cadmium toxicity. Metallothionein, a high affinity metal binding protein, is believed to be the primary biological detoxification system for cadmium. Analysis of restriction enzyme digests of DNA from deoxyazacytidine-treated rats indicated that such treatment resulted in a hypomethylation of the MT gene, a condition frequently associated with enhanced expressibility. These results provide further evidence indicating the metallothionein gene is a key determinant in the susceptibility or resistance to cadmium carcinogenesis.

On the molecular level, it was shown that, like the rat, mouse and monkey testes, the hamster ovaries are deficient in metallothionein. The hamster ovary was previously shown to undergo an acute phase response to cadmium analogous to that seen with the testes, and tumorigenic response is currently being tested. An apparent absence of metallothionein was also seen in the rat prostate, a newly established target site of cadmium carcinogenesis. Thus it appears that there is a consistent absence of this defensive protein in important targets of cadmium toxicity and carcinogenesis. Hence, an inability to express the metallothionein gene is probably a key tissue susceptibility factor in cadmium carcinogenesis.

Publications:

Bhave MR, Wilson MJ, Waalkes MP. Methylation status of the metallothionein-I gene in liver and testes of mice: comparison of strains resistant and susceptible to cadmium. Toxicology (In Press).

Boorman G, Eustis S, Waalkes MP, Rehm S. Seminomas in rats. In: Jones TC, ed. Pathology of laboratory animals vol. 5; genital system. New York: Springer-Verlag, 1988;192-5.

Klaassen CD, Waalkes MP. Cadmium-binding proteins in rat testes: characterization and apparent source of the low-molecular weight-protein. Experientia 1987;52(suppl):273-80.

Rehm S, Waalkes MP. Mixed sertoli-Leydig cell tumor and rete testis adenocarcinoma in rats treated with $CdCl_2$. Vet Path 1988;25:163-6.

Rehm S, Waalkes MP. Species and estrous cycle dependent ovarian toxicity induced by cadmium chloride in hamsters, rats, and mice. Fund Appl Toxicol (In Press).

Rehm S, Waalkes MP, Ward JM. Aspergillus rhinitis in Wistar (Crl:(WI)BR) rats. Lab Anim Sci (In Press).

Waalkes MP, Goering PL. The role of metal-binding proteins in metal toxicology. ISI Atlas of Science (In Press).

Waalkes MP, Miller MS, Wilson MJ, Bare RM, McDowell AE. Increased metallothionein gene expression in 5-aza-2'-deoxycytidine-induced resistance to cadmium cytotoxicity. Chem Biol Interact (In Press).

Waalkes MP, Perantoni AO. In vitro assessment of target cell specificity in cadmium carcinogenesis: interactions of cadmium and zinc with isolated interstitial cells of the rat testes. In Vitro Cell Develop Biol (In Press).

Waalkes MP, Perantoni AO, Bhave MR, Rehm S. Strain dependence in mice of resistance and susceptibility to the testicular effects of cadmium: assessment of the role of testicular cadmium-binding proteins. Toxicol Appl Pharmacol 1988;93:47-61.

Waalkes MP, Perantoni A, Palmer AE. Isolation and partial characterization of the low-molecular-weight zinc-, cadmium-binding protein from the testes of the patas monkey (Erythrocebus patas): distinction from metallothionein. Biochem J (In Press).

Waalkes MP, Rehm S, Perantoni A. Deficiency of metallothionein in the ovaries of Syrian hamsters: correlation with sensitivity to cadmium. Biol Reprod (In Press).

Waalkes MP, Rehm S, Riggs C, Bare RM, Devor DE, Poirier LA, Wenk ML, Henneman JR, Balaschak MS. Cadmium carcinogenesis in the male Wistar [Crl:(WI)BR] rat: dose-response analysis of tumor induction in the prostate, the testes and at the injection site. Cancer Res (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05524-02 LCC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Effects of Chemical Carcinogens on Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. S. Miller	Senior Staff Fellow	LCC	NCI
Others:	J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
	L. M. Anderson	Expert	LCC	NCI
	R. A. Lubet	Expert	LCC	NCI
	D. P. Chauhan	Visiting Fellow	LCC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.00	1.25	0.75

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

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

Previous studies have shown that treatment of a rat hepatoma cell line with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) caused concurrent decreases of the steroid-inducible levels of both tyrosine aminotransferase (TAT) enzyme activity and TAT-specific RNA. These studies have been extended and it has now been shown that MNNG treatment had no effect on the RNA levels of the constitutive housekeeping gene α-tubulin nor on the steroid- and metal-inducible metallothionein (MT) gene. Although attempts were made to measure the levels of the glucocorticoid receptor by both biochemical and molecular methods, receptor levels were too low (< 100 fmol/mg protein) to quantitate accurately. However, the lack of effect of MNNG on MT RNA levels suggests that the inhibitory effect of the carcinogen is not mediated through alterations in receptor level or affinity. The steroid hormone system has also been employed to examine the effects of an oncogene on expression of a cytoskeletal protein. A polyoma virus middle T gene has been recombined with a steroid-inducible promoter, rendering the oncogene sensitive to induction by steroids. It has been shown in two separately transfected clones that increases in the levels of the middle-T gene cause increases in α-tubulin gene expression. Also being studied is the role of drug metabolism in determining susceptibility to chemical carcinogens. The induction kinetics of cytochromes P1- and P3-450 have been determined in fetal mice after exposure to various inducers. The P-450s exhibited both tissue- and inducer-dependent specificity. It was also demonstrated that P3-450 is induced during the fetal period. Finally, a long-term carcinogenicity study has been initiated to determine if alterations in expression of the genes regulating drug metabolic enzymes affect the toxicological and carcinogenic consequences of diethylstilbestrol (DES) administration. The biochemical studies, as well as the chemical treatment of all the animals for the 2-year carcinogenicity study, have been completed.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. S. Miller	Senior Staff Fellow	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
R. A. Lubet	Expert	LCC	NCI
D. P. Chauhan	Visiting Fellow	LCC	NCI

Objectives:

To examine the mechanisms(s) by which chemical carcinogens alter the level of expression of various gene products; examination of the role of genetically regulated drug metabolism in determining susceptibility to carcinogenic chemicals.

Major Findings:

Effects of carcinogens on steroid-inducible gene expression: We have been using steroid-inducible genes as a model system in which to study the mechanism(s) by which chemical carcinogens alter gene expression. Steroids are important regulatory hormones which act as enhancer elements to increase the transcription rate of genes located downstream from the hormone-receptor binding site(s) on DNA and are probably the best understood eukaryotic gene regulatory system, making this an ideal system in which to study the mechanism(s) by which carcinogens can alter the levels of expression of various genes. Previous studies have shown that treatment of the Fao clone of Reuber H35 hepatoma cells with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) caused concurrent decreases in the levels of steroid-inducible tyrosine aminotransferase (TAT) enzyme activity and total TAT RNA, suggesting that MNNG inhibited the accumulation of TAT RNA by acting at a pre-translational step. Further studies have demonstrated that MNNG treatment had no effect on total RNA levels of the constitutive housekeeping gene, α-tubulin, nor did it alter the basal, DEX-inducible, or Cd-inducible total RNA levels of the metallothionein (MT) gene. Although attempts were made to measure the levels of the glucocorticoid receptor by both biochemical (steroid binding assays) and molecular (Northern blotting) methods, the levels of receptor were too low (<100 fmol/mg) to quantitate accurately. The lack of effect of MNNG on DEX-inducible MT RNA levels suggests that the inhibitory effect of the carcinogen are not mediated by alterations in the glucocorticoid receptor. Current studies in progress are examining the effects of MNNG on chromatin structure.

Polyoma virus middle T (mT) gene enhances expression of the α-tubulin gene: Two cell lines, mT-1 and gen-1, have been obtained which were derived from F111 rat fibroblasts transfected with recombinant DNA molecules, placing the mT gene of polyoma virus downstream of a steroid-inducible promoter. This construct renders the heterologous mT gene sensitive to induction by glucocorticoid hormones. These cell lines were employed in gene dosage experiments to determine the effects of the ras-like mT gene product on cytoskeletal proteins. Other investigators have demonstrated in these cells that the mT gene causes changes in cell morphology and growth characteristics. We have found that the mT-1 and gen-1 cell lines contain higher levels of α-tubulin RNA compared to the untransfected parent F111 fibroblasts, which may partially explain the alterations in cell morphology that occur following transfection. Treatment of these cell lines with DEX, which

increases the amount of mT protein produced, results in a further increase in α-tubulin RNA and more pronounced alterations in cell morphology and growth characteristics. Studies currently in progress are examining the kinetics of α-tubulin RNA induction and will also determine whether tubulin protein levels are increased as well.

Role of drug metabolism in determining susceptibility to chemical carcinogens: Previous studies conducted in the PCS by Dr. Anderson (see project Z01CP05352-06) have shown that the incidences of liver and lung tumors in mice exposed transplacentally to 3-methylcholanthrene (MC) were significantly influenced by the sensitivity of both mothers and fetuses to induction of cytochrome(s) P-450, as measured by the aryl hydrocarbon hydroxylase (AHH) assay. In order to further delineate the biochemical and molecular processes underlying the observed biological effects, we determined the inductive effect of MC and β-naphthoflavone (βNF) on cytochromes P-450 in F_1 fetuses of responsive mothers. We found that MC causes maximal induction of AHH activity by 8 hr in both the liver and lung. βNF causes nearly maximal induction of AHH activity by 8 hr in the lung but has little effect on liver AHH activity at this time. Maximal induction with βNF occurs by 24 hr in both organs. Addition of monoclonal antibody (MAb) 1-7-1, specific for the MC-inducible forms of cytochrome P-450 (P_1- and P_3-450), resulted in a 55 to 70% inhibition of AHH activity in both lung and liver asssays regardless of the inducing agent used, while having no effect on AHH activity from oil-treated mice. The enzyme data correlate very well with RNA blot analysis. MC causes maximal induction of P_1-450 RNA levels 4 hr after injection in both organs, whereas maximal levels of P_1-450 RNA are seen at 16 hr in fetal lung and liver following injection of βNF. However, the ratio of P_1-450 RNAs present at 2 vs 16 hr in βNF-treated lung appears greater than that in the liver. We have also shown that P_3-450 is coordinately induced with P_1-450 in the fetal liver. The results indicate that the increase in functional AHH activity is primarily due to induction of cytochrome P_1-450. The differences in induction kinetics observed for cytochromes P_1- and P_3-450 suggest that these enzymes exhibit both tissue-and inducer-dependent specificity, which may partially explain the differential effects of MC on tumor formation in target organs.

We have also initiated studies to determine if alterations in drug metabolic enzymes affect the toxicological and carcinogenic consequences of DES administration to neonatal rats. We have completed the first phase of a standard maximally tolerated dose protocol and have selected the doses of DES to be used in a long-term 2-year carcinogenicity study. We have also finished the biochemical analysis of the effects of the inducing agents on metabolic capacity. The appropriate dose combinations of inducing agent and DES have been selected, and all animals for the 2-year carcinogenicity study have been treated. A final expanded 5-week toxicology study is also currently in progress, and molecular studies of the underlying gene-regulatory phenomena are planned.

Publications:

Miller MS, Kono M, Wogan GN. Inhibitory effects of N-methyl-N'-nitro-N-nitrosoguanidine and benzo[a]pyrene-7,8-diol-9,10-epoxide on glucocorticoid-inducible polyoma virus middle-T gene expression in rat mT-1 cells. Carcinogenesis 1987;8:1159-63.

ANNUAL REPORT OF

THE LABORATORY OF EXPERIMENTAL CARCINOGENESIS
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY

October 1, 1987 through September 30, 1988

The major mission of the Laboratory of Experimental Carcinogenesis (LEC) is to conduct innovative and productive research aimed at elucidating mechanism(s) of malignant transformation in human and animal cells by chemical carcinogens and other cancer causing agents. From its inception, this laboratory has sought to accomplish this goal by an integrated multidisciplinary approach to cancer research. The present structure of LEC combines expertise in the diverse disciplines of cell biology, chemical and viral carcinogenesis, molecular biology, protein and nucleotide chemistry, and computer science. A fundamental aspect of our multidisciplinary approach to cancer research stems from the strong belief that the neoplastic process must be studied at all levels of biological complexity ranging from the intact organism to defined in vitro systems in order to identify and characterize the critical cellular and genetic factors in cancer development. The selection of the rat liver model as the major experimental system to study the mechanism of chemical carcinogenesis is a reflection of this research approach.

The integrated efforts of the LEC scientists have resulted in a number of unique accomplishments during the last year. These accomplishments in the major areas of investigation within LEC are summarized in the following sections.

Application of Two-Dimensional Gel Electrophoresis in Studies on Neoplastic Development

(1) Advances in the Computerized Analysis of Two-Dimensional Gels. The computer system, developed in this laboratory, for analyzing two dimensional gels has been dubbed ELSIE 4. It has been distributed to several other laboratories in the United States and Europe. In the past year our efforts have focused on the development of programs and techniques to aid in the analysis of experiments utilizing quantitative two-dimensional gel electrophoresis. Several projects in this laboratory make use of ELSIE 4 for the analysis of experiments.

Because of the number of laboratories that use ELSIE 4, and because we plan to upgrade our facility in the next year to use more powerful computers, we have spent some effort to ensure that the system will run, with minimal modification, on a wide variety of computer systems--that is, to make ELSIE 4 highly portable. The software can be run on any mid-sized computer system running under the UNIX (TM--AT&T Bell Laboratories) operating system. We have brought the system up, in whole or in part, on computer systems made by SUN Microsystems, Digital Equipment Corporation, Apollo Computers, Masscomp and Unisys. We have also put most of the system up on a personal computer, the IBM PS/2 model 60, running under MS/DOS (Microsoft Corp.). The major difficulty in porting ELSIE 4 is that its sophisticated graphics system is difficult to bring up on the different graphics hardware available on various computers. However, recent advances in developing a graphics standard for computers promises to allow us to create a completely portable system in the future.

Efforts are continuing to develop techniques and tools that will allow us to automatically analyze experiments that use multiple two-dimensional gels. Collaborators in Geneva have developed new techniques aimed at classifying sets of gels and identifying what spots are most characteristic of a particular sample. These techniques, heuristic clustering, and correspondence analysis, are under examination in this laboratory.

(2) Analysis of Polypeptides in Transformed and Untransformed Rat Liver Epithelial (RLE) Cells. The RLE cell line was derived from neonatal rat liver. These cells appear to be very much like normal rat liver cells. They have a normal chromosome count (2N=42), and, except for a duplication in the q arm of chromosome #1, the chromosomes appear to be karyotypically normal. These cells have been single-cell cloned and a number of samples have been stored away at low passage. These cells represent a homogeneous, clonal single-cell system and thus provide what we believe is the simplest, cleanest, and most straightforward model for the study of transformation.

Different retroviruses containing transforming oncogenes, such as v-*ras*, v-*raf*, and v-*myc* have been used to infect and transform these cells. Cells transformed by v-*ras* have been single-cell cloned and characterized. These cells grow at high efficiency in soft agar and are very tumorigenic in nude mice. Although more elongated than normal RLE cells, two different *ras*-transformed morphologies, one more flattened than the other, have been isolated. Karyotype analysis reveals an inversion in the q arm of chromosome #5 in all *ras*-transformed cells. One of the cell lines is also trisomic in chromosome #12. Normal RLE cells are inhibited by epidermal growth factor (EGF) at concentrations above 1 ng/ml, while the transformed cells are unaffected by EGF at concentrations up to 100 ng/ml. Two-dimensional gel electrophoresis show that there are a large number of quantitative, but few qualitative, differences between the normal and transformed RLE cells. Differences of tenfold, or more, are common. We plan to compare these patterns with those generated by the other transforming oncogenes in hopes of identifying polypeptides whose expression is altered in a similar manner by the different modes of transformation. If such proteins are detected, it may be possible to characterize and identify them by micro-sequencing directly off the two-dimensional gels using peptide sequencing technology available in our laboratory.

(3) Cellular Proteins in Oncogene-Transformed Rat Liver Epithelial Cells. In order to identify the cellular proteins that are associated with neoplastic development, we have used an in vitro transforming system consisting of helper-dependent retroviruses containing either v-*raf* or v-*raf*/v-*myc* as the transforming agents and a RLE cell line as the reporter cell. Both v-*raf* (3611) and v-*raf*/v-*myc* (J2) viruses were efficient in vitro transforming agents of the RLE cells and the infected cells formed tumors upon transplantation in nude mice. Neither the uninfected nor helper virus-infected RLE cells formed tumors in nude mice. Cellular protein patterns in normal RLE cells, helper-infected (RLE-H), 3611 (RLE-3611T) and J2 (RLE-J2)-transformed RLE cells have been analyzed by 2D-PAGE. Comparison of the autoradiograms from the RLE-H, RLE-3611T and RLE-J2, showed proteins which are either suppressed or induced and were therefore selected for closer study and purification. Polypeptides 1 - 4 (Mr/pI) (1. 145/5.9-7.2; 2. 130/6.5-7.5; 3. 100/4.0; 4. 30/6.0) were found be present in negligible amounts

in the tumorigenic cells from both autoradiograms and silver stained 2-dimensional polyacrylamide gels and polypeptides 5 - 9 (Mr/pI) (5. 27/5.0; 6. 26/4.9; 7. 25/4.9; 8. 25/5.2; 9. 27/6.6) were found to be present in negligible amounts in the non-tumorigenic RLE-H cells. Protein 1 appears to be associated with both soluble and membrane fractions; polypeptides 2, 3, 4, 8 and 9 appear to be associated with membrane and polypeptides 5, 6 and 7 appear in the cytosolic fraction.

(4) Polypeptide Modulation in Michigan Cancer Foundation (MCF)-7 Cells by Estrogen and Growth Factors. The purpose of this project was to utilize the established human mammary tumor cell lines (MCF-7, MCF-7gpt [produced by transfection with the Eco-gpt selectable gene marker], MCF-7*ras* [produced by transfection with Eco-gpt and the v-H*ras* oncogene] and LY2) to investigate the effect of antiestrogens, estrogen and other growth factors on the polypeptide expression of both cellular and secreted proteins in these cells. The growth factors IGF-I (insulin like growth factor-I) and TGF-alpha (transforming growth factor alpha) are able to elicit many of the growth stimulating responses of estrogen when applied to the MCF-7 human mammary tumor cell line. Analysis of the 2-dimensional polyacrylamide gels from cellular proteins of the MCF-7gpt and MCF-7*ras* cells that have been treated with 17-beta-estradiol or an ethanol vehicle showed 12 polypeptides, which were consistently altered. Of these, four polypeptides were clearly affected the same way by *ras* transfection and estrogen stimulation. Sixteen secreted polypeptides were altered by at least twofold subsequent to either estrogen or v-H*ras*. Opposing effects of estrogen and v-H*ras* were observed for a number of polypeptides, but synergistic effects of v-H*ras* and estrogen were seen for three polypeptides detected by autoradiography. Studies to ascertain the effects of the growth factors IGF-I and TGF-alpha on the expression and secretion of these polypeptides are in the final stages of analysis. Additional to this are experiments using the anti-estrogen LY117018 to assess the effect on polypeptide expression and secretion from the antiestrogen-resistant cell line LY2 and the MCF-7 cells.

(5) Cellular Polypeptides Associated with Metastasis of Rat Mammary Tumor Cells. This project was initiated to identify those polypeptides that specifically relate to the metastatic process as the first step toward their purification and identification. Utilization of metastasizing and non-metastasizing cells derived from the same parent population of tumor cells is fundamental to this project. We have confirmed that the TMT-081-MS cells do metastasize in syngeneic rats and that the TMT-081-NM cells do not metastasize in syngeneic rats. Radiolabelling of these cells with 14-C amino acids has revealed several qualitative and quantitative differences in their polypeptide patterns. The most intensely labelled spots that occurred in the TMT-081-MS cells were (MW/pI) 67/5.5 and 50/4.5 and the most intense spots that occurred only in the TMT-081-NM cells were 46/6.7 and 38/6.1. When a 32-P-radiolabel was used with these cells, a number of polypeptides could be observed that were not located from the 14-C labelling. The most intensely 32-P-labelled spots occurring only in the metastatic cells were 98/4.7 and 24/4.5. Those polypeptides that were approximately threefold greater in intensity in the metastatic cells compared to the non-metastatic cells were 14/5.2, 15/5.4 and 12/6.3. The polypeptides of 40/5.9 and 40/5.8 were at least threefold greater in the intensity of 32-P label compared to the corresponding polypeptides in the metastatic cells. A number of subclones of the metastasizing TMT-081-MS cells have been derived from metastasis in Wistar/Furth

rats. These subclones possess metastatic capacity and will be used in further experiments.

(6) Isolation & Characterization of Proteins from Two-Dimensional Polyacrylamide Gels. The purpose of this project is to develop the analytical technology required for the elution and subsequent microsequencing of proteins from two-dimensional polyacrylamide gels. A number of "interesting" protein spots have been defined whose regulation is markedly altered during the multistep process of neoplastic transformation. Initially, microscale procedures aimed at the recovery and sequence analysis of these proteins from one-dimensional SDS-PAGE were investigated. Electroelution and passive extraction techniques were found to be suitable only when large amounts (> 500 pmoles) of protein were available. Electroblotting techniques in which the proteins are transferred to quaternary ammonium-derivatized glass fiberpaper were modified such that 50 pmoles of soybean trypsin inhibitor applied to a 1D-SDS-polyacrylamide gel could subsequently be correctly sequenced to 17 cycles. Other supports for protein electroblotting and subsequent microsequence analysis were investigated and Immobilon-P was found to be the support of choice. Using this support electrophoretic conditions were optimized to allow the N-terminal sequence analysis of up to 20 residues from 50-100 pmoles of protein applied to a 2D-gel. With 1D-PAGE it was possible to obtain some sequence information from as little as 5 pmoles protein. This procedure was successfully applied to the sequence analysis of a number of previously uncharacterized membrane glycoproteins. Work is currently underway to apply this technique to the analysis of other unknown proteins whose expression is altered during carcinogenesis and also to improve the sequencing yields of proteins isolated from two-dimensional polyacrylamide gels.

(7) Plasma Membrane Proteins in Normal and Neoplastic Rat Hepatocytes. Our previous investigations of glycoproteins isolated from the plasma membranes of normal and neoplastic rat livers revealed many qualitative and quantitative differences when analyzed by 2D-PAGE. The main goal of this project is to purify and characterize the specific glycoproteins whose expression is markedly altered during chemically induced hepatocarcinogenesis in order to understand their role either as markers or causal agents during cell transformation. Results obtained are as follows: The previous isolation procedure was modified to more efficiently purify the glycoproteins of interest. The new method utilizes modified conditions for the Concanavalin-A (Con A) affinity chromatography and fast protein liquid chromatography (FPLC) using a Superose-12 column. This procedure provides a fraction that is enriched in the various glycoproteins of interest and enabled the final purification to be achieved by a single 2D-PAGE experiment. These separated components were transferred to an Immobilon-P membrane by electroblotting. It was necessary to optimize the conditions of the 2D-PAGE and electroblotting for these specific glycoproteins. In addition, the best procedure for amino acid sequencing (gas phase) from these membranes was established. Using these techniques, it was possible to determine the N-terminal amino acid sequence for 4 of the 9 glycoproteins analyzed. The remaining 5 components of interest were not sequencable in this manner, presumably because of blocked N-termini. A peptide, comprised of the first 13 N-terminal residues of rat liver membrane protein (RLMP-1), was synthesized, purified, and used to produce rabbit antibodies for use in the larger scale purification of the whole protein and in various biological studies. The glycoproteins from normal and transformed RLE cells are currently being characterized by the 2D-PAGE system.

Studies on Molecular Aspects of Chemical Hepatocarcinogenesis

(1) Cellular Evolution of Chemically Induced Rat Hepatomas. Recently studies on the fate and the possible role of oval cells in both normal liver biology and hepatocarcinogenesis have been conducted. After the administration of 2-acetylaminofluorene (AAF) for two weeks combined with partial hepatectomy (PH), only oval cells are able to proliferate and incorporate radiolabeled thymidine at day 7 after PH. However, at the later time points (9 to 13 days after PH) the label was present in the newly formed basophilic hepatocytes, which is an indication that oval cells are precursor cells for hepatocytes. At a high dose level of AAF, oval cell differentiation to hepatocytes was delayed and metaplastic differentiation to intestinal and biliary epithelial cells was frequently observed. No reutilization of labeled thymidine from dying cells by the regenerating hepatocytes was observed. Lack of glucose-6-phosphatase activity and presence of alpha-fetoprotein (AFP) gene message in oval cells and early basophilic hepatocytes is a further indication that oval cells are precursor cells for hepatocytes. Differentiation of oval cells to hepatocytes after AAF administration follows a pattern similar to that observed in the embryogenic liver. After AAF administration TGF-beta was present in the mesenchymal cells of the liver and in the oval cell compartment. In hepatocellular carcinoma TGF-beta was present only in stromal cells. In the fibrotic liver produced by carbon tetrachloride administration, collagen genes and TGF-beta were expressed in the cell population lining the peripheral hepatocytes of the pseudolobules.

(2) Genetic Determinants in Chemical Hepatocarcinogenesis. We have continued to utilize experimental hepatocarcinogenesis in the rat as a model to study the mechanism of neoplastic development with particular emphasis on defining the possible role of a set of oncogenes that are commonly associated with the process of hepatocarcinogenesis in vivo. Previous results had consistently shown up-regulation of the expression of myc and raf oncogenes during chemical hepatocarcinogenesis. In order to examine the transforming potential of these and other oncogenes in the liver system, we have established an in vitro transformation system consisting of a retroviral vector containing relevant oncogenes and a RLE cell line as the reporter cell. The main findings include: (1) v-raf, H-v-ras and a combination of v-raf and v-myc are potent transforming agents in the RLE cells; (2) different tumor types were observed following transplantation of the infected cells. The most undifferentiated tumors originated from the v-raf-infected cells whereas transformation with v-raf/f-myc combination resulted in hepatocellular carcinoma; (3) transformation of RLE cells with v-raf and v-H-ras resulted in increased expression of the multidrug-resistant (MDR-1) gene and the development of multidrug resistance; and (4) we have established that TGF-beta-1 is capable of differentiating the RLE cells towards the adult hepatocyte phenotype. Furthermore, transformation of the RLE cells blocks the TGF-beta-induced differentiation of these cells.

(3) Phenotypic Alterations Induced by Ras Oncogene in Rat Liver Cells. Studies on primary human and rodent hepatocellular carcinomas have strongly suggested that a transforming ras gene may participate in the initiation and/or maintenance stages of liver neoplasia. However, the function and exact phenotypic alterations caused by expression of a ras oncogene in the liver has yet to be described. To define the function and phenotypic characteristics associated with a transforming ras gene in liver, we have utilized a retroviral shuttle vector system to deliver an inducible ras oncogene into normal liver epithelial cells (RLE cells). The Moloney murine sarcoma virus based vector is composed of a

neomycin resistance gene (NEO)(transcriptionally derived from the 5′ long terminal repeat (LTR) and transforming Ha-ras gene under the transcriptional control of the glucocorticoid-inducible LTR of the mouse mammary tumor virus (MMTV) (Cell 27: 245, 1981). Southern, Northern and Western blot analysis confirmed stable proviral integration, ras gene transcription with polyadenylation and translation into a 21 K dalton protein with a specific mutation in codon 12. Northern and S1 nuclease analysis confirmed that dexamethasone induced ras transcripts from the MMTV LTR by 15-fold. Phenotypic alterations specifically associated with ras expression in these normal liver epithelial cells include: (1) alterations in growth kinetics, (2) ability to grow in soft agar and produce tumors in nude mice, (3) increases in metabolic rate determined by 2-deoxyglucose uptake, and (4) becoming positive for gamma-glutamyltranspeptidase activity.

(4) Effect of Ethanol on ODC and Proto-oncogene Expression in Liver Regeneration. The effect of ethanol consumption on liver regeneration has been found to exert significant but inconsistent changes of several metabolic pathways. The present project examined the effect of chronic ethanol consumption on ornithine decarboxylase activity (ODC), polyamine biosynthesis and proto-oncogene expression in the rat liver after PH. Chronic exposure to ethanol has been reported to increase both the activity and half-life of ODC, yet significantly inhibit post-hepatectomy DNA synthesis and restitution of liver mass. After using an established model of chronic ethanol feeding, animals underwent 2/3 partial hepatectomy and were sacrificed at 30 min, 1,3,6,12,18 and 24 hours thereafter. ODC activity increased significantly in both ethanol and pair-fed animals at 3 to 6 hours post-PH. However, the ethanol-treated group exhibited a fivefold less increase in activity as compared to the pair-fed animals. This decrease was paralleled by a similar change in putrescine activity, a major polyamine product of ODC activity. By 24 hours, ODC activity was similar in both groups. Total ODC protein content remained invariant to partial hepatectomy and/or chronic ethanol ingestion. In contrast, measurement of ODC mRNA levels revealed a 10-fold increase at 12 and 24 hours but no significant difference between pair-fed and alcohol-treated animals. Examination of a number of proto-oncogenes including c-myc, c-fos, v-raf, H-ras, c-mos, and v-erb B revealed no significant differences in expression between the two groups. Similar results were noted when expression of MDR-1, albumin, alpha-fetoprotein, and glutathione S-transferase-P genes was examined. The mRNA levels of the ethanol-inducible cytochrome P-450 was slightly increased in the ethanol-treated animals. The results suggest that in the present model, chronic alcohol ingestion induces diminished ODC activity in the early time points post-PH without affecting ODC protein content or mRNA levels. Proto-oncogene expression appears to be invariant to chronic alcohol ingestion under the conditions described.

(5) Neoplastic Development in Transgenic Mice. The overall objective of this project is to employ the transgenic mouse system to study neoplastic development in vivo. This system provides a new way of investigating tissue-specific and developmental stage-specific regulation of both natural and manipulated gene sequences introduced into the germ line of an animal. We are currently focusing on the neoplastic development in the liver employing the v-raf oncogene as the transforming agent. The v-raf under the control of liver-specific promoters from

both albumin and alpha-fetoprotein genes has been microinjected into the 1-cell embryo, and we are in the process of establishing transgenic lines from the progeny.

Growth Control of Normal and Transformed Hepatocytes

(1) Purification and Characterization of a Rat Hepatic Proliferation Inhibitor. The aim of this project is to isolate and characterize a protein from adult rat liver that produces a reversible inhibition of the proliferation of liver-derived cells. An improved analytical-scale purification procedure was recently developed that produces a preparation with a specific activity about 1000-fold greater than previously reported. This procedure has been modified for the large-scale purification of the growth inhibitor from 12,000 livers. At the present time a preparation (4 mg total protein) with an ID50 of 1-5 ng/ml has been obtained. The preparation contains at least two separate growth inhibitory polypeptides, which appear to have similar activities. 2D-PAGE analysis demonstrates that the preparation contains at least 20-30 polypeptides and current efforts are focused on obtaining a pure preparation of the inhibitory activities such that amino acid sequence analysis can be performed. Biological and physicochemical characterization of the liver-derived growth inhibitor(s) has demonstrated that it is different from any known well-characterized growth inhibitory polypeptides including TGF-beta, tumor necrosis factor, interferon and mammary derived growth inhibitor.

(2) Characterization of a Rat Liver-Derived Growth Inhibitor. The principle objective of this project is to further characterize the protein isolated from rat liver that reversibly inhibits the proliferation of RLE cells at extremely low concentrations (ID50 = 0.2 ng/ml). The biological effects of this inhibitor protein (designated as LDGI) were compared with those of TGF-beta and recombinant tumor necrosis factor-alpha (rTNF-alpha) in a variety of normal and malignant cell culture systems. RLE cells were highly sensitive to the antiproliferative effects of LDGI and TGF-beta but were relatively insensitive to the cytostatic effects of rTNF-alpha. Aflatoxin B_1 (AFB_{-1})-transformed RLE cells were resistant to the antiproliferative effects of TGF-beta and rTNF-alpha but showed sensitivity to the growth inhibitory effects of LDGI. Clones isolated from AFB-1-transformed RLE cells exhibited either sensitivity similar to parental AFB-1-transformed RLE cells or complete resistance to growth inhibition by LDGI. However, none of these clones exhibited sensitivity to the antiproliferative effects of TGF-beta or rTNF-alpha. Rat hepatoma (UVM7777) cells were insensitive to the cytostatic effects of all the three growth modulators. Human breast carcinoma (MCF-7) and rat hepatoma (Reuber) cells were extremely sensitive to rTNF-alpha, exhibited low sensitivity to LDGI, but were resistant to the antiproliferative effects of TGF-beta. The rate of DNA synthesis in rat kidney fibroblasts (NRK) and human foreskin fibroblasts was stimulated two- to threefold in response to LDGI, TGF-beta and rTNF-alpha treatment. The growth rate of RLE cells transformed with a retrovirus containing both v-*myc* and v-*raf* (J2) was stimulated by both LDGI and TGF-beta.

Studies on Suppressor tRNAs

(1) Opal Suppressor Phosphoserine and the 21st Naturally Occurring Amino Acid. Two opal suppressor phosphoseryl-tRNAs and the corresponding gene have been isolated and characterized in this laboratory. They have several unique features

which set them apart from all other eukaryotic tRNAs: (1) they are 90 nucleotides in length and thus are the longest eukaryotic tRNAs sequenced to date; (2) they are phosphorylated on their serine moiety to form phosphoseryl-tRNA; (3) they have few modified bases compared to other tRNAs; (4) they are encoded by a single gene even though several pyrimidine transitions occur post-transcriptionally and one of the transitions occurs in the anticodon; and (5) the primary transcript arises, unlike any other known tRNA, without processing on the 5′ side of the gene product. The genes encoding the opal suppressor tRNAs which have been isolated and sequenced from human, rabbit, chicken, Xenopus and nematode genomes are transcribed in vivo in Xenopus oocytes and in vitro in HeLa cell extracts. Two regulatory sites occur upstream of the gene which are involved in its expression. The phosphoserine moiety of phosphoseryl-tRNA is incorporated into the active site of glutathione peroxidase where selenocysteine normally occurs in the protein. Experiments are in progress to determine if phosphoserine or selenocysteine is the 21st naturally occurring amino acid. The glutathione peroxidase gene maps to human chromosome 3, while other loci on chromosomes 21 and X are probably processed pseudogenes.

(2) Amino Acid at the Suppression Site in Rabbit beta-Globin Readthrough Protein. Rabbit beta-globin readthrough protein is the only known naturally occurring readthrough protein in higher eukaryotes which does not involve a viral system. Since suppressor tRNAs have been used in gene therapy experiments and have been implicated in inhibiting viral expression, it is important to characterize the nonsense suppressor tRNA involved in the expression of this unique protein. Experiments were devised, therefore, to isolate this protein from rabbit reticulocytes and to identify the amino acid at the readthrough site. Specific antibodies against this protein were prepared by synthesizing a 22 amino acid peptide which corresponds to the readthrough portion of the beta-globin readthrough protein, coupling the peptide to the KLH protein and injecting the conjugated protein into a sheep. Specific antibodies were produced which were purified and used to isolate the readthrough protein. The protein was purified to homogeneity by electrophoresis on a polyacrylamide gel and the purified product is presently being sequenced through the readthrough site.

(3) Aminoacyl-tRNAs in HIV and other Retroviral Infected Cells. Transfer RNA was isolated from HIV-1 (human immunodeficient virus-1), HIV-2 (human immunodeficient virus-2), HTLV-1 (human T-cell leukemia virus) and BLV (bovine leukemia virus)-infected and -uninfected cells and the elution profiles of aminoacyl-tRNAs from infected and uninfected cells compared by reverse phase chromatography. In each case examined, Asn-tRNA, which normally contains the highly modified Q base in the 5′ position of its anticodon, was deficient in this base in infected cells. In addition, Phe-tRNA from HIV-1 and HIV-2 infected cells lacked the highly modified Wye base on the 3′ side of its anticodon. Interestingly, one or both of these tRNAs occur at or near the ribosomal frameshift site in expression of the gag-pol poly protein of each retrovirus. The possible role of the hypomodified base in ribosomal frameshifting is presently being investigated.

Protein Structure and Function

(1) Structural and Physicochemical Studies of Proteins. This project involves studies on the chemical structure and physicochemical characteristics of certain natural biopolymeric materials and their synthetic analogs with the aim of

relating the resulting structural information to their biological mode of action, such as cell growth regulation, cell transformation or differentiation. The modern methods of mass spectrometry, nuclear magnetic resonance and circular dichroism spectroscopies and various chemical methods are being applied. Projects include: (1) development of the fast atom bombardment mass spectrometric method of analyzing disulfide-linked dimeric peptides and of epidermal growth factor (EGF) and TGF-alpha related cysteine containing cyclic disulfides. In our approach reductive pyridinoethylation of the peptides yielded derivatives that were better suited for molecular weight and amino acid sequence analysis. In addition a large number of synthetic peptides were analyzed with the molecular weight limit of 2500 for ascertaining the correctness of the synthesis and the oxidation state. (2) Five peptides related to the RGDS segment of the cell attachment factor, fibronectin, were synthesized. This hydrophilic segment in fibronectin interacts with cellular receptors. Circular dichroism studies indicate various molecular conformations for these peptides, the spectrum of the native pentamer indicating a beta turn conformation. The anti-sense peptide, WTVPTA, to the native hexamer, GRGDSP, was also synthesized. Analytical affinity chromatography using the immobilized anti-sense column demonstrated a dissociation constant of 9x10-4 for the latter two peptides.

(2) Computer-Assisted Design of Recognition Peptides. It is now possible to design ex novo recognition peptides, called anti-sense peptides, based on the anti-sense DNA sequence of a gene coding for a target protein. In several cases tested so far, anti-sense peptides have been found to bind to the corresponding sense peptides, with rather profound implications for use in affinity technology to isolate and purify polypeptides of biological relevance. The major limitation for the general application of this new macromolecular recognition system is the lack of DNA sequence information, especially in the case of novel uncharacterized proteins. The main objective of this study is to devise a method to generate recognition peptides using only partial amino acid sequence information. Results obtained so far indicate that it is possible to design recognition peptides starting only from limited sequence information using values of individual amino acid hydropathy. Such ex novo sequence-directed recognition peptides, once immobilized on solid support, maintained their binding properties and proved to be useful in preliminary purification of target proteins.

Studies on Chromatin Structure, Glucocorticoid Receptor and Gene Expression

(1) Molecular Basis of Steroid Hormone Action. The mouse mammary tumor virus (MMTV) system has been studied for some time as a model for positive regulation of transcription by steroid hormones. Using a series of cell lines in which MMTV LTR fusion genes are amplified on extrachromosomally replicating bovine papillomavirus (BPV) "minichromosomes," we demonstrated by an exonuclease protection assay that factors bind to the MMTV promoter in vivo in response to hormone stimulation. These factors were identified in crude nuclear extracts from mouse cells as NF1 (-80 to -56 region) and F-i (-42 to +1 region). Activation of transcription at the MMTV promoter therefore appears to result from recruitment of preformed transcription factors to the promoter by the steroid receptor. The proopiomelanocortin (POMC) gene is negatively regulated by the same steroid receptor. We have prepared POMC-MMTV-BPV chimeric episomes and demonstrated positive regulation of MMTV and negative regulation of POMC on the same circular template. In addition, three factors that bind specifically to the POMC promoter have been identified in crude nuclear extracts.

(2) Chromatin Structure and Gene Expression. The genetic information in mammalian cells exits is organized into a highly condensed nucleoprotein structure whose basic repeating subunit is the nucleosome. Although the major function of this structure is usually thought to be packaging the very large amounts of DNA into a minimal volume, an important and unanswered question concerns whether the interaction of transacting gene regulatory factors with their DNA-binding sites is affected by the organization of these sites in chromatin. We have shown that nucleosomes are phased across the steroid-regulated MMTV promoter. The sites to which steroid-receptors bind are displayed on the surface of nucleosome B in this phased array. Hormone activation of the promoter leads to active displacement of this nucleosome, in contrast to other systems where nucleosome deposition is thought to inhibit factor access. A receptor activated by a steroid antagonist, dexamethasone-21 mesylate, although translocated to the nucleus, is unable to interact productively with MMTV chromatin.

(3) In Vivo Protein-DNA Interactions Probed by UV-cross-linking. The interaction of specific DNA-binding proteins with their recognition sites in the eukaryotic chromosome is central to the mechanisms of gene regulation that occur in these cells. The techniques used to study these interactions rely primarily on methodology designed to detect preferential binding between proteins at various levels of purity from broken cell preparations and purified DNA sequences. We found that two tight-binding transcription factors are excluded from MMTV chromatin in vivo (see Project Z01CP04986-09 LEC). These findings suggest that the mere presence in the nucleus of the factors requisite to initiate transcription in vitro is not a sufficient condition for initiation to occur in vivo. Ultraviolet DNA-protein cross-linking has been utilized to address this problem. Using amplified minichromosomes based on the BPV vector (see Project Z01CP05450-02 LEC), we have detected specific cross-links between minichromosome DNA, two types of proteins, RNA polymerase II and histones, and have shown that loading of pol II on the minichromosome is hormone-dependent. Attempts are underway to determine if the interaction of single-copy regulatory proteins, such as the glucocorticoid receptor, with the template in vivo can be successfully monitored by these techniques.

(4) Conditional Expression of Mammalian Genes. The controlled expression of genetic information in cells in culture and in transgenic animals is an essential tool in the study of gene function in vitro, and eventually will prove central to the treatment of disease by introduced genetic material (gene therapy). We showed previously that conditional expression of the v-*ras*-H oncogene from the glucocorticoid-responsive MMTV promoter could result in a regulated cell phenotype, and demonstrated that regulated "phenotype switching" can be employed to study the oncogenic process in whole animals. The oncogenic potential of NIH 3T3 fibroblasts carrying the hormone-inducible v-*ras*-H oncogene was markedly enhanced when p21 expression was induced prior to inoculation of cells into the animal. These experiments underscored the potential applications of this technology in studying various processes in the intact animal. Utilizing these principles we are now developing a series of retrovirus-based vectors that will permit us to explore the application of this approach to gene therapy in the human.

Studies on Food-Derived Mutagens and Cytochrome P-450

(1) Metabolism, mutagenicity and DNA adduction of IQ. 2-Amino-3-methyl-imidazole(4,5-f)quinoline (IQ) is among a number of heterocyclic arylamines found in cooked foods that have been shown to be potent carcinogens in rodent bioassays. We have initiated work involving (1) synthesis of N-OH-IQ, and N-acetoxy-IQ and IQ-N-sulfate, the reactive metabolites of IQ; (2) synthesis and characterization of the major DNA-IQ adduct; (3) examination of DNA-IQ adducts in monkeys by the 32P-postlabeling method; (4) IQ metabolism in monkeys; and (5) the role of specific cytochromes P-450 in the metabolic activation of IQ. N-OH-IQ was a direct mutagen in Salmonella TA98 and was capable of covalently binding to DNA without further activation. N-OH-IQ is also metabolized by mammalian O-acetyltransferase and sulfotransferase to N-acetoxy-IQ and IQ-N-sulfate, respectively. The C8-guanine-IQ adduct was synthesized and shown to be formed in vitro from either N-OH-IQ or N-acetoxy-IQ reacting with DNA. Using the 32P-postlabeling assay, eight DNA-IQ adducts including C8-guanine-IQ, were found in the liver of Cynomolgus monkeys fed IQ that were identical to those found in the liver of rats fed IQ. Analysis of DNA modified in vitro with N-OH-IQ showed seven adducts, including the C8-guanine-IQ adduct, that were identical to those found in vivo. Thus, N-OH-IQ appears to be responsible for all adducts found in vivo except one. DNA-IQ adducts were also detected in kidney, colon, stomach and bladder, and these adducts were identical in all organs examined. We examined the specificity of metabolic activation of IQ and a number of other mutagenic/-carcinogenic heterocyclic arylamines from cooked foods by employing a novel test system combining human cells individually expressing either recombinant cytochrome P1-450 or P3-450 as the bioactivation system with Salmonella typhimurium to score mutant. Our results show that cytochrome P3-450, a single isoform of the cytochrome P-450 super-gene family, is responsible for the bioactivation of the heterocyclic arylamine food mutagens to N-hydroxylamines.

(2) Role of Cytochrome P-450 Genes in Mutagenesis and Carcinogenesis. The cytochrome P-450 supergene family codes for enzymes which metabolize a wide array of both xenobiotics including drugs and carcinogens, and endobiotics such as steroids and prostaglandins. In animals, multiple forms of these enzymes exhibiting broad and overlapping substrate specificities are expressed simultaneously either constitutively or after administration of inducers. To define the contribution of a given cytochrome P-450 to the metabolism of specific drugs and carcinogens, it is important to express these enzymes individually in cells which lack endogenous background for these enzymes. Toward this goal we have begun to develop expression systems in which an individual cytochrome P-450 protein is synthesized from their coding DNA sequences. Success in this effort will enable us to define the contribution of each of these enzymes to mutagenesis and to cell transformation by chemical carcinogens. For this purpose, we have constructed infectious recombinant vaccinia virus and infectious recombinant retrovirus containing the full-length coding cDNA sequences for mouse cytochromes P1-450 and P3-450. Human and mouse cells infected with the recombinant viruses expressed high levels of the authentic size proteins that were enzymatically active and displayed substrate specificities diagnostic of the respective enzymes. Preliminary experiments indicate that cytochrome P3-450 selectively activates heterocyclic arylamines and cytochrome P1-450 preferentially activates aromatic hydrocarbons, and this property appears to be mutually exclusive at limiting substrate concentrations. Furthermore,the cytochrome P3-450-activated

food mutagens formed specific adducts with the DNA of cells expressing the P3-450. The DNA adducts detected were identical to those formed in mouse or rat liver after the in vivo administration of the food mutagens.

(3) Coinduction of Multidrug-Resistance (MDR-1) and Selective P450 Cytochromes. The levels of mRNA for both MDR-1 and selective cytochrome P-450 genes were determined in adult rat liver following administration of various natural and synthetic xenobiotics. MDR-1 was induced following administration of aflatoxin B_1, AAF, N-hydroxy-2-acetylaminofluorene (N-OH-AAF), isosafrole, phenothiazine, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), but not after phenobarbital (PB) or 7-hydroxy-2-acetylaminofluorene (7-OH-AAF) treatment. Cytochrome P-450 isoform **d** was induced by TCDD, isosafrole, phenothiazine and AAF, while cytochrome P-450 isoform **b** was induced by PB and to a lesser extent by isosafrole. Furthermore, aflatoxin, AAF and TCDD induced MDR in the B6 mouse but not in the D2 (Ah receptor deficient) mouse, which suggests that MDR induction via aflatoxin may be by a ligand-responsive transcription factor, possibly the Ah receptor or another cytosolic receptor. Taken together, the above data have placed MDR into a set of programmed "alarm response" genes such as glutathione-S-transferase and UDP-glucuronyl-transferase aimed at protecting the organism against the harmful effects of xenobiotics.

In addition to their intramural research efforts that have been summarized above, investigators within the LEC serve on editorial boards of major journals in their field and are involved as consultants or advisors on various national and international committees in the areas of chemical and biological carcinogenesis. Furthermore, the LEC scientists participate to a considerable degree in collaborative efforts with scientists both within the NCI and throughout the country, and the international scientific community.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05553-01 LEC

PERIOD COVERED

October 31, 1987 through September 30, 1988

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

Role of Cytochromes P-450 Genes in Mutagenesis and Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Battula	Expert	LEC	NCI
Others:	G. K. Townsend	Biologist	LEC	NCI
	E. G. Snyderwine	Guest Researcher	LEC	NCI
	S. S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (if any)

Department of Pathology, Medical College of Ohio, Toledo (H. A. J. Schut)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The cytochrome P-450 supergene family codes for enzymes which metabolize a wide array of both xenobiotics including drugs and carcinogens, and endobiotics such as steroids and prostaglandins. In animals, multiple forms of these enzymes exhibiting broad and overlapping substrate specificities are expressed simultaneously, either constitutively or after administration of inducers. To define the contribution of a given cytochrome P-450 to the metabolism of specific drugs and carcinogens, it is important to express these enzymes individually in cells which lack endogenous background for these enzymes. Toward this goal we have begun to develop expression systems in which an individual cytochrome P-450 protein is synthesized from their coding DNA sequences. Success in this effort will enable us to define the contribution of each of these enzymes to mutagenesis and to cell transformation by chemical carcinogens. For this purpose, we have constructed infectious recombinant vaccinia virus and infectious recombinant retrovirus containing the full length coding cDNA sequences for mouse cytochrome P1-450 and P3-450. Human and mouse cells infected with the recombinant viruses expressed high levels of the authentic size proteins that were enzymatically active and displayed substrate specificities diagnostic of the respective enzymes. Preliminary experiments indicate that cytochrome P3-450 selectively activates heterocyclic arylamines and cytochrome P1-450 preferentially activates aromatic hydrocarbons; and this property appears to be mutually exclusive at limiting substrate concentrations. Furthermore, the cytochrome P3-450-activated food mutagens formed specific adducts with the DNA of cells expressing the P3-450. The DNA adducts detected were identical to those formed in mouse or rat liver after the in vivo administration of the food mutagens.

(3) Human breast carcinoma (MCF-7) and rat hepatoma cells (Reuber) showed extreme sensitivity to rTNF-α (ID50 = 10 units/ml, and 20 units/ml, respectively), low sensitivity to LDGI (ID50 = 1 ng/ml), but were resistant to the cytostatic effects of TGF-β.

(4) The rate of DNA synthesis in rat kidney fibroblasts (NRK) and human foreskin fibroblasts (HFS-4) was stimulated by two- to threefold in response to TGF-β, LDGI, and rTNF-α treatment.

(5) RLE cells infected with retroviral construct J2 containing sequences for v-raf and v-myc oncogenes, unlike parental RLE cells, exhibited a two- to threefold increase in the rate of DNA synthesis after exposure to TGF-β and LDGI.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Mrunal S. Chapekar	Senior Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Peter P. Roller	Section Chief	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Anthony C. Huggett	Visiting Associate	LEC	NCI

Objectives:

The objectives of this project are to characterize the rat liver-derived growth inhibitor by comparing its biological effects with those of the known growth inhibitors, TGF-β and rTNF-α, and to investigate biochemical mechanisms involved in the growth regulating effects of this inhibitor in rat liver epithelial cell system.

Methods Employed:

The growth modulations of LDGI, TGF-β, and rTNF-α were assessed employing the semiautomated assay system which enabled us to measure DNA content and synthesis in monolayer cells in 96-well microtiter plates. The DNA content was determined using fluorescent dye Hoechst 33342 and DNA synthesis was measured by incorporation of ^{3}H thymidine in cellular DNA. The rate of DNA synthesis in cells was expressed as ^{3}H thymidine DPM incorporated for two hours per fluorescence unit of DNA.

The microcinematography technique was employed to examine cell cycle specificity of LDGI.

The tyrosine kinase activity in cells is measured by nondenaturing polyacrylamide gel assay system.

Major Findings:

(1) Normal RLE cells were highly sensitive to the antiproliferative effects of LDGI (ID50 = 0.2 ng/ml) and TGF-β (ID50 = 0.3 ng/ml), but were relatively insensitive to the cytostatic effects of rTNF-α (ID50 > 5000 units/ml).

(2) AFB_1-transformed RLE cells exhibited sensitivity to the growth inhibitory effects of LDGI (ID50 = 1.5 ng/ml), but were relatively resistant to the cytostatic effects of TGF-β and rTNF-α. Among the clones isolated from AFB_1-transformed RLE cells, clone C2 exhibited sensitivity to the growth inhibitory effects of LDGI (ID50 = 1.5 ng/ml), but was resistant to the antiproliferative effects of TGF-β and rTNF-α.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05503-02 LEC

PERIOD COVERED

October 1, 1987 to September 1988

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

Characterization of a Rat Liver-Derived Growth Inhibitor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Mrunal S. Chapekar	Senior Staff Fellow	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Peter P. Roller	Section Chief	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Anthony C. Huggett	Visiting Associate	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.35	1.35	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The principle objective of this project is to further characterize the protein isolated from rat liver that reversibly inhibits the proliferation of rat liver epithelial (RLE) cells at extremely low concentrations (ID50 = 0.2 ng/ml). The biological effects of this inhibitor protein (designated as LDGI) were compared with those of transforming growth factor-beta (TGF-beta) and recombinant tumor necrosis factor-alpha (rTNF-alpha) in a variety of normal and malignant cell culture systems. RLE cells were highly sensitive to the antiproliferative effects of LDGI and TGF-beta but were relatively insensitive to the cytostatic effects of rTNF-alpha. Aflatoxin B_1 (AFB-$_1$)-transformed RLE cells were resistant to the antiproliferative effects of TGF-beta and rTNF-alpha but showed sensitivity to the growth inhibitory effects of LDGI. Clones isolated from AFB-1-transformed RLE cells exhibited either sensitivity similar to parental AFB-1-transformed RLE cells or complete resistance to growth inhibition by LDGI. However, none of these clones exhibited sensitivity to the antiproliferative effects of TGF-beta or rTNF-alpha. Rat hepatoma (UVM7777) cells were insensitive to the cytostatic effects of all three growth modulators. Human breast carcinoma (MCF-7) and rat hepatoma (Rueber) cells were extremely sensitive to rTNF-alpha, exhibited low sensitivity to LDGI, but were resistant to the antiproliferative effects of TGF-beta. The rate of DNA synthesis in rat kidney fibroblasts (NRK) and human foreskin fibroblasts was stimulated two- to threefold in response to LDGI, TGF-beta and rTNF-alpha treatment. The growth rate of RLE cells transformed with a retrovirus containing both v-*myc* and v-*raf* (J2) was stimulated by both LDGI and TGF-beta.

by selective precipitation of the adduct with antibody to PolII. In addition, we have shown that the number of PolII molecules per minichromosome is markedly increased after stimulation by hormone, as expected.

We have employed an alternate approach to detect global interactions between minichromosome DNA and any protein. Restriction endonuclease chromatin fragments were electrophoresed and analyzed by Southern blotting. Identical fragments migrated more slowly after hormone stimulation, indicating an increased number of proteins in contact with the episome.

These experiments indicate that the minichromosome system sufficiently amplifies and enriches MMTV long terminal repeat (LTR) chromatin to permit detection of specific UV-induced protein-DNA covalent adducts. Efforts to extend this approach to the detection of single-copy regulatory molecules (such as steroid receptors) on the minichromosomes have, to date, yielded inconsistent data. There is an indication that single-copy molecules can be detected, but problems with incomplete restriction of the in vivo UV-damaged DNA render the experimental interpretation difficult.

Current experimentation is focused on two approaches to alleviate these problems. First, technology to more completely purify the DNA without protein-denaturing steps are under investigation. Secondly, intracellular concentrations of steroid receptors are being elevated by introduction of a cloned receptor driven from strong, constitutive promoters. Both of these steps should enhance our ability to detect the desired interactions in vivo.

Publications:

Richard-Foy H, Sistare FD, Riegel AT, Simmons SS, Hager GL. Mechanism of dexamethasone 21-mesylate antiglucocorticoid action. II. Receptor-antiglucocorticoid complexes are unable to interact productively with MMTV chromatin in vivo. Mol Endocrinol 1987;1:659-65.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gordon Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Anna Tate Riegel	Visiting Fellow	LEC NCI
Diana S. Berard	Microbiologist	LEC NCI

Objectives:

The primary objective of this project is to develop a methodology for the unambiguous detection of protein-DNA interactions in the living cell. Currently utilized technology in this problem area addresses primarily the interaction of proteins with DNA in broken cell, or highly purified preparations, and has led to a rapid accumulation of information on factors that bind to putative DNA regulatory sequences. These approaches are flawed, however, in that factors which potentially interact at a given target in the DNA can be identified, but the actual interaction in vivo remains hypothetical. We propose to adapt the technique of protein-DNA cross-linking with ultraviolet light to address this problem. The advantage in this technique is that a covalent bond is formed between the putative binding factor and DNA in vivo, permitting a rigorous analysis as to where the protein is and when it is there.

Methods Employed:

DNA sequences to be analyzed are introduced into murine cells by mobilization on the BPV 69% transforming fragment. Cell lines are isolated that uniquely contain non-rearranged, non-integrated copies of the chimeric constructions. These lines are then characterized for copy number, DNA structure, and transcriptional regulation.

DNA-protein cross-links are introduced in vivo by exposure to ultraviolet (UV) irradiation, either from a standard UV-mineral-light for long-term exposures (5-60 min) or from a high-intensity xenon flash lamp (40-1000 nanoseconds). Minichromosome chromatin from irradiated cells is prepared from nuclei of cells containing episomes by ammonium sulfate extraction.

Chromatin is digested with restriction endonucleases. The association of proteins with specific fragments is determined either by antibody precipitation of the protein-DNA adduct, or by Western blotting analysis of the protein-DNA adducts after electrophoresis and transfer to membranes.

Major Findings:

Our initial attempts to detect UV-induced protein-DNA cross-links in vivo involved RNA polymerase II and histones. Both molecules provide the advantage of several copies of the protein per individual minichromosome. A given protein-DNA adduct is detected with antisera specific to the protein. We were able to demonstrate specific binding of RNA polymerase II to minichromosomes chromatin

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05502-02 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

In Vivo Protein-DNA Interactions Probed by Ultraviolet-cross-linking

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Gordon Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Others:	Anna Tate Riegel	Visiting Fellow	LEC NCI
	Diana S. Berard	Microbiologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The interaction of specific DNA-binding proteins with their recognition sites in the eukaryotic chromosome is central to the mechanisms of gene regulation that occur in these cells. The techniques used to study these interactions rely primarily on methodology designed to detect preferential binding between proteins at various levels of purity from broken cell preparations and purified DNA sequences. We found (see Project Z01CP04986-09 LEC) that two tight-binding transcription factors are excluded from mouse mammary tumor virus (MMTV) chromatin in vivo. These findings suggest that the mere presence in the nucleus of the factors requisite to initiate transcription in vitro is not a sufficient condition for initiation to occur in vivo. Ultraviolet DNA-protein cross-linking has been utilized to address this problem. Using amplified minichromosomes based on the bovine papillomavirus (BPV) vector (see Project Z01CP05450-02 LEC), we have detected specific cross-links between minichromosome DNA two types of proteins, RNA polymerase II and histones, and have shown that loading of pol II on the minichromosome is hormone-dependent. Attempts are underway to determine if the interaction of single-copy regulatory proteins, such as the glucocorticoid receptor, with the template in vivo can be successfully monitored by these techniques.

not to the brain or liver. Cells in culture were radiolabelled with ^{14}C amino acids, ^{14}C mannose, ^{14}C galactose, or ^{32}P. Overlay comparison of the resultant autoradiograms from the ^{14}C amino acid labelling showed several distinct qualitative (10-14) and quantitative (15-18) differences to be readily apparent, indicating several differences in constitutive expression of polypeptides between the metastatic and non-metastatic cell types. The most intensely labelled spots that occurred only in the metastatic cells were (MW/pI) 67/5.5 and 50/4.5 and those that were observed only in the non-metastatic cells were 46/6.7 and 38/6.1. A large number of the polypeptides detected by ^{14}C amino acid labelling were also glycosylated and TMT-081-NM cells exhibited a greater extent of glycosylation. The autoradiograms resulting from ^{32}P labelling showed a marked difference in the polypeptide pattern produced from the two-dimensional electrophoresis of the ^{14}C amino acid labelling. This suggests that either many of these proteins labelled with ^{32}P are extremely stable and are synthesized at a very slow rate or that they are produced in such small, tightly regulated quantities as to be difficult to detect using ^{14}C amino acids. Considering the rate at which these cells grow (doubling time of approximately 36 hours), the former suggestion would appear much less probable. Comparing the ^{32}P autoradiograms between the TMT-081-MS and TMT-081-NM, 15-20 qualitative and 15-20 quantitative differences were readily observed in the polypeptide patterns. Those polypeptides that labelled most intensely with the ^{32}P and occurred only in the metastatic cells were 98/4.7 and 24/4.5, and the following polypeptides were approximately threefold greater in metastatic compared to non-metastatic cells: 15/5.4, 14/5.2 and 12/6.3. The polypeptides of 40/5.8 and 40/5.9 were at least threefold more intensely labelled in the non-metastatic compared to the metastatic cells.

Subclones have been derived from metastatic tumors arising from the TMT-081-MS cells in Wistar/Furth rats. Cells from these tumors were grown in culture and single cell clones obtained. These clones possess metastatic potential and will be used in further experiments. The use of these cells will allow multiple comparisons between the metastatic cells to more rapidly determine proteins that appear to be associated with the metastatic process.

This project is now at a stage where major steps can be made toward the identification and investigation of function of the regulatory proteins. Using the 2-dimensional gel electrophoresis system as an extremely sensitive, high resolution detector will enable most of those proteins which specifically relate to the metastatic cells to be located.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Peter J. Worland	Visiting Fellow	LEC NCI
Lori L. Hampton	Biologist	LEC NCI
Peter J. Wirth	Expert	LEC NCI

Objectives:

Breast cancer occurs at quite high rates in western females, comprising approximately 27% of all cancers and is responsible for 19% of all female cancer deaths in the United States. A large proportion of primary tumors can be treated effectively by combination of treatments--surgery, chemotherapy and radiotherapy--but metastatic tumor cells have often spread to other sites before they can be detected and treated. There has been considerable effort looking at the interaction of these cells within the basement membrane to determine how they escape from the primary site and then develop at a different locus. We have available to us two rat mammary tumor cell lines derived from the same parent population, but differ in that one (TMT-081-NM) is non-metastatic and the other (TMT-081-MS) is metastatic. These cells have been provided by Dr. Untae Kim of the Roswell Park Memorial Institute. Experiments utilizing 2-dimensional polyacrylamide gel electrophoresis and selective radiolabelling of cellular proteins with ^{14}C amino acids or ^{32}P to identify those proteins being turned over and those under phosphorylative control have been undertaken as the initial step to identify those proteins most closely associated with the metastatic process.

Methods Employed:

The principle methods employed are: (1) tissue culture techniques, (2) histochemical staining, (3) 2-dimensional gel electrophoresis, (4) autoradiography, (5) computer-assisted quantitation of autoradiograms and (6) animal husbandry.

Major Findings:

As it has been noted that metastatic cells tend to lose their metastatic potential in culture over extended periods of time, the metastasizing ability of the TMT-081-MS was compared against the TMT-081-NM. Two groups of 10W/Fu rats were injected subcutaneously in the right inguinal region with 2×10^7 cells of either type. Several aliquots of 2×10^6 cells of both cell types were stored frozen in liquid nitrogen for subsequent use. The tumors resulting from the TMT-081-NM cells had a latency of 9-10 days and grew extremely rapidly, with the rats being sacrificed because of poor condition at 35-40 days. The TMT-081-MS cells exhibited a longer latency of 20-23 days and these rats were sacrificed at 168 days for autopsy after it was observed that all rats had tumors in the right axillary lymph gland. Metastasis of the tumor occurred throughout the lymphatic system, the lungs, adrenals and thymus but

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05501-02 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cellular Polypeptides Associated with Metastasis of Rat Mammary Tumor Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Peter J. Worland	Visiting Fellow	LEC NCI
	Lori L. Hampton	Biologist	LEC NCI
	Peter J. Wirth	Expert	LEC NCI

COOPERATING UNITS (if any)

Department of Pathology, Roswell Memorial Park Institute, Buffalo, New York (Dr. Untae Kim)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	0.3

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

☐ (a1) Minors

☐ (a2) Interviews

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

This project was initiated to identify those polypeptides that specifically relate to the metastatic process as the first step toward their purification and identification. Utilization of metastasizing and non-metastasizing cells derived from the same parent population of tumor cells is fundamental to this project. We have confirmed that the TMT-081-MS cells do metastasize in syngeneic rats and that the TMT-081-NM cells do not metastasize in syngeneic rats. Radiolabelling of these cells with 14-C amino acids has revealed several qualitative and quantitative differences in their polypeptide patterns. The most intensely labelled spots that occurred in the TMT-081-MS cells were (MW/pI) 67/5.5 and 50/4.5 and the most intense spots that occurred only in the TMT-081-NM cells were 46/6.7 and 38/6.1. When a 32-P-radiolabel was used with these cells, a number of polypeptides could be observed that were not located from the 14-C labelling. The most intensely 32-P labelled spots occurring only in the metastatic cells were 98/4.7 and 24/4.5. Those polypeptides that were approximately threefold greater in intensity in the metastatic cells compared to the non-metastatic cells were 14/5.2, 15/5.4 and 12/6.3. The polypeptides of 40/5.9 and 40/5.8 were at least threefold greater in the intensity of 32-P label compared to the corresponding polypeptides in the metastatic cells. A number of subclones of the metastasizing TMT-081-MS cells have been derived from metastasis in Wistar/Furth rats. These subclones possess metastatic capacity and will be used in further experiments.

was accomplished by 2-dimensional polyacrylamide electrophoresis and the consequent patterns analyzed with computer assistance. E2 treatment of the MCF-7gpt cells reduced the number of differences found in the polypeptide patterns between MCF-7gpt and MCF-7*ras*. Twelve cellular polypeptides were consistently modulated by either E2 or v-H-*ras*, with 4 polypeptides clearly affected in the same way by both treatments. Polypeptides Gchc-0845 (54kD/pI6.9) and Gchc-0902 (52kD/pI6.3) were suppressed by E2 and v-H-*ras* while Gchc-1240 (34kD/pI4.4) and Gchc-1396 (23kD/pI5.3) were induced by E2 and v-H-*ras*. Sixteen secreted polypeptides were altered by at least twofold subsequent to E2 treatment or v-H-*ras* transfection. Transfection with v-H-*ras* had a wider range of effect than E2, stimulating the secretion of 8 polypeptides and suppressing the secretion of 7 other polypeptides compared to E2 increasing the secretion of 5 polypeptides and decreasing the secretion of a further 3 polypeptides, respectively. Synergistic effects by E2 and v-H-*ras* were noted for 3 of these polypeptides, with increased secretion of Gcls-175 (50kD/pI5.7), Gcls-320 (14<kD/pI3.6, pS2) and decreased secretion of Gcls-112 (76kD/pI6.9). Opposing effects of E2 and v-H-*ras* were seen for 7 polypeptides including the 48kD derivative of the 52K protein. These studies support the possibility that extremely few, but specific, polypeptides are regulated in association with quite diverse tumorigenic stimuli in MCF-7 human breast cancer cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Peter J. Worland	Visiting Fellow	LEC NCI
Peter J. Wirth	Expert	LEC NCI
Lori L. Hampton	Biologist	LEC NCI
Diane A. Bronzert	Biologist	LEC NCI
Robert B. Dickson	Senior Investigator	MB NCI
Marc E. Lippman	Section Chief	MB NCI

Objectives:

Hormonal control of cancer cell proliferation has recently received much attention with the proposal of autocrine or self-stimulating polypeptide growth factors. Growth of several types of cancer are known to be under endocrine control by steroid hormones: leukemia, prostate carcinoma, endometrial carcinoma and breast cancer. The latter is unique among the above-mentioned cancers in that its growth is strongly regulated in 1/3 of the clinical cases by estrogenic hormones or antiestrogenic antagonists. The MCF-7 cell line was originally derived from the pleural ascites of a metastasizing tumor and is responsive to the growth stimulation by estrogen. Several subclones have been derived from the MCF-7 cell line which include the control MCF-7gpt and the v-H-*ras* transfect MCF-7*ras* derived from the parent MCF-7 cells and the LY2 cell line which is resistant to the growth inhibitory effect of antiestrogens. The MCF-7*ras* transfect exhibits partial "estrogen escape" in that it can form tumors in nude mice without estrogen supplementation, in contrast to the MCF-7 cells. By treating the cells with estrogen, growth factors or antiestrogens and utilizing the resolving power of 2-dimensional polyacrylamide gel electrophoresis, it is intended to identify polypeptides associated with particular growth factors or antagonists. This will contribute significantly to the understanding of established tumor growth with possible future therapeutic benefits.

Method Employed:

The principle methods employed are: (1) tissue culture techniques, (2) 2-dimensional polyacrylamide gel electrophoresis, (3) autoradiography and (4) computer-assisted quantitation of autoradiograms.

Major Findings:

The polypeptide patterns of MCF-7 human breast cancer cells (MCF-7gpt) and a stably v-H-*ras*-transfected subclone (MCF-7*ras*) have been analyzed following estradiol (E2) treatment. Both E2 and v-H-*ras* transfection increase the tumorigenicity of MCF-7 cells. This study was therefore designed to ascertain if specific changes in polypeptides were common to both treatments and associated with the increased tumorigenicity. Separation of cellular and secreted proteins

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05500-02 LEC

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

Polypeptide Modulation in MCF-7 Cells by Estrogen and Growth Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Peter J. Worland	Visiting Fellow	LEC NCI
	Peter J. Wirth	Expert	LEC NCI
	Lori L. Hampton	Biologist	LEC NCI
	Diane A. Bronzert	Biologist	LEC NCI
	Robert B. Dickson	Senior Investigator	MB NCI
	Marc E. Lippman	Section Chief	MB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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SECTION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.5	0.3

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☐ (a1) Minors

☐ (a2) Interviews

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

The purpose of this project was to utilize the established human mammary tumor cell lines (MCF-7, MCF-7gpt [produced by transfection with the Eco-gpt selectable gene marker], MCF-7*ras* [produced by transfection with Eco-gpt and the v-H*ras* oncogene] and LY2) to investigate the effect of antiestrogens, estrogen and other growth factors on the polypeptide expression of both cellular and secreted proteins in these cells. The growth factors IGF-I (insulin like growth factor-I) and TGF-alpha (transforming growth factor alpha) are able to elicit many of the growth stimulating responses of estrogen when applied to the MCF-7 human mammary tumor cell line. Analysis of the 2-dimensional polyacrylamide gels from cellular proteins of the MCF-7gpt and MCF-7*ras* cells which have been treated with 17-beta-estradiol or an ethanol vehicle showed 12 polypeptides which were consistently altered. Of these, four polypeptides were clearly affected the same way by *ras* transfection and estrogen stimulation. Sixteen secreted polypeptides were altered by at least twofold subsequent to either estrogen or v-H*ras*. Opposing effects of estrogen and v-H*ras* were observed for a number of polypeptides, but synergistic effects of v-H*ras* and estrogen were seen for three polypeptides detected by autoradiography. Studies to ascertain the effects of the growth factors IGF-I and TGF-alpha on the expression and secretion of these polypeptides are in the final stages of analysis. Additional to this are experiments using the anti-estrogen LY117018 to assess the effect on polypeptide expression and secretion from the antiestrogen-resistant cell line LY2 and the MCF-7 cells.

Since metabolism of IQ to N-hydroxy-IQ appears to be a critical step in the activation of IQ to its carcinogenic form, studies concerning the role of cytochromes P-450 in the activation of IQ are being carried out. We examined the specificity of metabolic activation of IQ and a number of other mutagenic/carcinogenic heterocyclic arylamines from cooked foods by employing a novel test system combining human cells individually expressing either recombinant cytochrome P1-450 or P3-450 as the bioactivation system with Salmonella typhimurium to score mutations. Our results show that cytochrome P3-450, a single isoform of the cytochrome P-450 super-gene family, is responsible for the bioactivation of the heterocyclic arylamine food mutagens to N-hydroxylamines.

Disposition studies of IQ in monkeys indicate that IQ is metabolized to a number of more polar and non-mutagenic urinary metabolites. The urine from these monkeys, however, is mutagenic in the presence of an S9 activating system, and this mutagenicity is due to unmetabolized IQ. Urinary metabolites of IQ in monkeys are currently being purified by HPLC and identified by NMR and mass spectral analyses.

Publications:

Snyderwine EG, Roller PP, Adamson RH, Sato S, Thorgeirsson SS. Reaction of N-hydroxylamine and N-acetoxy derivatives of 2-amino-3-methylimidazolo[4,5-f]quinoline with DNA. Synthesis and identification of N-(deoxyguanosin-8-yl)-IQ. Carcinogenesis 1988;9:1061-5

Snyderwine EG, Wirth PJ, Roller PP, Adamson RH, Sato S, Thorgeirsson SS. Mutagenicity and in vitro covalent DNA binding of 2-hydroxyamino-3-methylimizazolo[4,5-f]quinoline. Carcinogenesis 1988;9:411-8.

Snyderwine EG, Yamashita K, Adamson RH, Sato S, Nagao M, Sugimura T, Thorgeirsson SS. Use of the ^{32}P-postlabeling method to detect DNA adducts of 2-amino-3-methylimidazolo[4,5-f]quinoline (IQ) in monkeys fed IQ: identification of the N-(deoxyguanosin-8-yl)-IQ adduct. Carcinogenesis (In Press).

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Elizabeth G. Snyderwine	Guest Researcher	LEC NCI
Peter P. Roller	Head, BPC	LEC NCI
Richard H. Adamson	Director	DCE NCI
Peter P. Wirth	Expert	LEC NCI
Narayana Battula	Expert	LEC NCI

Objectives:

IQ, a potent mutagen-carcinogen found in certain cooked foods, is metabolized by the cytochrome P-450 system to N-hydroxy-IQ, a reactive metabolite, capable of covalently modifying DNA. In light of the evidence that DNA adduction is a critical event in the process of carcinogenesis, one objective of this study is to characterize the major DNA-IQ adducts. A second objective is to examine the DNA adducts of IQ in monkeys fed IQ in order to assess the genotoxicity of IQ in non-human primates and thus by analogy, the potential genotoxicity towards man. Our third objective is to examine the disposition of IQ in monkeys and metabolism by the cytochrome P-450 system.

Methods Employed:

The principle methods employed are the following: (1) chemical synthesis, (2) HPLC, (3) Ames Salmonella mutagenicity assay, (4) nuclear mass resonance (NMR) and mass spectral analyses, (5) 32P-postlabeling assay, and (6) recombinant cytochromes P-450.

Major Findings:

N-Hydroxy-IQ was found to be mutagenic toward Salmonella TA98 in the absence of an S9 activating system, and capable of covalently binding to DNA without further activation. N-Hydroxy-IQ is also metabolized by mammalian O-acetyltransferase and sulfotransferase to N-acetoxy-IQ and IQ-N-sulfate, respectively, metabolites that are more reactive with DNA. The C8-guanine-IQ adduct was synthesized and shown by high performance liquid chromatography (HPLC) methods to form upon in vitro reaction of either N-hydroxy-IQ or N-acetoxy-IQ with DNA. Using the 32P-postlabeling assay, eight DNA-IQ adducts including C8-guanine-IQ, were found in the liver of Cynomolgus monkeys fed IQ that were identical to those found in the liver of rats fed IQ. The data suggest that monkeys will be as susceptible as rats to IQ-induced liver cancer. Analysis of DNA modified in vitro with N-hydroxy-IQ showed seven adducts, including the C8-guanine-IQ adduct, that were identical to those found in vivo. Thus, N-hydroxy-IQ appears to be responsible for all adducts found in vivo except one. DNA-IQ adducts were also detected in kidney, colon, stomach and bladder, and these adducts were identical in all organs examined.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05496-03 LEC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Metabolism, Mutagenicity and DNA Adduction of IQ

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Elizabeth G. Snyderwine	Guest Researcher	LEC NCI
	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
	Richard H. Adamson	Director	DCE NCI
	Peter P. Wirth	Expert	LEC NCI
	Narayana Battula	Expert	LEC NCI

COOPERATING UNITS (if any)

National Cancer Center Research Institute, Tokyo Japan (Drs. T. Sugimura, K. Yamashita, M. Nagao)

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0

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☐ (a1) Minors
☐ (a2) Interviews

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

2-Amino-3-methyl-imidazole(4,5-f)quinoline (IQ) is among a number of heterocyclic arylamines found in cooked foods that have been shown to be potent carcinogens in rodent bioassays. We have initiated work involving (1) synthesis of N-OH-IQ, and N-acetoxy-IQ and IQ-N-sulfate, the reactive metabolites of IQ; (2) synthesis and characterization of the major DNA-IQ adduct; (3) examination of DNA-IQ adducts in monkeys by the 32P-postlabeling method; (4) IQ metabolism in monkeys; and (5) the role of specific cytochromes P-450 in the metabolic activation of IQ. N-OH-IQ was a direct mutagen in Salmonella TA98 and capable of covalently binding to DNA without further activation. N-OH-IQ is also metabolized by mammalian O-acetyltransferase and sulfotransferase to N-acetoxy-IQ and IQ-N-sulfate, respectively. The C8-guanine-IQ adduct was synthesized and shown to be formed in vitro from either N-OH-IQ or N-acetoxy-IQ reacting with DNA. Using the 32P-postlabeling assay, eight DNA-IQ adducts including C8-guanine-IQ, were found in the liver of Cynomolgus monkeys fed IQ that were identical to those found in the liver of rats fed IQ. Analysis of DNA modified in vitro with N-OH-IQ showed seven adducts, including the C8-guanine-IQ adduct, that were identical to those found in vivo. Thus, N-OH-IQ appears to be responsible for all adducts found in vivo except one. DNA-IQ adducts were also detected in kidney, colon, stomach and bladder, and these adducts were identical in all organs examined. We examined the specificity of metabolic activation of IQ and a number of other mutagenic/carcinogenic heterocyclic arylamines from cooked foods by employing a novel test system combining human cells individually expressing either recombinant cytochrome P1-450 or P3-450 as the bioactivation system with Salmonella typhimurium to score mutations. Our results show that cytochrome P3-450, a single isoform of the cytochrome P-450 super-gene family, is responsible for the bioactivation of the heterocyclic arylamine food mutagens to N-hydroxylamines.

and the peptide containing the amino acid at the readthrough site isolated by HPLC. The sequence of this peptide will be determined to identify the amino acid at the readthrough site.

The readthrough protein has also been characterized in rabbit reticulocytes. Labeling the protein in reticulocytes with ^{35}S-methionine demonstrates that approximately 0.5% suppression of the beta-globin mRNA occurs naturally in reticulocytes. The presence of the beta-globin readthrough protein in reticulocytes was identified by Western blotting and by enhancement of its synthesis in response to an opal suppressor tRNA. The product synthesized in response to addition of an opal suppressor tRNA to reticulocytes was also isolated by the antibody-CNBr-activated Sepharose column.

Publications:

Hatfield DL, Thorgeirsson SS, Copeland T, Oroszlan S, Bustin M. Immunopurification of the suppressor tRNA dependent rabbit beta-globin readthrough protein. Biochemistry 1988;27:1179-83.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dolph L. Hatfield	Research Biologist	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Michael Bustin	Research Chemist	LMC NCI

Objectives:

The major goal of this project is to isolate the rabbit beta-globin readthrough protein from rabbit reticulocytes and identify the amino acid at the suppression site.

Specific steps to achieve this goal are: (1) prepare antibodies to the readthrough portion of the rabbit beta-globin polypeptide which results from suppression of the corresponding termination codon in rabbit reticulocytes, (2) purify and isolate antibodies specific to this protein, (3) use the purified antibodies in combination with polyacrylamide gel electrophoresis to isolate the readthrough protein, and (4) determine the amino acid at the readthrough site.

Methods Employed:

A peptide which is 22 amino acids in length and which corresponds to the portion of rabbit beta-globin polypeptide that results from readthrough was synthesized and coupled to the KLH protein by Peninsula Laboratories, Inc. A sheep was immunized by intradermal and intramuscular injection with the coupled protein. The sheep was bled at 3 weeks and at 3-week intervals thereafter. Antibodies were detected by the ELISA technique and were purified using CNBr-activated Sepharose. The purified antibodies were subsequently attached to CNBr-activated Sepharose in order to isolate the readthrough protein. The material obtained from the antibody-CNBr-activated Sepharose column was previously subjected to high performance liquid chromatography (HPLC) to isolate the readthrough protein, but more recently has been isolated in higher quantities by polyacrylamide gel electrophoresis.

Protein synthesis was carried out in the presence of rabbit reticulocyte lysates and ^{35}S-methionine, with or without an opal suppressor tRNA. The readthrough protein was characterized by polyacrylamide gel electrophoresis and by Western immunoblotting.

Major Findings:

Specific antibodies to the rabbit beta-globin readthrough protein were produced by immunizing a sheep with a peptide corresponding to the readthrough portion of this protein. The antibodies were purified and used in combination with polyacrylamide gel electrophoresis to isolate this unique protein from rabbit reticulocytes. The purified proteins are presently being digested with trypsin

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

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

Amino Acid at the Suppression Site in Rabbit beta-Globin Readthrough Protein

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Dolph L. Hatfield	Research Biologist	LEC NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC NCI
	Michael Bustin	Research Chemist	LMC NCI

COOPERATING UNITS (if any)

Bionetics Research, Inc. (Drs. Terry D. Copeland and Stephen Oroszlan)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

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

☐ (a1) Minors

☐ (a2) Interviews

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

Rabbit beta-globin readthrough protein is the only known naturally occurring readthrough protein in higher eukaryotes which does not involve a viral system. Since suppressor tRNAs have been used in gene therapy experiments and have been implicated in inhibiting viral expression, it is important to characterize the nonsense suppressor tRNA involved in the expression of this unique protein. Experiments were devised, therefore, to isolate this protein from rabbit reticulocytes and to identify the amino acid at the readthrough site. Specific antibodies against this protein were prepared by synthesizing a 22 amino acid peptide which corresponds to the readthrough portion of the beta-globin readthrough protein, coupling the peptide to the KLH protein and injecting the conjugated protein into a sheep. Specific antibodies were produced which were purified and used to isolate the readthrough protein. The protein was purified to homogeneity by electrophoresis on a polyacrylamide gel and the purified product is presently being sequenced through the readthrough site.

Wirth PJ, Vesterberg O. Rat liver cytosolic protein changes after ethanol exposure studies by two-dimensional electrophoresis. Electrophoresis 1988;9:47-53.

Wirth PJ, Yuspa S, Thorgeirsson SS, Hennings H. Induction of common patterns of polypeptide synthesis and phosphorylation of calcium and 12-O-tetradecanoyl-phorbol-13-acetate in mouse epidermal cell culture. Cancer Res 1987;47:2831-8.

technique, whereas the expression of TAT and asialoglycoprotein receptor genes were absent. This is an indication that TGFβ enhances the differentiation of RLE cells towards the fully differentiated adult hepatocyte.

(4) Coinduction of MDR-1 and Selective P-450 Cytochromes. The levels of mRNA for both MDR-1 and selective cytochrome P-450 genes were determined in adult rat liver following administration of various natural and synthetic xenobiotics. MDR-1 was induced following administration of aflatoxin B1, 2-acetylaminofluorene (AAF), N-hydroxy-2-acetylaminofluorene (N-OH-AAF), isosafrole, phenothiazine, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), but not after phenobarbital (PB) or 7-hydroxy-2-acetylaminofluorene (7-OH-AAF) treatment. Cytochrome P-450 isoform d was induced by TCDD, isosafrole, phenothiazine and AAF, while cytochrome P-450 isoform **b** was induced by PB and to a lesser extent by isosafrole. Furthermore, aflatoxin, AAF and TCDD induced MDR in the B6 mouse but not in the D2 (Ah receptor-deficient) mouse, which suggests that MDR induction via aflatoxin may be by a ligand-responsive transcription factor, possibly the Ah receptor or another cytosolic receptor. Taken together, the above data have placed MDR into a set of programmed "alarm response" genes, such as glutathione-S-transferase and UDP-glucuronyl-transferase, aimed at protecting the organism against the harmful effects of xenobiotics.

Publications:

Dybing E, Dahl JE, Beland FA, Thorgeirsson SS. Formation of reactive 1-nitropyrene metabolites by lung microsomes and isolated lung cells. Cell Biol Toxicol 1986;2:341-455.

Huber B, Thorgeirsson SS. Analysis of c-*myc* expression in a human hepatoma cell line. Cancer Res 1987;47:3414-20.

Klinken SP, Holmes KL, Morse HC, Thorgeirsson SS. The effect of DMFO on the expression of c-*myc*, c-*myb* and p53 during proliferation and differentiation of Friend murine erythroleukemia cells. Exp Cell Res (In Press).

Thorgeirsson SS. Modulation of gene expression during early stages of chemical hepatocarcinogenesis. In King CM, Romano LJ and Schuetzle D, eds.: Carcinogenic and mutagenic responses to aromatic amines and nitroarenes. New York: Elsevier, 1988;315-20.

Thorgeirsson SS, Garfield SH, Huber BE, Burt RK. Acquisition of multidrug resistance in chemical hepatocarcinogenesis. 22nd Hoechst Symp on Toxicological and immunological aspects of drug metabolism and environmental chemicals. (In Press).

Thorgeirsson SS, Nagy P, Evarts RP. Cellular and molecular changes in early stages of hepatocarcinogenesis. Proc 4th Sardinia Intl Mtg on Models and mechanisms and chemical carcinogenesis, Italy. New York: Plenum Press (In Press).

Thorgeirsson SS, Wirth PJ. Biochemical marker alterations in hepatic preneoplasia and neoplasia. In Sirica A, ed.: The pathobiology of neoplasia. New York: Plenum Press (In Press).

in normal RLE cells. The transforming ability of 3611MSV, a raf-containing retrovirus; J5, a v-myc containing retrovirus, J2; a v-myc and v-raf-containing retrovirus; and pRNR16, a v-H-ras-containing-retrovirus, were tested. The RLE cells were infected in culture with 3611, J5, J2 and pRNR16 according to Miller et al. (Mol. Cell. Biol. 6: 2895, 1986). Gamma-glutamyltranspeptidase (GGT)-positive foci of altered cells developed within two weeks post-infection with J2, 3611 and pRNR16, but no foci were induced by J5. The J2 virus was 4-5 times more efficient in transforming the RLE cells than the 3611 and pRNR16 viruses. Cells isolated from 3611, J2 and pRNR16-induced foci and from cultures infected with J5 were injected s.c. in nude mice. The J2, 3611 and pRNR16-induced focal cells developed rapidly growing tumors, whereas the J5-infected cells did not form tumors. Both 3611 and pRNR16-induced tumors were morphologically characterized as poorly differentiated carcinomas. In contrast, J2-induced tumors had more differentiated morphology.

These findings demonstrate that v-raf and v-H-ras and, in particular, a combination of v-raf and v-myc are efficient transforming agents of the RLE cells, whereas c-myc alone is apparently incapable of transforming these cells. Furthermore, these studies indicate that the different tumor types derived from infected RLE cells reflect expression of selected oncogenes or a combination of oncogenes.

(2) Transformation of RLE Cells with v-H-raf or v-ras Causes Expression of MDR-1, GST-P and Increased Resistance to Cytotoxins

It is well documented that chemical carcinogenesis results in tumors that are resistant to the cytotoxic and growth inhibitory effects of carcinogens. Whether the multidrug resistance of chemically induced tumors is an integral component of transformation or simply selected for by chemical exposure and unrelated to transformation per se is unclear. Therefore, we have examined the relationship between transformation and multidrug resistance by employing the v-H-ras or v-raf oncogenes to transform RLE cells in vitro. The data indicate that transformation of RLE cells with v-H-ras or v-raf results in increased resistance to the cytotoxins adriamycin, vinblastine, and 2-acetylaminofluorene. This multidrug resistance is accompanied by increased expression of P-glycoprotein (MDR-1) and glutathione-S-transferase P (GST-P). Thus, neoplastic transformation of RLE cells with v-raf or v-H-ras, independent of chemical exposure, results in the expression of genes such as MDR-1 and GST-P which are known to confer upon cells a survival advantage to environmental stress.

(3) Transforming Growth Factor-β (TGFβ) Induced Differentiation of RLE Cells. The TGFβ is one of the best characterized growth factors. It regulates cell proliferation and differentiation and is essential for several biological functions in the presence of other growth factors. Its actual effect depends on the cell type, environment and the presence of other growth factors. The cytostatic effect of TGFβ on RLE cells was observed earlier in our laboratory (Cancer Res. 46: 4665-4671, 1986). However, TGFβ did not inhibit the proliferation of the chemically transformed RLE cells. When RLE cells were treated with TGFβ, an increased expression of albumin, α-fetoprotein (AFP) and multiple drug resistant gene-1 (MDR-1) were observed using the solution hybridization

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Peter Nagy	Visiting Fellow	LEC NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Susan H. Garfield	Chemist	LEC NCI
Brian Huber	Senior Staff Fellow	LEC NCI
Richard K. Burt	Biotechnology Research Fellow	LEC NCI
Kay Johnson	Biologist	LEC NCI

Objectives:

The main objective of this project is to define the genetic determinants for the initiation stage in hepatocarcinogenesis and subsequent evolution of liver tumors that are brought about by chemical carcinogens and other cancer-causing agents. We then plan to examine the in vivo progression of these initiated cells when subjected to a variety of promotion stimuli (phenobarbital, 2,3,7,8-tetrachloro-dibenzo-p-dioxin, etc.) We will employ the rat liver and established normal RLE cell line as a model for these studies.

The research is currently focused on: (I) transfecting RLE cells with molecular chimeras of retroviral-associated oncogenes that are associated with chemical hepatocarcinogenesis; (2) determining if morphologic transformation and tumor formation cells is realized upon activation of these genes; (3) characterizing the cytomorphological and cytochemical changes, should transformation occur, and contrasting these changes with those observed with chemically initiated hepatocytes; (4) examining the response of these virally transformed cells to known promoters of chemically induced hepatocarcinogenesis in both anchorage-dependent and -independent conditions, growth inhibitors and growth factors known to act upon rat hepatocytes; and (5) transplanting the virally transformed hepatocytes into isogenic hosts and characterizing the growth and progression toward primary tumor formation under stimuli of glucocorticoid and/or liver tumor promoters.

Methods Employed:

Methods used in these studies include: tissue culture techniques, radioisotopic measurements, enzyme assays, histochemical and immunohistochemical methods, and recombinant and molecular technology including DNA and RNA preparation, Northern and Southern blotting, construction of cDNA, genome libraries and nucleic acid hybridization, and construction of retroviral vector systems.

Major Findings:

(I) Transforming Oncogenes. To determine the possible contribution of v-*raf* and v-*myc* oncogenes as well as the activated H-*ras* in hepatocarcinogenesis, we have used retroviral vectors containing these genes to test their transforming ability

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05453-04 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Genetic Determinants in Chemical Hepatocarcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Affiliation
PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Peter Nagy	Visiting Fellow	LEC NCI
	Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
	Susan H. Garfield	Chemist	LEC NCI
	Brian Huber	Senior Staff Fellow	LEC NCI
	Richard K. Burt	Biotechnology Research Fellow	LEC NCI
	Kay Johnson	Biologist	LEC NCI

COOPERATING UNITS (if any)

Laboratory of Immunopathology, NIAID (Dr. H. C. Morse, III)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.7	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

We have continued to utilize experimental hepatocarcinogenesis in the rat as a model to study the mechanism of neoplastic development with particular emphasis on defining the possible role of a set of oncogenes that are commonly associated with the process of hepatocarcinogenesis in vivo. Previous results had consistently shown up-regulation of the expression of _myc_ and _raf_ oncogenes during chemical hepatocarcinogenesis. In order to examine the transforming potential of these and other oncogenes in the liver system we have established an in vitro transformation system consisting of a retroviral vector containing relevant oncogenes and rat liver-derived epithelial (RLE) cell line as the reporter cell. The main findings include: (1) v-_raf_, H-v-_ras_ and a combination of v-_raf_ and v-_myc_ are potent transforming agents in the RLE cells. (2) Different tumor types were observed following transplantation of the infected cells. The most undifferentiated tumors originated from the v-_raf_-infected cells whereas transformation with v-_raf_/f-_myc_ combination resulted in hepatocellular carcinoma. (3) Transformation of RLE cells with v-_raf_ and v-H-_ras_ resulted in increased expression of the MDR-1 gene and the development of multidrug resistance. (4) We have established that transforming growth factor-beta-1 (TGF-beta-1) is capable of differentiating the RLE cells towards the adult hepatocyte phenotype. Furthermore, transformation of the RLE cells block the TGF-beta induced differentiation of these cells.

Falzon M, Sanderson N, Chung SY. Cloning and characterization of rat homeobox containing DNA sequences. Gene 1987;54:23-32.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Brian Huber	Senior Staff Fellow	LEC NCI
K-H. Lin	Visiting Fellow	LEC NCI
Nancy Sanderson	Chemist	LEC NCI

Objectives:

New developments in the past few years have made it possible to generate transgenic mice which carry introduced foreign genes in their germ line genome. The technique involves microinjecting cloned DNA fragments into the pronucleus of a fertilized egg. A number of laboratories have reported that the introduced genes integrated into the host genome are stably transmitted to future generations following Mendelian inheritance. Many of the introduced genes are expressed in a tissue-specific manner and the patterns of gene expression are transmitted to the offspring in some lines of transgenic animals. The overall objectives of this study are to exploit the transgenic mouse system to introduce natural or manipulated gene sequences into the germ line of an animal and to alter its phenotype and genetic background. This provides us a new way to investigate tissue-specific and developmental stage-specific regulation of gene expression. Furthermore, the interaction of the introduced gene and its biological effects on the host animal can be monitored throughout normal and malignant development from embryogenesis to adulthood. The current proposal focuses on the neoplastic development in the liver following introduction of the v-raf oncogene under the control of liver-specific promoters into the 1-cell embryo.

Methods Employed:

(1) Recombinant DNA techniques, (2) DNA sequencing analysis, (3) microinjection, (4) gel electrophoresis and nucleic acid hybridization analysis, and (5) histology and in situ hybridization.

Major Findings:

We have created transgenic mice lines following microinjection of two recombinant DNA constructs into 1-cell stage embryos: (a) a v-raf oncogene, and (b) a genomic human keratin gene. The genomic localization and expression of the introduced genes are being analyzed.

Publications:

Albrechtsen R, Wewer UM, Thorgeirsson SS. De novo deposition of laminin-positive basement membrane in vitro by normal hepatocytes and during hepatocarcinogenesis. Hepatology 1988;8:538-46.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05452-04 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Neoplastic Development in Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Affiliation
PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Brian Huber	Senior Staff Fellow	LEC NCI
	K-H. Lin	Visiting Fellow	LEC NCI
	Nancy Sanderson	Chemist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	1.7	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The overall objective of this project is to employ the transgenic mouse system to study neoplastic development in vivo. This system provides a new way of investigating tissue-specific and developmental stage-specific regulation of both natural and manipulated gene sequences introduced into the germ line of an animal. We are currently focusing on the neoplastic development in the liver employing the v-raf oncogene as the transforming agent. The v-raf under the control of liver-specific promoters from both albumin and alpha-fetoprotein genes has been microinjected into the 1-cell embryo, and we are in the process of establishing transgenic lines from the progeny.

The steroid antagonist, dexamethasone-21 mesylate (DM), is unable to activate transcription of the MMTV promoter despite the apparent high affinity of DM-activated receptor for specific binding sites on pure DNA (our earlier findings). We find that DM-receptor complex migrates normally to the nucleus in minichromosome-containing cells, but is unable to induce DNaseI hypersensitivity at the MMTV LTR, or induce loading of the preinitiation complex at the MMTV promoter. Thus high-affinity, site-specific binding of a receptor-hormone complex is not a sufficient condition for in vivo activation. The productive interaction of the activated receptor with chromatin is a more complex process than simple DNA binding.

Publications:

Lichtler A, Hager GL. Induction of multiple replacement mutations by oligonucleotide-directed mutagenesis with extended mismatch primers. Gene Anal Tech 1987;4:111-6.

Richard-Foy H, Hager GL. Sequence specific positioning of nucleosomes over the steroid-inducible MMTV promoter. EMBO J 1987;6:2321-8.

Sistare FD, Hager GL, Simmons SS. Mechanism of dexamethasone 21-mesylate antiglucocorticoid action: I. Receptor-antiglucocorticoid complexes do not competitively inhibit receptor-glucocorticoid complex activation of gene transcription in vivo. Mol Endocrinol 1987;1:648-58.

Major Findings:

Previous efforts had focused on characterization of cell lines with the steroid-inducible MMTV long terminal repeat (LTR) promoter mobilized on BPV vectors, and the analysis of chromatin structure with micrococcal nuclease. The normal steroid transcriptional response was shown to take place in the episomal environment, validating the system as appropriate to study transcriptional regulation of the MMTV promoter with this system. Data with micrococcal nuclease suggested that nucleosomes were specifically positioned, or phased, over the MMTV promoter and associated regulatory regions. The micrococcal nuclease data was confirmed by repeating the phasing experiments with the chemical cleavage reagent, methidium propyl EDTA-FeII. The analysis of MMTV LTR chromatin with this reagent has also been completed, giving essentially the same result as was obtained with micrococcal nuclease. We therefore conclude that nucleosomes are in fact phased over this region of the MMTV genome. MMTV DNA always acquires this specific nucleoprotein organization, no matter how the DNA is introduced into the cell.

When the MMTV promoter is induced, this highly structured nucleoprotein complex is specifically altered. A broad region of hypersensitivity, detected either by DNaseI or MPE, develops across a region of the promoter that is normally associated with nucleosome B in the phased array. These results indicate that the steroid receptors that modulate MMTV transcription interact with a region of DNA that is displayed on the surface of a nucleosome, and suggest that receptor binding and promoter activation is accompanied either by nucleosome displacement, or by a major modification of nucleosome structure.

In several systems, it has been argued that specific nucleosome positioning over a protein binding site will prevent factor access to its recognition site in the absence of a nucleosome destablizing process such as DNA replication. To confirm the identity of the complex protecting region B from nucleolytic attack, we have performed in vivo cross-linking studies. These results support the conclusion that the B region structure is in fact a nucleosome. In addition, we find that the MMTV promoter can be normally induced in the absence of DNA synthesis, indicating that replication-associated nucleosome disruption is not required for receptor binding.

We conclude that one transcription factor, the glucocorticoid receptor, can bind to its recognition site organized on a nucleosome and actively displace the octamer core from the phased array. This is the only promoter system in which this process is known to occur. Other transcription factors that participate in the formation of the preinitiation complex, although present in the uninduced nucleus (see project Z01CP04986-09 LEC), are excluded from non-induced chromatin. Two models are consistent with these results. Synergistic interactions between soluble components of the initiation complex may provide sufficient energy to disrupt nucleosome structure and shift the equilibrium of the preinitiation complex to a promoter bound state. Alternatively, receptor-induced disruption of nucleosome structure could provide a modified template to which the other soluble components of the preinitiation complex could bind.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gordon Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Trevor Archer	Visiting Fellow	LEC NCI
Diana S. Berard	Microbiologist	LEC NCI
Anna Riegel	Visiting Fellow	LEC NCI

Objectives:

The primary objective of this project is to analyze the nucleoprotein structure of genes in mammalian cells subject to transcriptional control, and to elucidate the role, if any, of chromatin organization in mechanisms of gene regulation. To facilitate this objective, a variety of regulated genes are to be mobilized on episomal, bovine papillomavirus (BPV)-based vectors. This permits a detailed examination of nucleoprotein structure in an amplified, homogeneous chromatin population that is isolated from potential interfering effects of chromosomal chromatin. After isolation of cell lines containing well-characterized (and well-behaved) episomal chimeras, the analysis of chromatin structure involvement in transcriptional regulation is to be studied.

Methods Employed:

Genes responsive to transcriptional regulation are introduced into murine cells by mobilization on the BPV 69% transforming fragment. Cell lines are isolated that uniquely contain non-rearranged, non-integrated copies of the chimeric constructions. These lines are then characterized for copy number, DNA structure, and transcriptional regulation.

Minichromosomes for run-on transcription assays are prepared from nuclei of cells containing episomes by ammonium sulfate extraction. Purified episomes are incubated with labelled ribonucleotides, and RNA extension products are analyzed for sequence content by hybridization to single-stranded probes.

Steady-state levels of intracellular RNA are measured by quantitative S1 nuclease mapping with specific end-labelled RNA or DNA probes.

Nucleosome positioning is analyzed by incubation of nuclei with either micrococcal nuclease, restriction enzymes, or methidium propyl ethylenediaminetetraacetate-FeII (MPE). Each reagent cleaves chromatin preferentially at internucleosomal linker regions. The positions are then determined by indirect end-labelling analysis with probes directionally positioned relative to specific restriction sites.

Hypersensitive sites are detected either with DNaseI or MPE. The position of these sites is also determined by indirect end-labelling analysis.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05450-04 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Chromatin Structure and Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Gordon Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Others:	Trevor Archer	Visiting Fellow	LEC NCI
	Diana S. Berard	Microbiologist	LEC NCI
	Anna Riegel	Visiting Fellow	LEC NCI

COOPERATING UNITS (if any)

Universite de Paris XI, Dept de Chimie Biologique, Lab Hormones 94270, Bicetre France (Dr. Helene Richard-Foy); Laboratory of Chemistry, NIDDK (Drs. S. Stoney Simmons and F. Sistare)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.4	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The genetic information in mammalian cells exits is organized into a highly condensed nucleoprotein structure whose basic repeating subunit is the nucleosome. Although the major function of this structure is usually thought to be packaging the very large amounts of DNA into a minimal volume, an important and unanswered question concerns whether the interaction of transacting gene regulatory factors with their DNA-binding sites is affected by the organization of these sites in chromatin. We have shown that nucleosomes are phased across the steroid-regulated mouse mammary tumor virus (MMTV) promoter. The sites to which steroid-receptors bind are displayed on the surface of nucleosome B in this phased array. Hormone activation of the promoter leads to active displacement of this nucleosome, in contrast to other systems where nucleosome deposition is thought to inhibit factor access. Receptor activated by a steroid antagonist, dexamethasone-21 mesylate, although translocated to the nucleus, is unable to interact productively with MMTV chromatin.

applied to the gel. The electrophoretic transfer of proteins to pre-derivatized glass fiber paper proved more successful. High transblotting efficiencies were achieved (usually >80% depending on the amount and MW of the protein) using GF/C paper coated with quaternary ammonium functions and it was possible to obtain the correct sequence of the first 17 amino acids of soybean trypsin inhibitor after transblotting from a one-dimensional SDS-polyacrylamide gel following the loading of only 50 pmoles onto the gel. The use of alternative supports for protein transblotting was investigated. Both PCGM-1 glass fiber paper and Immobilon also proved successful for protein electro-blotting and sequencing, and blotting yields of greater than 80% were obtained if electroblotting times were optimized for the individual proteins. Initial sequencing yields for standard proteins were greater than 60% regardless of the support used for electroblotting. Due to ease of use, Immobilon-P was selected as the support of choice and this was used for further optimization of electro-phoresis conditions. The necessity of various precautions that reportedly reduce N-terminal blockage of proteins during electrophoresis were investi-gated. The use of radical scavengers, such as glutathione during electro-phoresis had no effect on subsequent sequencing yields of the transblotted proteins. In addition recrystallization of SDS was unnecessary and samples could be boiled prior to electrophoresis without any effect on their sequence analysis. Sequencing yields were also independent of the size, thickness or acrylamide content of the gels. However, the initial yields obtained on sequence analysis of proteins electroblotted from 2D-PAGE were generally about 25% less than the yields obtained on sequencing proteins from 1D-PAGE. Changing the source and pH range of the ampholines used, or pre-focusing the tube gels had no effect on this and we are currently investigating the reason for the apparent N-terminal blockage that occurs during isoelectric focusing. However, despite this problem we have been able to routinely sequence 10-20 residues of standard proteins applied to 2D-PAGE at the 50-100 pmole level. With 1D-PAGE we are able to sequence up to 10 residues of proteins applied to the gel at the 5 pmole level and up to 20 residues at the 20 pmole level. The methodology has been optimized for a number of standard proteins of varying hydrophobic character and molecular weights ranging from 14 to 120 kD and it has been successfully applied to the sequence analysis of a number of uncharacterized rat liver membrane glycoproteins. The methodology is now at a stage where it is being applied to the investigation of a number of other neoplasia-related polypeptides that have been identified by 2D-PAGE analysis. The microscale sequence analysis of amino-terminal blocked proteins remains a major problem and the possible use of SDS-PAGE or microbore HPLC for the separation of peptide fragments, following chemical or enzymatic fragmentation, prior to their direct sequence analysis are under investigation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Anthony C. Huggett	Visiting Associate	LEC, NCI
Betty R. Yu	Biologist	LEC NCI
Tim Benjamin	Chemist	LEC NCI
David Parmelee	Expert	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI

Objectives:

The purpose of this project is to isolate and characterize relevant proteins from polyacrylamide gels following preparative one- and two-dimensional gel electrophoresis (2D-PAGE) of relatively crude tissue preparations. The availability of partial amino acid sequence analysis information will allow the construction of DNA probes which will facilitate both gene cloning and gene expression studies.

Methods Employed:

The principal methods employed are: (1) one- and two-dimensional PAGE; (2) transblotting of proteins to derivatized glass fiber paper and polyvinylidene difluoride-membranes (Immobilon-P); (3) gas-phase amino acid sequence analysis; (4) electroelution and electroblotting of proteins; (5) microbore high performance liquid chromatography (HPLC); (6) microscale chemical and enzymic digestion of proteins; (7) amino acid analysis; and (8) fast atom bombardment mass spectrometry.

Major Findings:

The computer-assisted two-dimensional gel electrophoresis methodology developed within this laboratory has provided a highly sensitive procedure capable of resolving large numbers of proteins within relatively crude preparations. Not only does this method provide a technique for the identification of marker proteins and other proteins that may be causally related to the multistage process of neoplastic transformation but it also provides a preparative procedure for their purification prior to amino acid sequence analysis. A number of "interesting" proteins have been defined using this procedure which await the development of techniques to allow their identification.

The isolation and characterization of proteins from 2D-PAGE gels requires the development of a battery of microanalytical techniques due to the small quantity of protein present in a gel spot. Studies evaluating various techniques for the recovery of proteins from SDS-polyacrylamide gels have demonstrated that electroelution and passive diffusion procedures are unsuitable since the presence of gel contaminants and amino-terminal blocking of proteins necessitates that large amounts (> 500 pmoles) of sample must be

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05447-04 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Isolation & Characterization of Proteins from Two-Dimensional Polyacrylamide Gels

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Anthony C. Huggett	Visiting Associate	LEC	NCI
Others:	Betty R. Yu	Biologist	LEC	NCI
	Tim Benjamin	Chemist	LEC	NCI
	David Parmelee	Expert	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.4	0.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The purpose of this project is to develop the analytical technology required for the elution and subsequent microsequencing of proteins from two-dimensional polyacrylamide gels. A number of "interesting" protein spots have been defined whose regulation is markedly altered during the multistep process of neoplastic transformation. Initially, microscale procedures aimed at the recovery and sequence analysis of these proteins from one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were investigated. Electroelution and passive extraction techniques were found to be suitable only when large amounts (> 500 pmoles) of protein were available. Electroblotting techniques in which the proteins are transferred to quaternary ammonium-derivatized glass fiber paper were modified such that 50 pmoles of soybean trypsin inhibitor applied to a 1D-SDS-polyacrylamide gel could subsequently be correctly sequenced to 17 cycles. Other supports for protein electroblotting and subsequent microsequence analysis were investigated and Immobilon-P was found to be the support of choice. Using this support electrophoretic conditions were optimized to allow the N-terminal sequence analysis of up to 20 residues from 50-100 pmoles of protein applied to a 2D-gel. With 1D-PAGE it was possible to obtain some sequence information from as little as 5 pmoles protein. This procedure was successfully applied to the sequence analysis of a number of previously uncharacterized membrane glycoproteins. Work is currently underway to apply this technique to the analysis of other unknown proteins whose expression is altered during carcinogenesis and also to improve the sequencing yields of proteins isolated from two-dimensional polyacrylamide gels.

Studies have begun on investigating the nature of interaction of the native FN hexamer segment GRGDSP with its anti-sense peptide. An analytical affinity column was constructed with the anti-sense peptide WTVPTA covalently attached to it. Using this approach we have found a dissociation constant of $9x10^{-4}$ for GRGDSP/WTVPTA interaction. This result compares favorably with the inhibition constant of approximately $1x10^{-4}$ (50% inhibition) obtained for the pentamer GRGDS competing with native FN for the cellular receptor site (Akiyama and Yamada, 1985). Our current CD studies indicate that the anti-sense hexamer has a random conformation by itself, both in methanol and in aqueous pH 5.7 buffer. CD studies have been initiated in attempts to observe conformational changes as a consequence of sense/anti-sense peptide interactions. Conditions are being optimized to make progress in this area.

Publications:

Fales HM, Blum MS, Southwick EK, Williams DL, Roller PP, Don AW. Structure and synthesis of tetrasubstituted pyrazines in ants in the genus *Mesoponera*. Tetrahedron (In Press).

Falzon M, Vu VT, Roller PP, Thorgeirsson SS. Relationship between 7,12-dimethyl- and 7,8,12-trimethyl-benz(a)anthracene DNA adduct formation in hematopoietic organs and leukemogenic effects. Cancer Lett 1987;37:41-9.

Han K, Niu CH, Brooks RR, Ferretti JA, Roller PP. Structures of human TGF-alpha fragments using two-dimensional proton magnetic resonance spectroscopy and computer simulation. In: Marshall GR, ed. Peptides: chemistry and biology. Leyden, The Netherlands: ESCOM Publishers, 1988:581-3.

Han KH, Niu CH, Roller PP, Ferrett JA. Conformation of the second loop in human transforming growth factor-alpha studied by two-dimensional NMR spectroscopy. Biopolymers (In Press).

Huggett AC, Cone JL, Thorgeirsson SS, Roller PP. Novel synthesis and spectral characterization of an N-acetoxy-arylamine, N-acetoxy-2,4-dinitrophenylamine. J Org Chem 1987;52:4933-7.

Misra R, Pandey RC, Hilton BD, Roller PP, Silverton JV. Structure of fredericamycin A, an antitumor antibiotic of a novel skeletal type: spectroscopic and mass spectral characterization. J Antibiot 1987;40:786-802.

Niu CH, Han K, Roller PP. Conformational studies of the N-terminal segments of ras p21 proteins. In: Marshall GR, ed. Peptides: chemistry and biology. Leyden, The Netherlands: ESCOM Publishers, 1988;572-4.

(Ala^{21})-TGF-alpha(16-32) peptide that comprises the second loop of the native hTGF-alpha molecule. The peptide was found to be ellipsoidal with a beta turn at Gly19 and a bend formed by four residues from Gln26 to Lys29. These features are similar in EGF. A more contrasting difference between the structure of the structure of the second loop of EGF and that of TGF-alpha is that only in EGF does the second loop form an antiparallel beta sheet structure. The correlation of the conformations of the various growth factor peptide segments to their receptor binding capacities awaits further experimentation.

In methods development studies conditions have been worked out for the reductive alkylation of cyclic disulfide-containing peptides with diethithrietol/4-vinylpyridine. The reagent is specific for -SH groups. The derivatization increases the solubility of peptides in acidic and neutral medium, shows distinctive UV absorbance characteristics, and the linearized peptide allows for generation of sequence- specific ions in the mass spectra. In current efforts the benefit of using resin-bound reducing agent prior to alkylation is being explored. Such a procedure should produce a much more pure product, in effect improving the sensitivity of the method.

During the course of synthesizing peptides occasionally multiple products are formed. In those cases MW analysis of the various HPLC components provides a quick answer for selecting the desired peptide. Quite frequently peptides were found to be irreversibly dehydrated, most likely at the serine or threonine residues. Other problems found were, premature termination of the synthesis, incomplete removal of the (bromo)benzyloxycarbonyl protective group from tyrosine, and the presence of beta-aspartyl peptide (isopeptide) bonding in an Asp-Ser-containing hexamer.

(2) Conformational studies of the cell-binding peptide segment of fibronectin. The attachment of cells to the extracellular matrix components, such as fibronectin (FN), is an important process in embryogenesis, differentiation and tumor invasion. FN contains a short hydrophilic segment RGDS that interacts with specific receptors located in the plasma membranes. In order to study the structural and conformational requirements of this hydrophilic loop toward receptor binding, several peptide analogs were synthesized. These include the native FN pentamer segment GRGDS and hexamer GRGDSP, the biologically inactive glutamic acid analog GRGES, a D-alanine-containing pentamer GR(d-A)DS, and the beta bend-forming pentamer GRPDS. In addition, the anti-sense peptide WTVPTA, designed on the basis of the complementary rat DNA, has been synthesized. The latter peptide was recently shown to effectively bind to native FN with properties resembling the cellular receptor in terms of function, but not necessarily in its sequence (Proc. Natl. Acad. Sci. USA 85: 364, 1988).

Conformational studies using circular dichroism (CD) spectroscopy show that the native segment GRGDS has a more highly ordered secondary structure, possibly a "beta turn," compared to GRGES. Two-dimensional proton NMR studies are being carried out to ascertain such a conformation in the native pentamer. The CD spectrum of the native hexamer GRGDSP is very different from the spectrum of the native pentamer, being dominated by spectral features characteristic of the proline beta bend structure alone.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
Chien-Hua Niu	Volunteer Scientist	LEC NCI
Giorgio Fassina	Visiting Associate	LEC NCI
James G. Omichinski	IRTA Fellow	LEC NCI

Objectives:

To study, in some detail, the chemical structure and physicochemical characteristics of certain natural biopolymeric materials, with emphasis on peptides and proteins that play a role in cell growth regulation. To develop and apply the modern mass spectrometric methods to the solution of structural problems, particularly to accurate molecular weight determination and to the amino acid sequencing of peptides. To study the molecular conformation of cell recognition peptides, and their effect on binding to model cellular receptor mimicking peptides.

Methods Employed:

(1) Fast atom bombardment (FAB) ionization mass spectrometry; (2) proton and carbon-13 nuclear magnetic resonance (NMR) spectroscopy in the solution state; (3) circular dichroism spectropolarimetry; (4) ultraviolet spectroscopy; (5) chemical synthesis of peptides; (6) chemical derivatization and modification of peptides; (7) high pressure liquid chromatography; and (8) analytical affinity chromatography.

Major Findings:

(1) Chemical structure confirmation by FAB mass spectrometry and NMR. There are a number of situations where the FAB mass spectral method can provide a speedy method of solving peptide structural problems. Thus, molecular weight information, obtainable routinely with mass accuracy of one or better, can be helpful in confirming the identity of peptides, in providing complementary information to Edman sequencing on tryptic or other digested protein fragments, and in uncovering the presence of blocked or otherwise modified peptides. Our current capabilities allow molecular weight measurements on peptides containing up to 23 amino acid residues (i.e., to MW 2500).

Several disulfide (cysteine)-containing peptides were synthesized in our laboratory. These peptides were designed as structurally similar variants of EGF and of TGF-alpha segments. The natural growth factors contain three loops each. The four synthetic peptides, comprising one loop only, demonstrated varying degrees of stabilities with respect to sulfoxide formation and to oligomerization. Molecular weight analysis was found to be necessary before the conformational studies by 2D NMR could be undertaken. Studies have been completed on the

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05374-05 LEC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Structural and Physicochemical Studies of Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
Others:	Chien-Hua Niu	Volunteer Scientist	LEC NCI
	Giorgio Fassina	Visiting Associate	LEC NCI
	James G. Omichinski	IRTA Fellow	LEC NCI

COOPERATING UNITS (if any)

Laboratory of Chemistry, NHLBI (Dr. Kyouhoon Han)

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION
Biopolymer Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 1.4	PROFESSIONAL: 1.4	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

This project involves studies on the chemical structure and physicochemical characteristics of certain natural biopolymeric materials and their synthetic analogs with the aim of relating the resulting structural information to their biological mode of action, such as cell growth regulation, cell transformation or differentiation. The modern methods of mass spectrometry, nuclear magnetic resonance and circular dichroism spectroscopies and various chemical methods are being applied. Projects include: (1) development of a fast atom bombardment mass spectrometric method of analyzing disulfide-linked dimeric peptides and of epidermal growth factor (EGF) and transforming growth factor (TGF)-alpha-related cysteine-containing cyclic disulfides. In our approach reductive pyridinoethylation of the peptides yielded derivatives that were better suited for molecular weight and amino acid sequence analysis. In addition a large number of synthetic peptides were analyzed with the molecular weight limit of 2500 for ascertaining the correctness of the synthesis and the oxidation state. (2) Five peptides related to the RGDS segment of the cell attachment factor, fibronectin, were synthesized. This hydrophilic segment in fibronectin interacts with cellular receptors. Circular dichroism studies indicate various molecular conformations for these peptides, the spectrum of the native pentamer indicating a beta turn conformation. The anti-sense peptide, WTVPTA to the native hexamer GRGDSP was also synthesized. Analytical affinity chromatography using the immobilized anti-sense column demonstrated a dissociation constant of 9x10-4 for the latter two peptides.

inhibitor. Most recent work is focused on the large-scale purification of the liver-derived growth factor. Livers from 12,000 adult rats (mixed sex and strain) have been used. Following homogenization and protein precipitation steps, the preparations were subjected to diethyl amino ethyl (DEAE)-cellulose chromatography. The inhibitory activity eluted at about 0.05 M NaCl with an ID_{50} of about 500 ng/ml. FPLC-gel filtration was employed as the next step and the activity eluted at a MW of 20-25 kD and with an ID_{50} of about 50 ng/ml. The chromatofocusing step used as the next step in the analytical-scale purification proved to be unsuitable for the large-scale purification due to the coelution of a major protein impurity with the peak of growth inhibitory activity. Various alternative chromatographic procedures were examined for suitability as the next step for the large-scale purification. These included reverse phase HPLC, Mono S cation FPLC, Mono Q anion FPLC and alkyl superose FPLC. Each procedure resulted in a purification of the inhibitory activity and it was noted that at least two peaks of inhibitory activity could be observed eluting from each of these columns if conditions were optimized. Mono S cation-exchange chromatography proved to be the most suitable procedure for further purification at this stage and the two growth inhibitory fractions produced had similar ID_{50}s (about 10-20 ng/ml). Alkyl superose FPLC was used as the next step and the resulting inhibitory activity had an ID_{50} of about 1-5 ng/ml. About 4 mg of protein has been obtained at this stage of the purification. Analysis of these preparations by 2D-PAGE indicates the presence of at least 30 proteins. Recent work is primarily focused on the further purification of the liver-derived growth inhibitor in order to obtain pure HPI in quantities sufficient for microsequence analysis. Parallel studies are currently being undertaken in order to determine other possible sources of the inhibitory activity. Human platelets, a rich source of growth regulatory polypeptides, did not contain HPI; and conditioned media from various normal and transformed rat liver epithelial cells are being examined. Initial results indicate that the normal cells do not secrete this factor.

Publications:

Huggett AC, Krutzsch HC, Thorgeirsson SS. Characterization of an hepatic proliferation inhibitor. Effect of HPI on the growth of normal liver cells: Comparison with TGF-beta. J Cell Biochem 1987;35:305-14.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Anthony C. Huggett	Visiting Associate	LEC	NCI
Caroline P. Ford	Microbiologist	LEC	NCI
Betty Yu	Biologist	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The purpose of this project is to isolate and characterize, both biologically and structurally, a growth inhibitory polypeptide from normal adult rat liver. The aim is to investigate the role of this factor in normal cells and also in the neoplastic process.

Methods Employed:

The principal methods employed in these studies include: (1) use of tissue homogenization, protein precipitation, standard column chromatographies, fast protein liquid chromatography (FPLC), microbore high performance liquid chromatography (HPLC) and 1D- and 2D-sodium dodecyl sulfate (SDS)-PAGE to purify the growth inhibitor from the liver of adult rats; (2) use of an immortalized line of neonatal rat liver epithelial cells for the bioassay of growth inhibitors using analysis of DNA content and DNA synthesis; (3) culture of various cell types for the characterization of growth regulatory activity; (4) use of neutralizing antibodies directed against other growth inhibitors to discriminate inhibitory activities.

Major Findings:

Methodology for the analytical-scale isolation of a highly purified growth inhibitor (HPI) from 1000 adult rat livers has been developed. The resulting preparation (1 μg total protein), although still not homogeneous, was highly active as an inhibitor of the proliferation of rat liver epithelial (RLE) cells and of mitogen-stimulated DNA synthesis in primary hepatocyte cultures (ID_{50}: 50 and 250 pg/ml, respectively). It is active as a growth inhibitor on RLE cells in their log-phase of growth and is effective in serum-free and serum-supplemented media. In addition it has been shown to be a growth regulator for other non-liver and non-epithelial cell types and has a growth inhibitory effect on some transformed cells. The factor responsible for these activities was shown to be an acid- and heat-labile polypeptide with a molecular weight in the range 17-25 kD and a pI of 5.5. Physicochemical and biological characterization of the growth inhibitor demonstrated that it is distinct from any of the well-characterized growth inhibitory polypeptides previously reported including TGF-βs, tumor necrosis factor (TNFs) and interferon (IFNs). Western analysis and cell proliferation studies utilizing a neutralizing antibody directed against MDGI have shown that HPI is different from this recently described growth

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05373-05 LEC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Purification and Characterization of a Rat Hepatic Proliferation Inhibitor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Anthony C. Huggett	Visiting Associate	LEC	NCI
Others:	Caroline P. Ford	Microbiologist	LEC	NCI
	Betty Yu	Biologist	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION
Biopolymer Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 1.0	OTHER: 1.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The aim of this project is to isolate and characterize a protein from adult rat liver that produces a reversible inhibition of the proliferation of liver-derived cells. An improved analytical-scale purification procedure was recently developed that produces a preparation with a specific activity about 1000-fold greater than previously reported. This procedure has been modified for the large-scale purification of the growth inhibitor from 12 x 1000 livers. At the present time a preparation (4 mg total protein) with an ID50 of 1-5 ng/ml has been obtained. The preparation contains at least two separate growth inhibitory polypeptides which appear to have similar activities. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis demonstrates that the preparation contains at least 20-30 polypeptides and current efforts are focused on obtaining a pure preparation of the inhibitory activities so that amino acid sequence analysis can be performed. Biological and physicochemical characterization of the liver-derived growth inhibitor(s) has demonstrated that it is different from any known well-characterized growth inhibitory polypeptides including transforming growth factor-beta (TGF-beta), tumor necrosis factor (TNF), interferon (IFN) and mammary derived growth inhibitor (MDGI).

separate sites in the 5′ flank for transcription. One is a TATA box coupled with an upstream GC rich region and the second occurs in an AT rich region which is present within nucleotides -62 to -97. The nature of transcription of the Xenopus gene in vivo suggests that the latter regulatory site may be developmentally expressed.

The bovine GPx gene was obtained from Dr. G. Mullenbach (Chiron Corp) and subcloned into an expression vector for making GPx mRNA. Phosphoseryl-$tRNA_{UGA}$ donates its phosphoserine to this polypeptide in a cell-free protein synthesis system in response to a UGA codon which occurs at the active site of GPx. Selenocysteine occurs at the active site of GPx. Experiments are in progress to determine if phosphoserine or selenocysteine is the 21st naturally occurring amino acid.

The human GPx gene was also obtained from Dr. G. Mullenbach and used as a probe to hybridize to DNA isolated from human rodent somatic cell hybrids. The probe hybridized to chromosome 3, 21 and X. Experiments using the 3′ flanking region and a GPx gene intron as probes suggests that the gene is located in chromosome 21 and that the loci on chromsomes 21 and X are processed pseudogenes.

<u>Publications</u>:

Lee BJ, de la Pena P, Tobian JA, Zasloff M, Hatfield D. Unique pathway of expression of an opal suppressor phosphoserine tRNA. Proc Natl Acad Sci 1987;84:6384-8.

McBride OW, Rajagopalan M, Hatfield, D. Opal suppressor phosphoserine tRNA gene and pseudogene are located on human chromosomes 19 and 22, respectively. J Biol Chem 1987;262:11163-6.

the 5′ flanking region and within the opal suppressor tRNA gene, additional genomic libraries screened, new opal suppressor tRNA genes isolated and deletions and new genes sequenced by standard techniques.

Major Findings:

Four opal suppressor tRNA genes and two pseudogenes have previously been sequenced from human, rabbit, chicken, and Xenopus genes. The pseudogenes occur in mammalian genomes and the gene appears to occur in single gene copy in each of these organisms. This past year an opal suppressor tRNA gene was isolated and sequenced from a nematode, C. elegans. Its structure differs substantially from that of the vertebrate gene demonstrating that this gene has undergone substantial evolutionary change. An examination of the distribution and evolutionary origin of the opal suppressor gene in nature shows that under less stringent conditions of hybridization it is present in genomic DNAs of representatives of the Monera (Clostridum), protist (Ochromonas, L. major and Physarum), fungi (yeast) and plant (wheat germ) kingdoms. It is also present in the genomic DNAs of representatives of many phyla of the animal kingdom. The gene occurs in the Phyla Chordata (tunicates, amphioxus, lamprey, hag fish, horned shark, winter flounder, Xenopus, chicken and bovine), Arthropoda (Drosophila and horse shoe crab), Mollusca (clam, oyster and snail), Annelidal (oligochaets and polychaets), Aschelmenthes (Ascarus and C. elegans) and Porifera (sponge). In most cases, only one or two DNA fragments from each organism hybridized to the probe, with the exception of polychaet DNA which contained several positive fragments. These data show that the gene is widespread in nature and that it apparently has undergone evolutionary change. In addition, we have detected the gene product(s) in the seryl-tRNA populations of fish, Saccoglossus, earthworms and snails. Comparison of the sequences of the opal suppressor phosphoserine tRNAs to those of 478 other tRNAs by a computer program demonstrated that there is no evolutionary linkage between the opal suppressors and any known tRNA sequence. These observations demonstrate that the opal suppressor tRNAs evolved independently of other tRNA genes and that it is apparently a very ancient gene.

Transcription of the human, rabbit, chicken and Xenopus genes were examined in vivo (in Xenopus oocytes) and in vitro (in HeLa cell extracts). They begin transcription, unlike any known tRNA, at the first nucleotide within the gene. They terminate transcription at a T cluster in the 3′ flank. The trailer sequence is cleaved by a purified 3′ processing enzyme. The mature tRNA is transported from the nucleus of the cytoplasm in Xenopus oocytes. The 5′ triphosphate on the mature tRNA is transported from the nucleus and is preserved in the cytoplasm, suggesting that it may have a role in the function of this tRNA. The Xenopus gene is severalfold more efficiently transcribed both in vivo and in vitro, while the human and rabbit genes are transcribed about equally and more efficiently than the chicken gene. Since the Xenopus and chicken genes are identical in sequence and differ from those of human and rabbit by a single pyrimidine transition at position 11, the gene sequences do not account for variations in transcription levels. Exchange of the flanking regions between chicken and Xenopus genes and characterization of the transcription efficiencies of a number of deletions within the 5′ flanking region demonstrate that all of the regulatory sites for expression of this gene occur upstream. There are two

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dolph L. Hatfield	Research Biologist	LEC NCI
Byeong Jae Lee	Visiting Fellow	LEC NCI
Malini Rajagopalan	Visiting Fellow	LEC NCI
O. Wesley McBride	Chief, Cellular Regulation Section	DCBD NCI

Objectives:

The major goals of the project are to understand the structure, expression, function and evolutionary origin of the opal suppressor tRNA genes and the role that the products of these genes have in protein phosphorylation.

Specific steps to achieve these goals are: (1) to isolate and characterize opal suppressor tRNA genes from genomes of a wide variety of organisms; (2) to sequence the genes and their flanking DNA segments; (3) to investigate the structure of the genomic regions that contain these genes with respect to transcription and evolutionary conservation; (4) to study the control of transcription by using in vivo and in vitro transcription systems; (5) to use in vivo transcription systems to study processing and localization of the tRNA product; (6) to make site-specific mutations in the internal control region and in the anticodon region of the tRNA genes and to replace the 5′ flanking sequence with that of another tRNA gene in order to understand better the expression and cellular function of these genes; (7) to subclone the opal suppressor tRNA gene which has the 5′ flanking sequence replaced so that it will make a product in high yields and subclone the ochre suppressor tRNA gene which has been generated by site-specific mutagenesis into a mammalian cell line in order to determine the effects of these suppressors on cellular function (i.e., if the gene products are phosphorylated on the serine moiety and if phosphoserine is incorporated directly into protein); (8) to investigate the distribution and evolutionary origin of this unique gene in nature; and (9) to determine if this phosphoseryl-tRNA is responsible for the occurrence of selenocysteine in glutathione peroxidase (GPx) which occurs at the active site of GPx and is known to be coded by UGA.

Methods Employed:

Genomic DNAs from a wide variety of organisms were obtained from a number of laboratories or were isolated by standard techniques of preparing DNA. These DNAs were digested with restriction enzymes and electrophoresed on agarose gels, Southern blotted and hybridized with an appropriate probe under very stringent and under less stringent conditions of hybridization.

Transcription of the opal suppressor tRNA genes were carried out in the presence of extracts of HeLa cells which were used as a source of RNA polymerase III. The opal suppressor phosphoserine tRNA genes were also injected into Xenopus oocytes,

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05317-05 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Opal Suppressor Phosphoserine and the 21st Naturally Occurring Amino Acid

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Dolph L. Hatfield	Research Biologist	LEC NCI
Others:	Byeong Jae Lee	Visiting Fellow	LEC NCI
	Malini Rajagopalan	Visiting Fellow	LEC NCI
	O. Wesley McBride	Chief, Cellular Regulation Section	DCBD NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Two opal suppressor phosphoseryl-tRNAs and the corresponding gene have been isolated and characterized in this laboratory. They have several unique features which set them apart from all other eucaryotic tRNAs: (1) they are 90 nucleotides in length and thus are the longest eucaryotic tRNAs sequenced to date; (2) they are phosphorylated on their serine moiety to form phosphoseryl-tRNA; (3) they have few modified bases compared to other tRNAs; (4) they are encoded by a single gene even though several pyrimidine transitions occur post-transcriptionally and one of the transitions occurs in the anticodon; and (5) the primary transcript arises, unlike any other known tRNA, without processing on the 5′ side of the gene product. The genes encoding the opal suppressor tRNAs which have been isolated and sequenced from human, rabbit, chicken, Xenopus and nematode genomes are transcribed in vivo in Xenopus oocytes and in vitro in HeLa cell extracts. Two regulatory sites occur upstream of the gene and are involved in its expression. The phosphoserine moiety of phosphoseryl-tRNA is incorporated into the active site of glutathione peroxidase where selenocysteine normally occurs in the protein. Experiments are in progress to determine if phosphoserine or selenocysteine is the 21st naturally occurring amino acid. The glutathione peroxidase gene maps to human chromosome 3, while other loci on chromosomes 21 and X are probably processed pseudogenes.

Publications:

Hager GL. MMTV as a model for gene expression in mammary tissue. In: Lippman ME, Dickson R, eds. Breast cancer: cellular and molecular biology. Martinus Nijhoff B.V., 1988;267-81.

internal MMTV promoter, thus separating expression of the convected gene from transmission, which was based on standard MuLV LTRs.

In addition, we examined the potential for modulating v-$\underline{ras}^{H}$ oncogene expression in the whole animal at levels with measurable biological impact. Expression of this gene has previously been correlated both with tumorigenic potential and metastatic progression of transformed cells. We employed an in vivo lung metastasis assay to monitor the metastatic potential of cells whose v-$\underline{ras}^{H}$ oncogene expression levels have been manipulated in vitro. In these studies, we utilized an NIH 3T3-derived cell line (433) that is conditionally transformed by the v-$\underline{ras}^{H}$ oncogene; that is, levels of p21 gene transcription are sufficiently low in the absence of hormone stimulation such that the cell is morphologically normal. When the p21 protein level is induced by activating the MMTV promoter with hormone, cells rapidly acquire the characteristics of transformed cells. We found that induction of v-$\underline{ras}^{H}$ protein by glucocorticoids markedly affects the metastatic potential of the cells.

These experiments led to two important conclusions. First, a direct correlation between the level of p21 expression and metastatic potential of the cells was established. Secondly, these experiments illustrated the utility of the central objective of this project, that is, the ability to express genes in the whole animal environment at levels relevant to their biologic activity and to control such expression levels.

In current experiments, the primary objective has been to extend vector development to include expression of the ligand-responsive promoter-inducing protein. That is, expression of the steroid receptor protein that activates the promoter of interest is to be incorporated into the vector itself. This modification will allow us to introduce genes into cells and tissues under control of an inducing protein (receptor) not necessarily expressed in those cells. For example, incorporation of the progesterone receptor into the vector would render expression of the MMTV-driven gene responsive to progesterone. Since many cells do not contain functional levels of progesterone receptor, responsiveness of the convected gene would also be vector-dependent. This would open the way to eventual targeting of the convected receptor to only the promoter of interest by genetic modification of the receptor.

We are also examining the utility of this vector concept in the repression of gene expression by antisense mRNA formation. The reversion of oncogene expression by the introduction of antisense RNA cassettes represents one avenue by which oncogenic transformation could be reversed. The application of the inducible vector concept to this problem would permit transient activation of an inducible antisense message for the period of time necessary to accomplish phenotype reversion, followed by withdrawal of the inducer. This represents a more feasible approach to antisense RNA therapy, compared to antisense expression from a constitutive promoter.

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Diana S. Berard	Microbiologist	LEC NCI
Ronald G. Wolford	Microbiologist	LEC NCI

Objectives:

The primary objective of this project is to develop and evaluate methods for the controlled expression of genes in mammalian cells, and to explore the application of these principles to gene therapy. Hormone-regulation of steroid-receptor-inducible promoters is the model under investigation. Tester genes fused to these promoter are introduced into cells either by DNA transfection or by retroviral transmission. Current efforts are directed at developing reagents that are efficient in terms of vector convection and predictable in terms of their subsequent regulatory behavior.

Methods Employed:

Vectors containing selectable genes fused to regulated promoters are constructed and characterized by standard recombinant DNA techniques.

Gene expression cassettes are introduced into mammalian cells either by standard DNA-transfection (CaPO4) methodology, or by insertion of the cassette into a defective retrovirus recombinant, and subsequent infection of cells.

Mammalian cell lines containing expression cassettes to be evaluated are isolated by marker selection, cloned and established by conventional tissue culture protocols.

The status of chimeric DNA acquired in a given cell line is determined by conventional DNA technology, i.e., Southern blotting, restriction mapping, and DNA sequencing.

The expression patterns of genes introduced into cells are characterized by standard molecular biology technology, including Northern blotting, S1 nuclease mapping of RNA-DNA hybrids, primer extension, and enzymatic assay of genes such as chloramphenicol acetyl transferase, luciferase, and alkaline phosphatase.

Major Findings:

Previous efforts had focused on parameters involved in the construction of stable retrovirus chimeras carrying the v-*ras*^{H} oncogene driven from the MMTV promoter in a variety of configurations, and utilizing the neomycin resistance gene (neo) from the Tn5 transposon as a dominant coselection marker. A series of vector constructions was characterized in which the v-*ras*^{H} oncogene was promoted from an

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NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05283-06 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Conditional Expression of Mammalian Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Others:	Diana S. Berard	Microbiologist	LEC NCI
	Ronald G. Wolford	Microbiologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.1	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The controlled expression of genetic information in cells in culture and in transgenic animals is an essential tool in the study of gene function in vitro, and eventually will prove central to the treatment of disease by introduced genetic material (gene therapy). We showed previously that conditional expression of the v-ras-H oncogene from the glucocorticoid-responsive mouse mammary tumor virus (MMTV) promoter could result in a regulated cell phenotype, and demonstrated that regulated "phenotype switching" can be employed to study the oncogenic process in whole animals. The oncogenic potential of NIH 3T3 fibroblasts carrying the hormone-inducible v-ras-H oncogene was markedly enhanced when p21 expression was induced prior to inoculation of cells into the animal. These experiments underscored the potential applications of this technology in studying various processes in the intact animal. We are now developing a series of retrovirus-based vectors utilizing these principles that will permit us to explore the application of this approach to gene therapy in the human.

Publications:

Hochstrasser DF, Harrington MG, Hochstrasser A-C, Miller MJ, Merril CR. Methods for increasing the resolution of two-dimensional protein electrophoresis. Anal Biochem (In Press)

Huber BH, Wirth PJ, Miller MJ, Glowinski IB. Comparison of gene expression in preneoplastic and neoplastic rat liver to adult, fetal regenerating and tumor promoted liver. Cancer Res 1988;48:2382-7.

Miller MJ. Analysis of sets of two-dimensional gel electrophoretograms: building a database, correction of errors, analysis of data. In: Schafer-Neilsen C, ed. Electrophoresis'88. Weinheim, VCH:Verlagsgesellschaft (In Press)

Miller MJ. Computer analysis of two-dimensional gel electrophoretograms. In: Dunn MJ, ed. Advances in electrophoresis. Weinheim, VCH:Verlagsgesellschaft, (In Press)

Miller MJ. Sensitivity of RNA synthesis to actinomycin D inhibition is dependent on the frequency of transcription: a mathematical model. J Theor Biol 1987;129:289-99.

Miller MJ, Schwartz DM, Thorgeirsson SS. Inter- and intraclonal variability of polypeptides synthesized in a rat hepatoma cell line: quantitative two-dimensional gel analysis. J Biol Chem (In Press)

Olson AD, Miller MJ. Elsie 4: quantitative computer analysis of sets of two-dimensional gel electrophoretograms. Anal Biochem 1988;169:49-70.

Digital Equipment Corporation, Apollo Computers, Masscomp and Unisys. We have also put most of the system up on a personal computer, the IBM PS/2 model 60, running under MS/DOS (Microsoft Corp.). The major difficulty in porting ELSIE 4 is that its sophisticated graphics system is difficult to bring up on the different graphics hardware available on various computers. However, recent advances in developing a graphics standard for computers promises to allow us to create a completely portable system in the future.

Efforts are continuing to develop techniques and tools that will allow us to automatically analyze experiments that use multiple two-dimensional gels. Collaborators in Geneva have developed new techniques aimed at classifying sets of gels and identifying what spots are most characteristic of a particular sample. These techniques, heuristic clustering, and correspondence analysis are under examination in this laboratory.

(2) Analysis of Polypeptides in Transformed and Untransformed Rat Liver Epithelial (RLE) Cells. The RLE cell line was derived from neonatal rat liver. These cells appear to be very much like normal rat liver cells. They have a normal chromosome count (2N=42), and, except for a duplication in the q arm of chromosome #1, the chromosomes appear to be karyotypically normal. These cells have been single-cell cloned and a number of samples have been stored away at low passage. These cells represent a homogeneous, clonal single-cell system and thus provide what we believe is the simplest, cleanest, and most straightforward model for the study of transformation.

Different retroviruses containing transforming oncogenes, such as v-*ras*, v-*raf*, and v-*myc* have been used to infect and transform these cells. Cells transformed by v-*ras* have been single-cell cloned and characterized. These cells grow at high efficiency in soft agar and are very tumorigenic in nude mice. Although more elongated than normal RLE cells, two different *ras*-transformed morphologies, one more flattened than the other, have been isolated. Karyotype analysis reveals an inversion in the q arm of chromosome #5 in all *ras*-transformed cells. One of the cell lines is also trisomic in chromosome #12. Normal RLE cells are inhibited by epidermal growth factor (EGF) at concentrations above 1 ng/ml, while the transformed cells are unaffected by EGF at concentrations up to 100 ng/ml. Two-dimensional gel electrophoresis shows that there are a large number of quantitative, but few qualitative, differences between the normal and transformed RLE cells. Differences of tenfold, or more, are common. We plan to compare these patterns with those generated by the other transforming oncogenes in hopes of identifying polypeptides whose expression is altered in a similar manner by the different modes of transformation. If such proteins are detected, it may be possible to characterize and identify them by micro-sequencing directly off the two-dimensional gels using peptide sequencing technology available in our laboratory.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Mark J. Miller	Senior Staff Fellow	LEC NCI
Arthur David Olson	Computer Programmer	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Lori Hampton	Biologist	LEC NCI

Objectives:

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

Major Findings:

From its inception, the major objective of the Laboratory's computer facility has been to further expand and develop the two-dimensional gel analysis system in order to facilitate the use of this important research technique in the analysis of the neoplastic process.

(1) Advances in the Computerized Analysis of Two-Dimensional Gels. The computer system, developed in this laboratory, for analyzing two dimensional gels has been dubbed ELSIE 4. It has been distributed to several other laboratories in the United States and Europe. In the past year our efforts have focused on the development of programs and techniques to aid in the analysis of experiments utilizing quantitative two-dimensional gel electrophoresis. Several projects in this laboratory make use of ELSIE 4 for the analysis of experiments.

Because of the number of laboratories that use ELSIE 4, and because we plan to upgrade our facility in the next year to use more powerful computers, we have spent some effort to ensure that the system will run, with minimal modification, on a wide variety of computer systems--that is, to make ELSIE 4 highly portable. The software can be run on any mid-sized computer system running under the UNIX (TM -- AT&T Bell Laboratories) operating system. We have brought the system up, in whole or in part, on computer systems made by SUN Microsystems,

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05263-07 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Computer Analysis of Carcinogenesis by Two-Dimensional Gel Electrophoresis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Mark J. Miller	Senior Staff Fellow	LEC NCI
Others:	Arthur David Olson	Computer Programmer	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI
	Lori Hampton	Biologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.1	0.7

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The objective of this project is to study the mechanism of carcinogenesis using quantitative two-dimensional gel electrophoresis. This technique allows the separation of total cellular polypeptides on a single gel and lets us examine both qualitative and quantitative changes in the pattern of protein synthesis as the cell undergoes neoplastic transformation. Research is focused on (1) continued development of the computer system (dubbed ELSIE 4) used to automatically analyze gels and (2) use of ELSIE 4 to analyze experiments requiring computerized analysis of two-dimensional gels. In the past year we have continued developing software tools to aid the investigator in identifying interesting spots. Statistical tests designed to search for spots that may vary over the course of an experiment have been refined, and new tests, such as heuristic clustering, have been included. The system is now running on a number of mid-sized computers as well as the IBM PS/2, model 60 personal computer. The ELSIE 4 system is being used in a number of projects in the Laboratory, including one designed to study the effects of different transforming oncogenes on the synthesis of proteins in rat liver-derived epithelial (RLE) cells. RLE cells have a normal chromosome count (2N=42), the chromosomes appear karyotypically normal except for a duplication in q arm of chromosome #1. Different retroviruses containing transforming oncogenes, such as v-*ras*, v-*raf*, and v-*myc* have been used to infect and transform these cells. The v-*ras*-transformed cells have been single-cell cloned. All transformed cell clones tested grow in soft agar and are highly tumorigenic. Two-dimensional gels have been run on the normal and transformed cells and a number of significant variations in polypeptide synthesis have been noted.

technique in both experimental fibrosis and in transformed rat livers produced by the Solt-Farber method. These studies revealed that: (i) Type IV, III and I collagen genes were expressed in the regenerating and in inflammatory livers, but were absent in the necrotic areas following carbon tetrachloride administration; (ii) There was a prominent expression of collagen genes in hepatic fibrosis in the cell population lining the peripheral hepatocytes of the pseudolobules. Thus, it is likely that fibroblasts rather than hepatocytes play the main role in carbon tetrachloride-induced hepatic fibrosis; (iii) Transformed hepatocytes in hepatocellular carcinoma do not express collagen genes, but may interact with extracellular matrix-producing cells; (iv) TGF-beta was expressed in the stromal cells in hepatic fibrosis.

Publications:

Evarts RP, Nagy P, Marsden E, Thorgeirsson SS. In situ hybridization studies on expression of albumin and alpha-fetoprotein during early stage of neoplastic transformation in rat liver. Cancer Res 1987;47:5469-75.

Evarts RP, Nagy P, Marsden E, Thorgeirsson SS. A precursor-product relationship exists between oval cells and hepatocytes in rat liver. Carcinogenesis 1987;8:1737-1740.

Nagy P, Evarts RP, Marsden E, Roach J, Thorgeirsson SS. Cellular distribution of c-myc transcripts during chemical hepatocarcinogenesis. Cancer Res (In press).

Wirth PJ, Rao MS, Evarts RP. Coordinate polypeptide expression during hepatocarcinogenesis: comparison of the Solt-Farber and Reddy models. Cancer Res 1987;47:2829-51.

In addition differentiation of oval cells to intestinal lining cells was common at a high dose level of AAF. The total liver radioactivity after thymidine administration remained the same between days 9 and 13 after partial hepatectomy, which is an indication that no reutilization of labeled thymidine by the proliferating hepatocytes was occurring. Other indications of precursor product relationship between oval cells and hepatocytes after AAF administration are the presence of albumin and lack of glucose-6-phosphatase both in oval cells and early basophilic hepatocytes. A significantly higher expression of the AFP gene was found in early basophilic hepatocytes than in the old acidophilic hepatocytes when the Magiscan Image Analysis System was used for quantitation of the number of silver grains per cell. We regard the observed changes in albumin and AFP gene expression and concomitant histochemical changes as evidence that oval cells are progenitors of regenerating hepatocytes when proliferation of normal hepatocytes is prevented by AAF.

(2) Most of the hepatocytes are positive for oval cell antibody in the embryonic liver at day 19. A few days after the birth this positive reaction for oval cell antibody disappears concomitantly with the decrease of the expression of AFP and increase in the expression of albumin. Thus, the pattern of gene expression is similar to that observed in the regeneration following AAF administration combined with partial hepatectomy. Similar changes in gene expression are not observed in the regeneration after partial hepatectomy alone. Tyrosineamino-transferase (TAT) and tryptophan oxygenase (TO) are reexpressed at days 2 and 14, respectively, after the birth. The expression of these genes will be followed in early preneoplastic livers and in the hepatocellular carcinomas using the in situ hybridization technique.

(3) After administration of AAF combined with partial hepatectomy, a prominent oval cell proliferation and subsequent differentiation to hepatocytes were observed (Carcinogenesis 8,1737-1740,1987). Under these conditions we demonstrated an increased level of TGF-beta mRNA using Northern blot analysis. The in situ hybridization technique revealed that the message was localized in the mesenchymal cells of the liver and in the oval cell compartment. We also demonstrated TGF-beta mRNA in the fetal liver. From these data we propose that the TGF-beta has a regulatory function in the differentiation of hepatocytes in vivo (see project Z01CP05453-04). When preneoplastic and neoplastic lesions were produced by the Solt-Farber method, the TGF-beta message did not return to the level observed in control livers. It was expressed in both oval type cells and in mesenchymal cells of the liver tissue. However, in hepatocellular carcinoma TGF-beta was expressed only in stromal cells. We could not detect the TGF-beta message in the carcinoma cells. Since the TGF-beta inhibits the proliferation of the normal hepatocytes and epithelial cells, whereas initiated cells are resistant, it may have an intrinsic promoter role in the carcinogenesis process helping the expansion of the initiated cells.

(4) Liver cirrhosis is one of the most common lesions among the chronic liver diseases, and is closely related to the complication of hepatocellular carcinoma. To elucidate how the fibrotic process and carcinogenesis of the liver interact with each other, the expression of collagen genes (types I, III and IV) was studied by Northern blot analyses and by using the in situ hybridization

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on the Project:

Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Peter Nagy	Visiting Fellow	LEC NCI
Harushige Nakatsukasa	Expert	LEC NCI
Elizabeth Marsden	Biologist	LEC NCI

Objectives:

The objective of this project is to characterize the cellular evolution of chemically-induced murine hepatomas and to identify the contributions of different genes and gene products to the phenotypes of the transformed cells. Topics under investigation are: (1) the evolution of oval cells, the putative hepatic stem cells, via transitional cells, to fully differentiated hepatocytes and possible precursor cells of neoplastic lesions; (2) both temporal and cell-specific distribution of gene transcripts among normal, regenerating, preneoplastic, neoplastic and fibrotic liver; (3) contribution of growth factors to the differentiation of primitive nonparenchymal liver epithelial cells; (4) the role of collagen in chemical hepatocarcinogenesis; (5) the mechanism of hepatic fibrosis; (6) involvement of TGF-beta in hepatic fibrosis.

Methods Employed:

(1) In situ hybridization for spatial localization of mRNA. (2) Immunocytochemistry for identification of cells positive for oval cell antibody, vimentin and cytokeratins. (3) Preparation of poly(A)RNA. (4) Techniques used in molecular biology including Northern blotting and solution hybridization. (5) Preparation of riboprobes using ^{3}H and ^{35}S-labeled uracil triphosphate. (6) Quantitation of in situ hybridization data by using the Magiscan Image Analysis System.

Major Findings:

(1) A long-term administration of carcinogens usually produces a prominent oval cell proliferation. Oval cell proliferation is also noted when mature hepatocytes are unable to proliferate due to the nutritional and or toxic effects of chemicals. The physiological function and ultimate fate of oval cells has remained a source of considerable controversy. It is believed that oval cells and bile ductal cells are related; however, the question regarding the precursor-product relationship with normal hepatocytes is unknown. We produced oval cell proliferation by AAF administration and partial hepatectomy. We demonstrated the transfer of radiolabeled thymidine from the oval cells to newly formed basophilic hepatocytes. The occurrence of labeled basophilic hepatocytes was delayed when a high dose of AAF was administered to the animals. However, even at later time points (13 days after partial hepatectomy and 6 days after thymidine administration), hepatocytes in the basophilic areas became labeled.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05262-07 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cellular Evolution of Chemically Induced Rat Hepatomas

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Affiliation
PI:	Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC NCI
	Peter Nagy	Visiting Fellow	LEC NCI
	Harushige Nakatsukasa	Expert	LEC NCI
	Elizabeth Marsden	Biologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	2.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

We have continued to use the rat liver as a model for both in vivo and in vitro studies on the mechanism of chemically-induced tumor formation. Recently studies on the fate and the possible role of oval cells in both normal liver biology and hepatocarcinogenesis have been conducted. After the administration of 2-acetyl-aminofluorene (AAF) for two weeks combined with partial hepatectomy (PH), only oval cells are able to proliferate and incorporate radiolabeled thymidine at day 7 after PH. However, at the later time points (9 to 13 days after PH) the label was present in the newly formed basophilic hepatocytes, which is an indication that oval cells are precursor cells for hepatocytes. At a high dose level of AAF, oval cell differentiation to hepatocytes was delayed and metaplastic differentiation to intestinal and biliary epithelial cells was frequently observed. No reutilization of labeled thymidine from dying cells by the regenerating hepatocytes was observed. Lack of glucose-6-phosphatase activity and presence of alpha-fetoprotein (AFP) gene message in oval cells and early basophilic hepatocytes is a further indication that oval cells are precursor cells for hepatocytes. Differentiation of oval cells to hepatocytes after AAF administration follows a pattern similar to that observed in the embryogenic liver. After AAF administration TGF-beta was present in the mesenchymal cells of the liver and in the oval cell compartment. In hepatocellular carcinoma, transforming growth factor-beta (TGF-beta) was present only in stromal cells. In the fibrotic liver produced by carbon tetrachloride administration, collagen genes and TGF-beta were expressed in the cell population lining the peripheral hepatocytes of the pseudolobules.

Major Findings:

Binding of the glucocorticoid receptor at sequences critical for hormone regulation was shown to result in the hormone-dependent establishment of a stable transcription factor complex at the promoter. We determined that the factors responsible for the exonuclease-resistant complex established at the hormone-activated promoter are apparently equal in abundance and DNA-binding affinity in crude extracts from non-stimulated cell nuclei. The interaction of these factors with naked DNA in vitro has been partially characterized in crude and fractionated nuclear extracts.

Accessibility of promoter chromatin was probed by digestion in whole nuclei with a variety of restriction endonucleases. We demonstrated a hormone-dependent increase in accessibility of the sequences closely associated with transcription factor binding sites. Furthermore, mutations which result in increased activity of the non-stimulated promoter in the presence of an upstream enhancer element were found to cause increased restriction enzyme sensitivity of the promoter and increased transcription factor binding in the absence of hormone. These results suggest that transcription factor binding sites are sequestered by nucleoprotein structure in the inactive promoter and that activation occurs in part by receptor-mediated alterations in local nucleoprotein structure.

Taken together, these findings have major implications both for steroid hormone-regulated promoters and other regulated eukaryotic genes. The glucocorticoid regulatory element of MMTV shares some qualities with the class of elements known as enhancers. A fundamental understanding of the mechanism by which binding of the steroid receptor molecule at its cis-regulatory sequence recruits transcription factors to form the active promoter complex may therefore be central to a future understanding of mechanisms of enhancer activation. Nucleoprotein structure and its maintenance, perhaps by specific negative modulatory factors, may be a critical determinant in regulation of gene expression.

Publications:

Charron J, Richard-Foy H, Berard DS, Hager GL, Drouin J. Persistent gene-specific glucocorticoid inhibition of transcription in the presence of a contiguous glucocorticoid-inducible gene. EMBO J (In Press)

Cordingley MG, Hager GL. Binding of multiple factors to the MMTV promoter in crude and fractionated nuclear extracts. Nucleic Acids Res 1988;16:609-28.

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Michael Cordingley	Visiting Associate	LEC NCI
Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Anna Tate Riegel	Visiting Fellow	LEC NCI
Diana S. Berard	Microbiologist	LEC NCI
Ronald Wolford	Microbiologist	LEC NCI

Objectives:

(1) Analysis of positive regulation of mouse mammary tumor virus (MMTV) transcription by steroid hormones, and negative regulation of proopiomelanocortin (POMC); characterization of nuclear DNA-binding factors specific for MMTV and POMC regulatory sequences.

(2) Determination of the molecular mechanism responsible for glucocorticoid receptor-mediated induction or repression of transcription at hormonally regulated promoters.

(3) Application of the methodologies developed for the study of hormone action to the study of other transcription regulatory systems important in cell growth.

Methods Employed:

Molecular chimeras between the MMTV long terminal repeat (LTR), or the POMC promoter and various reporter genes are used to assay expression from hormonally regulated promoters in transient expression assays. Reporter genes include the v-ras gene of Harvey murine sarcoma virus (HaMuSV), the chloramphenicol acetyl transferase (CAT) gene from the bacterial transposon Tn9, the luciferase gene (LUC), and the alkaline phosphatase (AP) gene.

Molecular clones of these chimeric molecules provide probes with which to measure steady state transcript levels by nuclease S1 analysis or transcription rates by transcription extension assays in isolated nuclei.

Cell lines containing these chimeric genes amplified on extrachromosomally replicating bovine papillomavirus (BPV) vectors are utilized for high resolution detection of nuclear factors bound at promoter and regulatory sequences in chromatin in vivo by an exonuclease protection assay in isolated nuclei.

DNase-I "footprinting" techniques are used to detect and characterize specific DNA-binding factors present in crude and fractionated nuclear extracts.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04986-11 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Basis of Steroid Hormone Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	Michael Cordingley	Visiting Associate	LEC NCI
Others:	Gordon Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
	Anna Tate Riegel	Visiting Fellow	LEC NCI
	Diana S. Berard	Microbiologist	LEC NCI
	Ronald Wolford	Microbiologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	2.0	1.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The mouse mammary tumor virus (MMTV) system has been studied for some time as a model for positive regulation of transcription by steroid hormones. Using a series of cell lines in which MMTV long terminal repeat (LTR) fusion genes are amplified on extrachromosomally replicating bovine papillomavirus (BPV) "minichromosomes," we demonstrated, by an exonuclease protection assay, that factors bind to the MMTV promoter in vivo in response to hormone stimulation. These factors were identified in crude nuclear extracts from mouse cells as NF1 (-80 to -56 region) and F-i (-42 to +1 region). Activation of transcription at the MMTV promoter therefore appears to result from recruitment of preformed transcription factors to the promoter by the steroid receptor. The proopiomelanocortin (POMC) gene is negatively regulated by the same steroid receptor. We have prepared POMC-MMTV-BPV chimeric episomes and demonstrated positive regulation of MMTV and negative regulation of POMC on the same circular template. In addition, three factors that bind specifically to the POMC promoter have been identified in crude nuclear extracts.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. Battula	Expert	LEC	NCI
G. K. Townsend	Biologist	LEC	NCI
E. G. Snyderwine	Guest Researcher	LEC	NCI
S. S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The overall goal of this project is to express cytochrome P-450 enzymes individually and to define their contribution to mutagenesis and chemical carcinogenesis. Specific aims are (a) to express the coding DNAs of cytochrome P-450 in recombinant vaccinia viruses and recombinant retroviruses; (b) to analyze the expressed products for post-transcriptional processing, subcellular localization and enzymatic activities; (c) to test the magnitude and the nature of binding of the enzyme-activated carcinogen to the cellular macromolecules; (d) to test for mutations and cell transformation after exposure to carcinogens of cells expressing the P-450 enzymes and (e) to characterize structure function relationships by site-directed mutagenesis.

Methods Employed:

Construction of recombinant viruses requires extensive use of recombinant DNA methodology, DNA separation procedures based on hydrodynamic properties and electrophoretic mobilities, DNA transfections, cell culture techniques, virological procedures and genetic selection of cells and viruses. Detection and isolation of expression products requires use of different blotting methods, subcellular fractionation procedures, protein separations by electrophoresis and column chromatography, immunological procedures employing monoclonal and polyclonal antibodies. Functional evaluation of the expressed proteins requires a variety of enzymatic assays such as aryl hydrocarbon hydroxylase, ethoxyresorufin O-deethylase, 7-ethoxycoumarin O-deethylase and acetanilide hydroxylase, spectrophotometry, spectrofluorimetry and high pressure liquid chromatography.

Major Findings:

Infectious recombinant vaccinia viruses and retroviruses containing a selectable reporter gene marker and full length cDNAs for either mouse cytochromes P1-450 or P3-450 were constructed and characterized. These recombinant viruses have a broad host range and can infect a variety of mammalian cells.

Human and murine cells infected with the infectious recombinant vaccinia virus efficiently expressed their respective proteins. Characterization of the newly synthesized proteins by immunochemical, spectral and enzymatic analysis indicates that the polypeptides expressed were authentic in size, have incorporated heme and the holoenzyme displayed catalytic activities characteristic of the respective cytochrome P-450 enzymes.

Mammalian cells infected with the infectious cytochrome P3-450 recombinant retrovirus have stably integrated the P3-450 DNA sequences into the host cellular DNA. The infected clones constitutively expressed new protein products indistinguishable from the cytochrome P3-450 found in the mouse liver microsomes. Enzymatic analysis of the proliferating cells in situ and of the cell homogenates in vitro showed catalytic properties diagnostic of cytochrome P3-450.

We have initiated studies to examine the consequences of expression of these pure enzymes in their native microsomal environment. Cytochrome P3-450 selectively catalyzed mutagenic activation of heterocyclic arylamines found in cooked foods. Cytochrome P1-450 preferentially activated the aromatic hydrocarbon benzo(a)-pyrene 7,8 diol. Thus, these enzymes are selective in the choice of their substrates and appear to exhibit mutually exclusive activities at limiting substrate concentrations.

Clones expressing cytochrome P3-450 were challenged with the food mutagen IQ and the specific IQ-DNA adducts were analyzed by ^{32}P postlabeling assays. Five specific IQ-DNA adducts were detected. The adducts were identical to those formed in mouse or rat liver after the in vivo administration of IQ. Thus, clones constitutively expressing cytochrome P-450s provide an excellent model system to study the mechanism of activation of putative human mutagens and carcinogens.

Publications:

Gelboin HV, Park SS, Battula N. DNA recombinants and monoclonal antibody directed methods for determining cytochrome P-450 specificity. Biochem Pharm 1988;37:98-102.

Gelboin HV, Gonzalez FJ, Park SS, Sagara J, Battula N. Cytochrome P-450 function analysis with monoclonal antibodies and cDNA expression vectors. In: Torino FB, ed. Chemical carcinogenesis: models and mechanism. Plenum Press (In Press).

Patents:

Battula N, Gelboin HV, Gonzalez FJ, Moss B. US Patent (Pending): Construction of Infectious Recombinant Vaccinia Virus Containing DNA Coding Sequences for Cytochromes P-450 and Their Expression as Active Enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05554-01 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Effect of Ethanol on ODC and Proto-oncogene Expression in Liver Regeneration

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Clifford J. Steer	Expert	LEC NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC NCI

COOPERATING UNITS (if any)

VA Medical Center, Wash. D.C. (Anna Mae Diehl)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The effect of ethanol consumption on liver regeneration has been found to exert significant but inconsistent changes of several metabolic pathways. The present project examined the effect of chronic ethanol consumption on ornithine decarboxylase activity (ODC), polyamine biosynthesis and proto-oncogene expression in the rat liver after partial hepatectomy (PH). Chronic exposure to ethanol has been reported to increase both the activity and half-life of ODC, yet significantly inhibit post-hepatectomy DNA synthesis and restitution of liver mass. After using an established model of chronic ethanol feeding, animals underwent 2/3 partial hepatectomy and were sacrificed at 30 min, 1,3,6,12,18 and 24 hours thereafter. ODC activity increased significantly in both ethanol and pair-fed animals at 3 to 6 hours post-PH. However, the ethanol-treated group exhibited a fivefold less increase in activity as compared to the pair-fed animals. This decrease was paralleled by a similar change in putrescine activity, a major polyamine product of ODC activity. By 24 hours, ODC activity was similar in both groups. Total ODC protein content remained invariant to partial hepatectomy and/or chronic ethanol ingestion. In contrast, measurement of ODC mRNA levels revealed a tenfold increase at 12 and 24 hours but no significant difference between pair-fed and alcohol-treated animals. Examination of a number of proto-oncogenes including c-<u>myc</u>, c-<u>fos</u>, v-<u>raf</u>, H-<u>ras</u>, c-<u>mos</u>, and v-<u>erb</u> B revealed no significant differences in expression between the two groups. Similar results were noted when expression of multidrug resistant (MDR-1), albumin, alpha-fetoprotein, and glutathione S-transferase-P genes was examined. The mRNA levels of the ethanol-inducible cytochrome P-450 was slightly increased in the ethanol-treated animals. The results suggest that in the present model, chronic alcohol ingestion induces diminished ODC activity in the early time points post-PH without affecting ODC protein content or mRNA levels. Proto-oncogene expression appears to be invariant to chronic alcohol ingestion under the conditions described.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Clifford J. Steer	Expert	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI

Objectives:

(1) To study the effect of chronic ethanol consumption on ODC enzyme activity and polyamine biosynthesis in regenerating rat liver stimulated by partial hepatectomy.

(2) Correlate ODC enzyme activity with ODC protein content and mRNA levels (static and kinetic) in the regenerating liver.

(3) Examine the effect of chronic ethanol consumption on proto-oncogene expression in the regenerating rat liver and correlate putative differences between the two groups to changes in ODC activity and/or polyamine biosynthesis.

Methods Employed:

(1) Wistar male rats were fed a 5% liquid chow diet in which ethanol contributed 36% of total calories. A second group (pair-fed) was fed isocaloric quantities of a similar diet without ethanol. Animals were maintained on a 12-hour light, dark cycle for the 6-week feeding period. Partial hepatectomies were performed so that each animal was sacrificed at approximately the same time in the a.m. (2) ODC activity was measured from liver homogenate by quantitation of $^{14}CO_2$ liberated from the ^{14}C-labeled substrate ornithine. ODC total protein was measured by ^{3}H-2-difluoromethylornithine (DFMO) binding according to established methods. (3) Polyamine levels were determined by reverse phase high pressure liquid chromatography. (4) Total RNA was isolated from flash-frozen liver by extraction with guanidine thiocyanate and purified by centrifugation through cesium chloride cushion. Polyadenylated RNA was isolated from total RNA by chromatography on oligo(dT)-cellulose. (5) mRNA expression was determined by Northern blot analysis.

Major Findings:

Short term exposure to ethanol has been shown to inhibit ODC activity as well as DNA synthesis and liver regeneration. More chronic exposure has been reported to increase ODC activity and therefore could conceivably stimulate DNA synthesis and regeneration. To explore this possibility, the effects of chronic ethanol consumption on ODC activity, polyamine synthesis and proto-oncogene expression were examined. Previous work has shown that chronic ethanol feeding failed to inhibit the induction of ODC by partial hepatectomy, yet significantly inhibited posthepatectomy DNA synthesis and liver regeneration. The present study has extended those findings and has shown that chronic ethanol consumption significantly delays the increase in both ODC activity and polyamine

biosynthesis induced by partial hepatectomy, in particular, between 3 and 6 hours. At the 24-hour point, both the ethanol and pair-fed group exhibited similar levels of ODC activity and polyamine synthesis. Total ODC protein, as determined by DFMO binding was unaffected by either the chronic ethanol consumption or the partial hepatectomy. In contrast, Northern blot analysis revealed a significant increase in mRNA for ODC at 12-, 18- and 24-hour time points with perhaps a slight increase at 6 hours. No differences were noted between the ethanol and pair-fed groups at any of the time points examined. Nuclear run-on experiments are presently being done to establish the kinetics of the increase in ODC message.

Because of the significant role of proto-oncogenes in liver regeneration, we elected to also examine the effect of chronic ethanol consumption on their expression. We have examined a number of proto-oncogenes and no significant differences have been detected between the ethanol and pair-fed groups. Similar lack of differences were noted for the asialoglycoprotein receptor mRNA, as well as for MDR, albumin, alpha-fetoprotein, and glutathione S-transferase. Results for the epidermal growth factor receptor message and several others are pending. Acute ethanol ingestion and its effect on liver regeneration and proto-oncogene expression are in progress. However, it appears that no significant change in proto-oncogene expression is correlated with the biochemical changes in ODC activity, DNA synthesis or restitution of liver mass resulting from chronic ethanol consumption in the rat model.

Publications:

Steer CJ, Weiss P, Huber BE, Wirth PJ, Thorgeirsson SS, Ashwell G. Ligand induced modulation of the hepatic receptor for asialoglycoproteins in the human hepatoblastoma cell line, Hep G2. J Biol chem 1987;262:17524-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05555-01 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Aminoacyl-tRNAs in HIV and other Retroviral Infected Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Dolph L. Hatfield	Research Biologist	LEC NCI
Others:	Byeong J. Lee	Visiting Fellow	LEC NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Genetics, NICHHD (Drs. X. F. Feng, Judith Levin); Bionetics Research, Inc. (Drs. Alan Rein and Stephen Oroszlan)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Transfer RNA was isolated from HIV-1 (human immunodeficiency virus-1), HIV-2 (human immunodeficiency virus-2), HTLV-1 (human T-cell leukemia virus) and BLV (bovine leukemia virus)-infected and -uninfected cells and the elution profiles of aminoacyl-tRNAs from infected and uninfected cells were compared by reverse phase chromatography. In each case examined, Asn-tRNA, which normally contains the highly modified Q base in the 5′ position of its anticodon, was deficient in this base in infected cells. Phe-tRNA from HIV-1- and HIV-2-infected cells, in addition lacked the highly modified Wye base on the 3′ side of its anticodon. Interestingly, one or both of these tRNAs occur at or near the ribosomal frameshift site in expression of the gag-pol poly protein of each retrovirus. The possible role of the hypomodified base in ribosomal frameshifting is presently being investigated.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dolph L. Hatfield	Research Biologist	LEC NCI
Byeong J. Lee	Visiting Fellow	LEC NCI

Objectives:

The major goals of this project are to determine if the aminoacyl-tRNAs in HIV and other retroviral-infected cells are altered and if such altered tRNAs have a role in the expression of the virus. Specific steps to achieve this goals are: 1) to isolate tRNA from HIV and other retroviral-infected cells and the corresponding uninfected cells; 2) to determine if differences in the tRNAs occur in infected and uninfected cells by comparing tRNA elution profiles on reverse phase chromatographic columns; and 3) to isolate and determine if altered tRNAs in infected cells may have a role in the expression of the virus.

Methods Employed:

HIV-1-, HIV-2-, HTLV-1- and BLV-infected cells and a control set of uninfected cells were grown under identical conditions. A matched set of uninfected cells corresponding to HTLV-1-infected cells were not available. Transfer RNA was extracted from cells, aminoacylated with radioactive amino acids by standard techniques and their elution profiles compared by reverse phase chromatography. Those tRNAs which were altered were purified by standard techniques.

A portion of the HIV genome encoding the gag-pol region was subcloned into an expression vector in order to prepare mRNA. The mRNA was then used to program rabbit reticulocyte lysates for investigating the role of the purified, hypomodified tRNAs in ribosomal frameshifting.

Major Findings:

Asn-tRNA and other tRNAs which normally contain Q base in the 5′ position of their anticodon (i.e., Asp-, His- and Tyr-tRNAs) were deficient in this base in each of the infected cells. Asn-tRNA occurs just before the upstream ribosomal frameshift sites in HTLV-1 and BLV and occurs one amino acid upstream of the ribosomal frameshift site in HIV-1. In addition, Phe-tRNA lacked the highly modified Wye base in HIV-1 and HIV-2. Wye base normally occupies the position immediately 3′ to the anticodon. Phe-tRNA occurs just before the ribosomal frameshift site in HIV-1 and HIV-2. The possible role of hypomodified tRNAs in ribosomal frameshifting is presently being examined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05556-01 LEC

PERIOD COVERED
March 27, 1988 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Computer-Assisted Design of Recognition Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Giorgio Fassina	Visiting Associate	LEC	NCI
Others:	James G. Omichinski	IRTA Fellow	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION
Biopolymer Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0

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

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

It is now possible to design ex novo recognition peptides, called anti-sense peptides based on anti-sense DNA sequence of a gene coding for a target protein. In several cases tested so far, anti-sense peptides have been found to bind to the corresponding sense peptides, with rather profound implications for use in affinity technology to isolate and purify polypeptides of biological relevance. The major limitation for the general application of this new macromolecular recognition system is the lack of DNA sequence information, especially in the case of novel uncharacterized proteins. The main objective of this study is to devise a method to generate recognition peptides using only partial amino acid sequence information. Results obtained so far indicate that it is possible to design recognition peptides starting only from limited sequence information using values of individual amino acid hydropathy. Such ex novo sequence-directed recognition peptides, once immobilized on solid support, maintained their binding properties and proved to be useful in preliminary purification of target proteins.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Giorgio Fassina	Visiting Associate	LEC NCI
James G. Omichinski	IRTA Fellow	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI

Objectives:

The main objectives of this project are 1) to devise a method to generate ex novo recognition peptides from amino acid sequence information of the target polypeptide, and 2) to use such recognition peptides immobilized on a solid support to isolate and purify novel polypeptides associated with biological relevant processes, and 3) to investigate their applicability as potential structure-function modifiers.

Methods Employed:

The principal methods employed are (1) computer-assisted modeling of peptide hydrophatic plots, (2) peptide synthesis and purification, (3) analytical high performance affinity chromatography, (4) circular dichroism, (5) enzymatic assays, (6) sequence analysis, and (7) one- and two-dimensional polyacrylamide gel electrophoresis (PAGE).

Major Findings:

Using values of individual amino acid hydropathy, it was possible to develop a computer-assisted method to generate hydrophatic complementary peptide. In the several systems tested so far, specific interactions were observed between target peptides and computer-designed recognition peptides. Once immobilized on a solid support, they maintained specific binding properties, indicating great potential as purification tools.

A 13 mer recognition peptide has been designed, synthesized and characterized which is able to interact specifically with the corresponding 13 mer target peptide, with relatively high affinity. The target peptide represents the first 13 residues of a hepatocyte plasma membrane glycoprotein partially sequenced after transblotting from two-dimensional sodium dodecyl sulfate (SDS) PAGE. The glycoprotein has been found to have lowered levels in neoplastic liver. Preliminary attempts to isolate this glycoprotein from crude liver extracts indicated the possibility to achieve severalfold enrichment in a single step, using an affinity column with the immobilized recognition peptide. To evaluate the generality of the phenomenon, a recognition peptide directed towards the 20 mer putative adenosine 5′-triphosphate binding site of the raf oncogene product was designed and its interaction properties compared with the corresponding anti-sense peptide deduced from DNA sequence. Amino acid sequence-directed recognition peptide demonstrated higher affinity for the target peptide than DNA sequence-generated anti-sense peptides. Currently, we are investigating the

possibility of using affinity columns with these peptides immobilized to attempt the purification of the _raf_ oncogene product. Furthermore, studies are underway to elucidate the mechanism of the interaction, define the chemical nature of the process, and exploit other potential uses of these molecules.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05557-01 LEC

PERIOD COVERED

October 13, 1987 September 30, 1988

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

Phenotypic Alterations Induced by Ras Oncogene in Rat Liver Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Brian Huber	Senior Staff Fellow	LEC NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC NCI
	Michael G. Cordingley	Visiting Associate	LEC NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Studies on primary human and rodent hepatocellular carcinomas have strongly suggested that a transforming ras gene may participate in the initiation and/or maintenance stages of liver neoplasia. However, the function and exact phenotypic alterations caused by expression of a ras oncogene in the liver has yet to be described. To define the function and phenotypic characteristics associated with a transforming ras gene in liver, we have utilized a retroviral shuttle vector system to deliver an inducible ras oncogene into normal rat liver epithelial cells (RLE cells). The Moloney murine sarcoma virus-based vector is composed of a neomycin resistance (NEO) gene (transcriptionally derived from the 5′ long terminal repeat (LTR) and transforming Ha-ras gene under the transcriptional control of the glucocorticoid inducible LTR of the mouse mammary tumor virus (MMTV) (Cell 27: 245, 1981). Southern, Northern and Western blot analysis confirmed stable proviral integration, ras gene transcription with polyadenylation and translation into a 21 K dalton protein with a specific mutation in codon 12. Northern and S1 nuclease analysis confirmed that dexamethasone induced ras transcripts from the MMTV LTR by 15-fold. Phenotypic alterations specifically associated with ras expression in these normal liver epithelial cells include: (1) alterations in growth kinetics; (2) ability to grow in soft agar and produce tumors in nude mice; (3) increases in metabolic rate determined by 2-deoxyglucose uptake; and (4) becoming positive for gamma-glutamyltranspeptidase activity.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Brian Huber	Senior Staff Fellow	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Michael G. Cordingley	Visiting Associate	LEC NCI

Objectives:

Studies on primary human and rodent hepatocellular carcinomas (HCCs) have strongly suggested that a transforming *ras* gene may participate in the initiation and/or maintenance stages of liver neoplasia. However, the function and exact phenotypic alterations caused by expression of a *ras* oncogene in the liver have yet to be described. To define the function and phenotypic characteristics associated with a transforming *ras* oncogene in the liver, we have utilized a retroviral vector system to deliver an inducible transforming *ras* gene into normal RLE cells. To obtain inducible expression of a *ras* oncogene, we utilized the system of Hager et al. (Cell 27: 245-255, 1981) in which the *ras* gene is coupled to the glucocorticoid responsive MMTV LTR.

Methods Employed:

Cells. RLE cells, a normal liver epithelial cell line established from a 10-day-old male Fischer rat, were supplied by J. McMahon, DCT, NCI, NIH.

Viral shuttle vectors. The viruses were constructed from the Moloney murine sarcoma virus using the G418-resistance gene, NEO, and MMTV LTR-*ras* gene cassette. Viruses were packaged using the Psi-2 packaging cell line.

Northern and Southern blotting. Poly(A^+)RNA was isolated with guanidine isothiocyanate, electrophoresed on denaturing formaldehyde gels and transferred to nitrocellulose. DNA was isolated using proteinase K and ribonuclease A, digested with restriction endonucleases, fractionated on agarose gels and transferred to Nytran membranes.

Immunoprecipitation/Western blot analysis. P21 was first immunoprecipitated with a mutation-nonspecific antibody (AF). Immunoprecipitates were then fractionated in duplicate by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose. Duplicate blots were probed with either mutation-specific or mutation-nonspecific antibody.

S1 Nuclease. S1 nuclease protection assays were performed with a 247 bp MMTV LTR-labeled probe at 10 μg poly(A^+)RNA in the presence of 75% deionized formamide at 50°C for 17 hours. Reaction was terminated by 1000 U S1 nuclease followed by electrophoresis on 8 M urea, 7% acrylamide gel.

Major Findings:

A ras-containing retroviral vector was constructed and was used to efficiently infect normal rat liver epithelial cells. Conditional expression of the ras gene was achieved by utilizing the glucocorticoid-responsive MMTV-LTR promoter sequence. Phenotypic alterations specifically associated with expression of this transforming ras gene in these liver epithelial cells were determined. This system may allow for a comprehensive temporal analysis of the effects of a transforming ras oncogene expressed in liver epithelial cells. In addition, this system may allow for the identification of compounds which specifically interact and antagonize a transforming ras oncogene.

Based on Southern, Northern and Western blot analysis, the ras gene was shown to be stably integrated into the target cells in two copies, transcribed into RNA which was post-transcriptionally polyadenylated and translated into a functional protein of correct molecular weight containing a specific transforming mutation in codon 12. In the presence of an appropriate inducing agent, specific ras transcripts were shown to be upregulated in these target cells by approximately 15-fold. S1 nuclease analysis confirmed that these transcripts originated from the appropriate inducible promoter that was engineered in these vectors.

Phenotypic alterations specifically associated with ras expression in these epithelial cells were identified. They include alterations in growth kinetics, increase in glucose transport, morphological changes, ability to grow in soft agar, expression of gamma-glutamyl transpeptidase, lactate dehydrogenase K and nicotinamide adenine dinucleotide (NAD)-dependent 5,10-methylene tetrahydrofolate dehydrogenase isozymes and becoming tumorigenic in nude mice.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05558-01 LEC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cellular Proteins in Oncogene Transformed Rat Liver Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Peter J. Worland	Visiting Fellow	LEC NCI
	Lori Hampton	Biologist	LEC NCI
	Anthony C. Huggett	Visiting Associate	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda Maryland 20892

TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.8	OTHER: 0.2

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

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

In order to identify the cellular proteins that are associated with neoplastic development, we have used an in vitro transforming system consisting of helper-dependent retroviruses containing either v-raf or v-raf/v-myc as the transforming agents and rat liver epithelial (RLE) cell line as the reporter cell. Both v-raf (3611) and v-raf/v-myc (J2) viruses were efficient in vitro transforming agents of the RLE cells and the infected cells formed tumors upon transplantation in nude mice. Neither the uninfected nor helper virus-infected RLE cells formed tumors in nude mice. Cellular protein patterns in normal RLE cells, helper-infected (RLE-H), 3611 (RLE-3611T)- and J2 (RLE-J2)-transformed RLE cells have been analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Comparison of the autoradiograms from the RLE-H, RLE-3611T and RLE-J2, nine proteins which are either suppressed or induced were selected for closer study and purification. Polypeptides 1 - 4 (Mr/pI) (1. 145/5.9-7.2; 2. 130/6.5-7.5; 3. 100/4.0; 4. 30/6.0) were found be present in negligible amounts in the tumorigenic cells from both autoradiograms and silver-stained 2-dimensional polyacrylamide gels and polypeptides 5 - 9 (Mr/pI) (5. 27/5.0; 6. 26/4.9; 7. 25/4.9; 8. 25/5.2; 9. 27/6.6) were found to be present in negligible amounts in the non-tumorigenic RLE-H cells. Protein 1 appears to be associated with both soluble and membrane fractions; polypeptides 2, 3, 4, 8 and 9 appear to be associated with membrane and polypeptides 5, 6 and 7 appear in the cytosolic fraction.

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC NCI
Peter J. Worland	Visiting Fellow	LEC NCI
Lori Hampton	Biologist	LEC NCI
Anthony C. Huggett	Visiting Associate	LEC NCI

Objectives:

The search for proteins associated with the development of cancer requires comparison between normal and tumorigenic cells. In this laboratory, we have developed RLE cells that become tumorigenic when infected with viruses containing the oncogenes v-*raf* and a combination of v-*raf* and v-*myc*. These cells now allow us to make comparison of the polypeptide expression between the non-tumorigenic RLE control cells, RLE-H which were infected by virus alone, the tumorigenic RLE-3611T cells (RLE cells infected with v-*raf*) and the RLE-J2 cells (RLE cells infected with v-*raf* and v-*myc*).

Methods Employed:

The principle methods employed are: (1) tissue culture techniques, (2) animal husbandry, (3) histochemical staining, (4) 2-dimensional polyacrylamide gel electrophoresis and (5) autoradiography.

Major Findings:

The RLE-J2 cells were the only cells capable of forming colonies in soft agar with an efficiency of approximately 47% (232 ± 11 colonies/500 cells plated per culture dish), the RLE-3611T cells formed one to two colonies that were only detectable by microscopic analysis after 21 days. When these cells (RLE-H, RLE-3611T and RLE-J2) were injected into nude mice, the RLE-H cells did not cause any detectable tumor growth on autopsy of the nude mice after 4 weeks, F3611T formed tumors in the nude mice after 4 days and had grown to a size of 4 mm diameter after 7 days, FJ2 cells rapidly formed tumors in the nude mice which were at a diameter of 4-8 mm after 7 days.

After metabolically radiolabelling protein from the RLE-H, RLE-3611T and RLE-J2 subclones with 14C amino acids, proteins were separated by 2-dimensional polyacrylamide gel electrophoresis. Comparison of the resultant autoradiograms indicated that nine polypeptides could be distinguished between the non-tumorigenic and tumorigenic cell types. Repetition of the experiment on two subsequent occasions confirmed the presence of these polypeptides in the respective cell types. Silver staining and fractionation into membrane and cytosol allowed estimation of their relative amounts and their approximate cellular location. Polypeptides appearing on the 2-dimensional gels from the

non-tumorigenic cells were (Mr/pI) 1. 145/5.9-7.2; 2. 130/6.5-7.5; 3. 100/4.0; 30/6.0 and polypeptides appearing principally in the tumorigenic cells were 5. 27/5.0; 6. 26/4.9; 7. 25/4.9; 8. 25/5.2; 9. 27/6.6. Polypeptides 5-8 were observed in the non-tumorigenic cells, but to a greatly reduced extent. Polypeptides 1-3 are in a region of the 2-dimensional gel that will allow simple, partial purification prior to the determination of their N-terminal sequence.

This project is rapidly approaching a point where N-terminal sequence data on at least three polypeptides that are clearly down-regulated in the tumorigenic cells would appear attainable. This information should allow us to design experiments to determine the functional aspects of these polypeptides.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05559-01 LEC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Plasma Membrane Proteins in Normal and Neoplastic Rat Hepatocytes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	David C. Parmelee	Expert	LEC NCI
Others:	Timothy Benjamin	Chemist	LEC NCI
	Chien-Hua Niu	Volunteer Scientist	LEC NCI
	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI
	Anthony C. Huggett	Visiting Associate	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 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 (Use standard unreduced type. Do not exceed the space provided.)

Our previous investigations of glycoproteins isolated from the plasma membranes of normal and neoplastic rat livers revealed many qualitative and quantitative differences when analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The main goal of this project is to purify and characterize the specific glycoproteins whose expression is markedly altered during chemically induced hepatocarcinogenesis in order to understand their role either as markers or causal agents during cell transformation. Results obtained are as follows: The previous isolation procedure was modified to more efficiently purify the glycoproteins of interest. The new method utilizes modified conditions for the Concanavalin-A (Con A) affinity chromatography and fast protein liquid chromatography (FPLC) using a Superose-12 column. This procedure provides a fraction that is enriched in the various glycoproteins of interest and enabled the final purification to be achieved by a single 2D-PAGE experiment. These separated components were transferred to an Immobilon-P membrane by electroblotting. It was necessary to optimize the conditions of the 2D-PAGE and electroblotting for these specific glycoproteins. In addition, the best procedure for amino acid sequencing (gas phase) from these membranes was established. Using these techniques, it was possible to determine the N-terminal amino acid sequence for 4 of the 9 glycoproteins analyzed. The remaining 5 components of interest were not sequencable in this manner, presumably because of blocked N-termini. A peptide comprised of the first 13 N-terminal residues of the fragment RLMP-1 was synthesized, purified, and used to produce rabbit antibodies for use in the larger scale purification of the whole protein and in various biological studies. The glycoproteins from normal and transformed rat liver epithelial (RLE) cells are currently being characterized by the 2D-PAGE system.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

David C. Parmelee	Expert	LEC NCI
Timothy Benjamin	Chemist	LEC NCI
Chien-Hua Niu	Volunteer Scientist	LEC NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Anthony C. Huggett	Visiting Associate	LEC NCI

Objectives:

The main objective of this project is to isolate, purify, and structurally characterize the specific glycoproteins whose expression is qualitatively and quantitatively altered during chemically induced hepatocarcinogenesis in order to understand their biological functions during cell transformation.

Methods Employed:

The principle methods employed are (1) affinity chromatography utilizing Con-A, (2) FPLC, (3) 2D-PAGE, (4) electroblotting, (5) gas-phase protein sequencing, (6) high performance liquid chromatography (HPLC), and (6) peptide synthesis.

Major Findings:

(1) A modified isolation procedure provides a fraction that is enriched in the various glycoproteins of interest and enables the final purification to be achieved by a single 2D-PAGE experiment. These separated components were transferred to an Immobilon-P membrane by electroblotting. The conditions for the 2D-PAGE and electroblotting were optimized for these particular glycoproteins.

(2) The best procedures for amino acid sequencing (gas phase) from the Immobilon membranes were established. Using these techniques, it was possible to determine the N-terminal amino acid sequence for 4 of the 9 glycoproteins analyzed as shown in Table I. The remaining five components of interest were not sequencable in this manner, presumably because they had blocked N-termini.

(3) A peptide, comprised of the first 13 N-terminal residues of the fragment RLMP-1, was synthesized, purified, and used to produce rabbit antibodies which will be helpful in the larger scale purification of the whole protein and in various biological studies.

(4) A similar 2D-gel pattern was observed for the glycoproteins from normal rat liver and from a normal rat liver epithelial cell line. However, several of the proteins from the neoplastic liver and transformed FJ-2 cell line were present in different amounts.

Table I

RLMP-	1 — 5 — 10 — 15	MW x 10_{-3}/PI coordinates
1[ab]	NH_2-PHe-ASN-LEU-ASP-ALA-GLU-ALA-PRO-ALA-VAL-LEU-SER-GLY-PRO-PRO-GLY-	145 / 4.5-4.6
2	(No sequence observed)[c]	143 / 4.7-4.8
3	NH_2--X[d]---X---X--ASP-LYS-LYS--X--ASP-VAL-GLY-THR-	70 / 5.0-5.1
4	NH_2-ALA-SER-GLU-ALA-ILE-LYS-GLY-ALA-MET-MET-GLY-ILE--X--LEU-	68 / 5.5-5.8
5	NH_2--X--TYR-THR-VAL-ASN-SER-ALA-TYR-GLY-ASP-THR-ILE-VAL-MET-PRO-	95 / 4.6-4.8
6	(No sequence observed)	83 / 4.8-4.9
7	(No sequence observed)	40 / 5.7
8	(No sequence observed)	155 / 5.0-5.1
9	(No sequence observed)	56 / 5.3

a Proteins are denoted (RLMP-1 - 5) as shown in Fig. 3.
b The amino acid residues are given in the three letter notation.
c Mechanical malfunctions were not responsible when a lack of sequence was observed, since the 5 pmoles of internal standard was being properly analyzed (see Materials and Methods).
d X indicates no residue was identifiable by the sequence procedure, indicating that the amino acid may be glycosylated or that it was a cysteine or tryptophan.

ANNUAL REPORT OF

THE LABORATORY OF EXPERIMENTAL PATHOLOGY
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

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; and (3) research on mechanisms of carcinogenesis correlating different levels of biological organization, from whole organisms (human and animal), organs and tissues, to the cellular, subcellular and molecular levels.

General Research Objectives

Present studies in the Laboratory of Experimental Pathology (LEP) are concerned with two correlated problems: (1) the pathogenesis of neoplastic disease, induced by chemical and physical factors, in epithelial cells studied in selected biological models in vivo and in vitro, particularly in the respiratory tract and the epidermis; and (2) the interactions of different factors in multifactorial carcinogenesis mechanisms, including the role of carcinogens, promoters, oncogenes, growth factors, cellular mediators and certain types of tissue injury.

In order to correlate mechanisms of carcinogenesis investigated at the cellular and molecular levels with the corresponding events in animal and human tissues and organs, it is important to connect these different levels of observation and to study the mechanisms of action of carcinogens in relevant biological systems. Such an approach requires the development of a range of biological models related to human cancer pathology and particularly to those epithelial target tissues from which most of the major forms of human cancer originate. A great deal of progress has occurred in this direction in the past two decades and experimental animal models have been established, by chemical induction, for most of the major types of human cancer. For many of these models adequate culture systems have been developed for the target tissues and cells, including both animal and human target cells. Pathogenetic mechanisms have been clarified through major advances in experimental pathology, cell biology, molecular biology and biochemistry. Work in the LEP has contributed substantially to this progress. The current LEP studies continue this approach.

Work in this fiscal year has been primarily devoted to the following activities: extensive histopathological evaluation and data analysis of recently conducted animal experiments on respiratory carcinogenesis by crystalline silica in different species and by multifactorial treatments; experimental cell biology studies on the response of recently established mouse epidermal cell lines in

serum-free media to growth factors and carcinogens; assays for induction of metastatic activity in nude mice of cell lines transformed by metals and other carcinogens; and completion of a preliminary study that identified preferential carcinogen binding and repair at DNAase I-hypersensitive sites in liver cell DNA in vivo and in the ras oncogene. The laboratory facilities were closed for relocation and complete renovation, and are expected to be ready to resume benchwork activity by the end of this fiscal year.

Results Obtained in the Current Year

(1) Respiratory carcinogenesis in vivo. The relationship between chronic pulmonary granulomatosis/fibrosis and the induction of cancer of the lung was investigated by the recently developed experimental model of lung carcinogenesis induced by crystalline silica particles. Crystalline silica, in the form of quartz, is the second most common mineral in the earth's crust. In fine particulate form of respirable size (about 1-5 μm) it is well known to induce a progressive obstructive pulmonary disease, silicosis, characterized by granulomatous and fibrogenic reactions. Recent findings of lung cancer induction in rats by silica have been consistent with reports of increased human lung cancer risk in silicotic subjects. It was hypothesized (Saffiotti, 1986) that the induction of carcinomas of the lung by silica may be mediated by the action of the silicotic granuloma on the adjacent epithelia of the peripheral airways. Extensive serial sacrifice studies in rats, hamsters and mice, following single intratracheal instillations of silica particles in the lung, have now demonstrated marked species differences in the structure and cellular composition of the granulomatous reaction, accompanied by fundamental species differences in the reaction of the epithelium of the bronchioles and alveoli. The rat shows an intense hyperplasia of the bronchiolar and alveolar (type II) epithelium, adjacent to silicotic lesions, starting within days of silica exposure. Subsequent diffuse epithelial hyperplasias and bronchiolar/alveolar adenomatoid lesions are followed by the appearance, beginning in less than one year, of carcinomas, mostly of the adenocarcinomatous type and often developing around fibrotic areas. The latter are comparable to the pulmonary "scar cancers" of human pathology, of which they represent a new, reproducible experimental model. In contrast, the epithelial reaction to silica in the lung is minimal in hamsters and limited to a transient hyperplasia of the bronchi and bronchioles in mice (including mice of the strain A, otherwise prone to lung adenoma induction by carcinogens). The results of several experiments showed induction of high incidences of lung cancer by silica in rats, with prevalence in females. The pathogenesis of the epithelial proliferation is being further studied in relation to the adjacent inflammatory granulomatous reaction. The present results support the view that the cellular mediators of inflammation, extensively secreted in the proximity of the peripheral airways, are the likely stimulus to epithelial proliferation and neoplastic growth.

A separate set of experiments revealed a marked enhancing effect of systemically administered (intraperitoneal) dimethylsulfoxide (DMSO) on the incidence of respiratory tract tumors (especially carcinomas) induced by repeated intratracheal instillations of benzo[a]pyrene/ferric oxide in hamsters. The DMSO effect was more marked when DMSO was given at the same time as the carcinogen, rather than after a five-day interval. Localization and binding of a dose of tritiated benzo[a]pyrene was not affected by concurrent DMSO administration.

(2) Studies on growth and transformation of cells in culture. Newly established cell lines of mouse epidermal keratinocytes in serum-free media were characterized. These lines, even at advanced passage levels, retain epithelial morphology and markers, respond to the induction of terminal differentiation by calcium and by serum, and remain non-tumorigenic. Their growth requirement for bovine pituitary extract could be replaced by increased bovine serum albumin levels or by somatomedin C. A cell line highly resistant to transforming growth factor-beta was isolated and characterized. Chemically induced toxicity and transformation were studied with different carcinogens, including arsenic and other metals, in these epithelial lines and in the mouse embryo cell line, BALB/3T3 clone A31-1-1, previously characterized in this laboratory. Particular studies are currently concerned with the requirements for the induction of a metastasizing phenotype as an end-point of transformation. All the tested chemically transformed BALB/3T3 cell lines were highly tumorigenic in nude mice, but they were not metastatic by either subcutaneous or intravenous assays. The induction of metastatic phenotype by transfection with the activated c-Ha-*ras*-1 oncogene (and the inhibition of this effect by the adenovirus type 2 Ela gene) as recently reported (Pozzatti et al., 1986) was shown to correspond to the activation of collagenase type IV production in the target cells. Current studies are addressed to investigate whether chemical carcinogens are capable of inducing the metastatic phenotype in these tumorigenic but non-metastatic cell populations.

(3) Studies on preferential DNA binding sites for carcinogens. Although binding of carcinogens to DNA and its repair have been traditionally determined in whole DNA, thereby providing only average distribution data in relation to DNA structure, recent investigations in several laboratories have revealed highly selective, non-random sites both for preferential binding and for repair, depending, for example, on the form of DNA packaging and on the state of actively transcribed genes versus inactive genes. Present investigations with the carcinogen benzo[a]pyrene in liver cells in vivo have identified highly selective early preferential binding to DNAase I-hypersensitive sites, accounting for about 80% of the DNA-bound carcinogen at early times (30 min). Binding at these sites, which are only a small fraction of the genome, therefore represents "hot spots" which were found to be rapidly repaired; thus, persisting adducts were located outside the DNAase I-hypersensitive sites. DNAase I-hypersensitive sites were identified in the c-Ha-*ras*-1 proto-oncogene and it was found that benzo[a]pyrene produces selective DNA damage at these sites in this gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04491-12 LEP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Quantitative Studies on Concurrent Factors in Neoplastic Transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: U. Saffiotti Chief LEP NCI

COOPERATING UNITS (if any)

Mario Negri Pharmacol. Res. Institute, Milan, Italy (F. Bertolero);
Inst. of Histology and Embryology, Univ. of Padua, Italy (S. Garbisa)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
0.2	0.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Studies were continued on mechanisms of transformation in the mouse embryo cell line, BALB/3T3 clone A31-1-1. Transformation was obtained with several metals. Studies with trivalent and pentavalent arsenic (As) showed that trivalent As was four times more active than pentavalent As. However, when correlated with the calculated As uptake and cell burden, toxicity and transformation frequency were found to be the same for both forms of As. The results of metabolic studies indicate that trivalent As is the form responsible for the toxic and transforming effects, independently of the valence state of the As exposure.

BALB/3T3 cells transformed by metals and by organic carcinogens, or spontaneously, when inoculated in nude mice were found to be tumorigenic but not metastasizing, even by intravenous administration. This finding is consistent with the lack of type IV collagenolytic activity observed in these transformed cell lines. Induction of metastatic activity by further treatment with the ras oncogene or with carcinogens is under investigation.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP NCI

Objectives:

(a) To study mammalian cell culture systems quantitatively for induction kinetics and mechanisms of cytoxicity, neoplastic transformation, mutagenicity and DNA damage and repair in response to different exposures to carcinogens. (b) To investigate the induction of invasiveness and metastatic activity as a marker of advanced transformation.

Methods Employed:

The BALB/3T3 clone A31-1-1 mouse embryo cell line was used under test conditions previously standardized in this laboratory for transformation assays with different carcinogens and for the induction of ouabain resistance. DNA damage and repair were determined by alkaline elution analysis and the removal of alkylated DNA adducts was measured by high performance liquid chromatography. Arsenic metabolism studies were conducted with ^{73}As sodium arsenite and arsenate, gamma-counting and paper electrophoresis. Degradation of labelled type IV collagen substrate was measured in control and chemically transformed BALB/3T3 cell lines, and the collagen degradation pattern was examined by gel electrophoresis. Assays for tumorigenic and metastatic activity were conducted in nude mice.

Major Findings:

Transformation was obtained with several metals. Trivalent arsenic (As) was found to be four times more active in inducing cytotoxicity and transformation than pentavalent As. Cellular uptake and metabolic studies showed that when correlated with the calculated As cell burden, toxicity and transformation frequency were found to be the same for both forms of As. The results indicated that trivalent As is the form responsible for the toxic and transforming effects, independently of the valence state of the As exposure.

BALB/3T3 cells transformed by As, chromium, cadmium, benzo[a]pyrene and N-methyl-N′-nitro-N-nitrosoguanidine, or spontaneously, when inoculated subcutaneously in nude mice were found to be tumorigenic, producing histologically typical fibrosarcomas. When cell suspensions were tested in nude mice for metastatic activity, either by subcutaneous or by intravenous inoculation, none of these tumorigenic cell lines was found to have metastatic activity, in agreement with the lack of type IV collagenolytic activity observed in these cell lines. Induction of metatastic activity by further treatment with the ras oncogene or with carcinogens is under investigation.

Publications:

Bertolero F, Pozzi G, Sabbioni E, Saffiotti U. Cellular uptake and metabolic reduction of pentavalent to trivalent arsenic as determinants of cytotoxicity and morphological transformation. Carcinogenesis 1987;8:803-8.

Garbisa S, Negro A, Kalebic T, Pozzatti R, Muschel RJ, Saffiotti U, Liotta LA. Type IV collagenolytic activity. Linkage with the metastatic phenotype induced by ras transfection. In: Liotta LA, et al, eds. Mechanisms of metastasis, New York: Plenum Press (In Press).

Garbisa S, Pozzatti R, Muschel RJ, Saffiotti U, Ballin M, Goldfarb RH, Khoury G, Liotta LA. Secretion of type IV collagenolytic protease and metastatic phenotype: Induction by transfection with c-Ha-ras but not with c-Ha-ras plus Ad2-Ela. Cancer Res. 1987;47:1523-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05265-07 LEP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Effects of Chemical Carcinogens on Transforming DNA Sequences and Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	M. I. Lerman	Expert	LIB	NCI
	S. F. Stinson	Biologist	DTP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The aim of this project is to identify, characterize, and clone those genes that drive the development of neoplasia and whose malignant potential results from changes caused by chemical carcinogens. DNAase I-hypersensitve (HS) sites were identified as targets for rapid binding and repair following in vivo exposure to benzo[a]pyrene (BP), both in total liver cell DNA and in the c-Ha-*ras*-1 proto-oncogene. The kinetics of BP adduct formation and repair were first determined in total liver DNA from hamsters given tritiated BP intraportally. Isolation of nuclei after selected BP treatment times showed that 80% of the adducts were DNAase I-HS at early times after BP exposure (30 min), whereas the adducts remaining when repair was nearly complete (60 min) were no longer DNAase I-HS. The Ha-*ras* gene was analyzed under the same conditions of BP exposure in hamster liver DNA and showed a marked DNAase I-HS response at the early time points (15-30 min), but not after repair completion (120 min), indicating that BP binding and repair occur preferentially at DNAase I-HS sites. DNAase I-HS sites were also found in the Ha-*ras* locus in human liver DNA, and human hepatoma DNA.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP	NCI
M. I. Lerman	Expert	LIB	NCI
S. F. Stinson	Biologist	DTP	NCI

Objectives:

The overall objective of this project is to identify and characterize genes that drive the development of neoplasia and whose malignant potential results from changes caused by chemical carcinogens. The immediate objective in the current year was to complete a preliminary study to define the role of DNAase I-hypersensitive sites in DNA damage and repair induced by a carcinogen in total cellular DNA and in the ras gene.

Methods Employed:

Brief treatment of nuclei with different low concentrations of DNAase I were used to define DNAase I-hypersensitive regions in the chromosome. These DNAase I-hypersensitive regions, when mapped, occur in the putative regulatory regions of actively expressed genes. Carcinogen treatment in vivo or in vitro, followed by DNAase I treatment of the nuclei, was used to determine whether or not carcinogens preferentially bind to DNAase I-hypersensitive regions. Labeled probes of known oncogenes were hybridized to blots of genomic DNA to study the effects of carcinogen and DNAase I treatments at the gene level.

Major Findings:

DNAase I-hypersensitive (HS) sites of the c-Ha-ras-1 proto-oncogene were identified as targets for rapid binding and repair following exposure to benzo[a]pyrene (BP). Initial experiments, using total liver DNA from hamsters treated with [^{3}H]BP by intraportal injections, showed the kinetics of BP adduct formation (maximum after 30 min; >80% repair after 60 min). Nuclei were isolated at selected times after BP treatment and were treated with increasing concentrations of DNAase I. At the time of maximum binding (30 min), 80% of the adducts were removed by DNAase I and thus located at DNAase I-HS sites, whereas the adducts remaining at 60 min, were no longer located at DNAase I-HS sites. The effects of BP exposure on the Ha-ras gene were then analyzed under the same conditions described above for total DNA in hamster liver cells. Early time points after BP injection (15-30 min) showed marked DNAase I sensitivity, whereas late points (120 min) were not different from untreated controls. BP treatment alone showed DNA breaks resulting in restriction enzyme bands of the same fragment length as those resulting from DNAase I alone, indicating again the activity of BP at DNAase I-HS sites. BP pretreatment markedly enhanced the effect of DNAase I at these sites. Thus, DNAase I-HS sites were shown to be preferential sites for BP binding and repair; and BP was shown to damage the ras gene at DNAase I-HS sites near the promoter region. Studies on human normal liver cells and human hepatoma cells showed DNAase I-HS sites in both, with differences in the banding pattern.

Publications:

Lerman MI, Norman RL, Stevens L, Stinson SF, Saffiotti U. DNAase I-hypersensitive sites of the c-Ha-*ras*-1 proto-oncogene as targets for rapid binding and repair of benzo[a]pyrene adducts. In: Castellani A, ed. DNA damage and repair. New York: Plenum Press (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CP05274-07 LEP

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Respiratory Carcinogenesis by Chemical and Physical Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	S. F. Stinson	Biologist	DTP	NCI

COOPERATING UNITS (if any)
Department of Pathology, University of Maryland, School of Medicine, Baltimore, MD (E. M. McDowell, K. P. Keenan)

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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

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

The role of chronic pulmonary granulomatosis/fibrosis in the induction of lung cancer was studied in a new experimental model, i.e., carcinogenesis by silica. Crystalline silica forms (quartz, hydrofluoric-acid-etched quartz, cristobalite and tridymite) in fine particles were instilled intratracheally in inbred rats, hamsters and mice. Long-term studies with quartz and hydrofluoric-acid-etched quartz showed that rats developed high incidences of pulmonary carcinomas (mostly adenocarcinomas) with prevalence in females. Serial sacrifice studies showed that this carcinogenic response of the rat is preceded by an intense hyperplasia of the bronchiolar and alveolar (type II) epithelia in areas adjacent to silicotic granulomatous lesions. The epithelial hyperplasia begins within days of single administrations of silica and progresses into proliferative hyperplasias and adenomatoid lesions, and ultimately into carcinomas. In contrast, the epithelial reaction was minimal in hamsters and transient in mice. Within the times so far observed, no carcinogenic response was found in the lungs of hamsters and mice, even in the adenoma-prone strain A. Marked species differences were identified in the structure and cellular composition of the granulomatous reaction. The correlation of epithelial proliferation with the adjacent inflammatory cells and their mediators is investigated in relation to host differences in the cellular pathogenesis of the epithelial and inflammatory/fibrogenic responses.

Dimethylsulfoxide (DMSO), given intraperitoneally, markedly enhanced respiratory carcinogenesis by intratracheal benzo[a]pyrene/ferric oxide (BP) in hamsters. The enhancement was higher when DMSO was given concurrently rather than five days after each BP dose. Autoradiographic analysis showed no influence of concurrent DMSO on localization and binding of a single BP dose in the whole respiratory tract.

GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP	NCI
S. F. Stinson	Biologist	DTP	NCI

Objectives:

The objective of this project is the elucidation of the pathogenetic mechanisms by which respiratory tract cancers, representing major forms of human cancer, are induced by chemical and physical factors, alone or in various combinations.

Animal models for the induction of respiratory carcinogenesis are being characterized, especially in relation to their human counterpart. Current studies have the objective of elucidating the relationship between chronic pulmonary granulomatosis/fibrosis and the induction of cancers of the lung in the recently developed experimental model of lung carcinogenesis by crystalline silica particles.

Methods Employed:

Lifetime and serial sacrifice studies, with histopathological investigation of respiratory tract pathology in inbred rats, hamsters, and mice. Intratracheal or intralaryngeal instillations of particulate suspensions are used to study respiratory tissue reactions by histologic, histochemical, autoradiographic and immunochemical methods and by scanning and transmission electron microscopy. Particulate materials currently investigated include crystalline silica (quartz, hydrofluoric acid-etched quartz, crystobalite, tridymite) and ferric oxide. Concurrent effects of the solvent, dimethylsulfoxide (DMSO), and polycyclic aromatic hydrocarbons are also being investigated.

Major Findings:

(1) Pulmonary carcinogenesis by crystalline silica. Extensive pathogenetic studies have been conducted on the relationship between granulomatous/fibrogenic responses induced by crystalline silica particles in the lung and the induction of epithelial hyperplasia and epithelial cancers. Serial sacrifice studies have provided the histopathogenetic basis for the evaluation of the granulomatous and epithelial lesions induced by single intratracheal instillations of silica particles in inbred animals of three species: Fischer 344 rats, 15.16/EHS:CR hamsters, and mice of the three strains, A/JCr, BALB/c and NCR:NU (athymic nude). In the current year, extensive study of the resulting histopathology has provided several major findings. All species and strains tested developed granulomatous silicotic lesions, but their structure, cellular composition and kinetic development were found to vary considerably from species to species. The reaction of the adjacent bronchiolar and alveolar epithelium also showed remarkable species differences, being minimal in the hamster, transient in the mouse and very intense and progressive in the rat. Of the three species, only the rat showed a marked carcinogenic response after the single exposure to

silica, reaching a high incidence of pulmonary carcinomas in the 11 and 17 months sacrifice groups. No tumor induction has been observed thus far in hamsters nor in mice of any of the tested strains, including mice of the strain A that are known for their high susceptibility to the induction of lung adenomas by a wide variety of carcinogens. In the rats, a much higher incidence of lung tumors was observed in females than in males, again indicating the determinant role of host factors in the carcinogenic response to silica. The induced lung tumors included a majority of adenocarcinomas, and some epidermoid carcinomas, mixed carcinomas, and adenomas. Many silica-induced adenocarcinomas have a wide fibrotic core, and appear to be a good model for human "scar cancer" in the lung. Further histochemical analysis is under way to characterize the cellular types involved in the preneoplastic and neoplastic reactions. These studies have documented the very early hyperplastic response of the epithelium of the peripheral airways to the silica particles in rats and the development of hyperplastic areas, bronchiolar adenomatoid lesions and, ultimately, carcinomas with various types of cellular differentiation. The interaction of the cellular components of the granulomatous reaction (macrophages, polymorphonuclear monocytes, lymphocytes, fibroblasts and mastcells) with the adjacent epithelial cells of the peripheral airways is under further study by immunohistochemical methods. The present findings confirm the interest of the silica model for the study of the role of cellular mediators of inflammation in respiratory carcinogenesis.

(2) <u>Enhancement of respiratory carcinogenesis by dimethylsulfoxide (DMSO)</u>. A marked enhancement by DMSO given systemically was found in the course of respiratory tract carcinogenesis induced by 12 weekly intratracheal instillations of benzo[a]pyrene/ferric oxide particles (BP/Fe_2O_3) in hamsters. Three groups of 50 (male and female) Syrian golden hamsters received 12 weekly intratracheal instillations of six mg BP/Fe_2O_3 in saline. Two of the groups also received intraperitoneal administrations of 0.1 ml DMSO given either concurrently with or five days after each intratracheal dose of BP/Fe_2O_3. The respiratory tumor incidence in groups receiving both BP/Fe_2O_3 and DMSO was significantly greater than in the group given only BP/Fe_2O_3. The incidence of all respiratory tumors was 76% for DMSO concurrent with BP/Fe_2O_3, 63% for DMSO five days after BP/Fe_2O_3, and 36% for BP/Fe_2O_3 without DMSO. The incidence of respiratory tract carcinomas showed an even greater relative effect (62%, 34%, and 22% respectively). The enhancing effect of DMSO was observed in all segments of the respiratory tract (larynx, trachea, and bronchi/lung). These results indicate that systemic DMSO markedly enhances respiratory carcinogenesis in this experimental model, and that the magnitude of the enhancement depends, in part, on the relative time of administration of the DMSO and the carcinogen.

Autoradiographic localization and binding of [^{3}H]BP/Fe_2O_3 in the hamster respiratory tract were analyzed at close intervals, up to one week after a single intratracheal administration, with or without concurrent intraperitoneal injection of DMSO. The pattern and level of BP binding was not altered by DMSO in any of the segments of the respiratory tract at any of the observed time points.

(3) <u>Multifactorial study of respiratory carcinogenesis</u>. Statistical analysis and data evaluation have been completed for this large in vivo study that has demonstrated the determinant role of mucosal injury in respiratory carcinogenesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05276-07 LEP

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Growth Control in Epithelial Cells and its Alteration in Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	M. E. Kaighn	Expert	LHC	NCI

COOPERATING UNITS (if any)

Mario Negri Pharmacol. Res. Institute, Milan, Italy and Dept. of Toxicology, Karolinska Institute, Stockholm, Sweden (F. Bertolero)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.7	0.3	0.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Mouse keratinocyte cell lines were obtained without initial crisis by use of a serum-free medium, LEP/MK2 (low-calcium Minimal Essential Medium with non-essential aminoacids, supplemented with eight factors). The lines are subtetraploid with random gain and loss of otherwise normal chromosomes. At higher passage numbers, the lines acquire greater independence from growth factors and become less sensitive to growth inhibitors; the requirement for bovine pituitary extract becomes replaceable with bovine serum albumin or with somatomedin C. Transforming growth factor beta (TGF-β) is a major growth inhibitor of these lines, without inducing terminal squamous differentiation. Cells exposed to 1000 pg/ml of TGF-β in medium LEP/MK2, and so maintained, developed into a TGF-β-resistant cell line (MK/2067C), which remains responsive to induction of terminal squamous differentiation by calcium and by serum. The TGF-β-resistant cell line is subtetraploid with three marker chromosomes. The effect of carcinogens and/or oncogenes is studied in these cell lines.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP	NCI
M. E. Kaighn	Expert	LHC	NCI

Objectives:

The overall objectives are to develop and characterize epidermal cell culture systems suitable for investigating chemical carcinogenesis and the sequence of changes produced by carcinogens in the control of growth and differentiation of epithelial cells. Mouse epidermal keratinocytes (MK) are currently used in these studies. Specific objectives include: (a) establishment of cell lines in serum-free media, (b) investigation of altered growth properties acquired by these cells with continued growth in culture and by exposure to growth factors, and (c) development of selective conditions for cells transformed by oncogenes or chemical carcinogens.

Methods Employed:

BALB/c newborn mouse epidermal cells are isolated by cold trypsinization as previously described and cultured in a serum-free medium developed in this laboratory. The growth medium, LEP/MK2, consists of Eagles's MEM with nonessential amino acids but without added calcium, supplemented with epidermal growth factor (5.0 ng/ml); insulin (5.0 μg/ml); transferrin (5.0 μg/ml); hydrocortisone (0.5 μM); ethanolamine and phosphoethanolamine (0.5 μM each); bovine serum albumin (100 μg/ml); and bovine pituitary extract (0.5%). Clonal growth is measured by colony-forming efficiency (CFE) and by average colony size after 7-10 days. The clonal growth rate is defined as population doublings per day (PD/d). For transformation experiments, toxicity is determined by clonal survival assays. The appearance of altered growth properties, ultrastructure (using both SEM and TEM) and karyological changes are studied by standard methods. Growth in soft agar and tumorigenicity in nude mice are used to demonstrate acquired neoplastic properties.

Major Findings:

Work on this project has yielded new information on the behavior of mouse keratinocytes (MK) in culture: (1) Continuous, spontaneously "immortalized" cell lines were established in serum-free medium. (2) The cell lines maintain typical morphology and keratin structures, and remain sensitive to induction of terminal differentiation by calcium and serum. (3) Whole serum, chelexed serum, and serum-derived factors inhibit growth of MK cells. (4) Bovine serum albumin (BSA) stimulates growth progressively even in the absence of bovine pituitary extract (BPE) and can partly replace the requirement for BPE in late passage cells. The BPE requirement can also be partly replaced by somatomedin C. With increasing passage levels, the cell lines become more responsive to growth stimulation by BPE and acquire partial independence from BPE requirement. (5) With continued passage, MK cells become less dependent on growth factors and less sensitive to growth inhibitors; however, they remain able to express their terminal

differentiation program. The chromosomal profiles of all tested lines showed a rapid shift to a subtetraploid karyotype (70-80 chromosomes). Most chromosomes remained normal when examined by Giemsa banding but there was a random loss and gain of individual chromosomes. These changes begin early in the culture, even in primary culture. (6) When inoculated in nude mice, all tested MK lines remain non-tumorigenic, even at late passage (tested as late as passage 118). (7) Transforming growth factor β (TGF-β) is a major growth inhibitor for MK cell lines, but its effects are not mediated by the induction of terminal squamous differentiation. After five passages in serum-free medium LEP/MK2, cells from the resulting line (MKDC4) were exposed to 1000 pg/ml of purified TGF-β in medium LEP/MK2. The surviving cells (40% after two days) were then regularly refed with the same TGF-β containing medium. After about a month, the cells formed a TGF-β-resistant cell line (MK/2057C). This cell line has been maintained through over 150 doublings and cryopreserved. The clonal growth responses of the parental and resistant lines to various factors were compared. The resistant line was less responsive to BPE; both fetal bovine serum and calcium induced terminal squamous differentiation in both lines. Giemsa banding showed that the resistant line was subtetraploid. Marker chromosomes, developed by passage 12, involved chromosomes 1, 14 and a centric fragment. Other chromosomes were normal, but chromosome 19 was overrepresented and chromosome 14 was underrepresented. The effect of several carcinogens is studied in these different MK cell lines. Toxicity dose-response data were obtained with such chemical carcinogens as N-methyl-N′-nitro-N-nitrosoguanidine, 7,12-dimethylbenz[a]anthracene, sodium arsenite and potassium chromate and transformation assays were started with these carcinogens and with transfected oncogenes.

Publications:

Bertolero F, Camalier RF, Kaighn ME, Saffiotti U. Spontaneous establishment and characterization of mouse keratinocyte cell lines in serum-free medium. In Vitro Cell Dev Biol (In Press)

ANNUAL REPORT OF

THE LABORATORY OF HUMAN CARCINOGENESIS
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The Laboratory of Human Carcinogenesis conducts investigations to assess (1) mechanisms of carcinogenesis in epithelial cells from humans and experimental animals, (2) experimental approaches in biological systems for the extrapolation of carcinogenesis data and mechanisms from experimental animals to the human situation, and (3) host factors that determine differences in carcinogenic susceptibility among individuals.

The scientific and managerial strategy of the Laboratory is reflected in its organization into three sections, i.e., In Vitro Carcinogenesis Section (IVCS), Molecular Genetics and Carcinogenesis Section (MGCS), and Biochemical Epidemiology Section (BES). Scientifically, the emphasis is on the role of inherited or acquired host factors as important determinants in an individual's susceptibility to carcinogens, cocarcinogens and tumor promoters. Our investigations of host factors involve interspecies studies among experimental animals and humans, cover the spectrum of biological organization ranging from molecular and cellular biology, pathology, epidemiology, and clinical investigations. Two sections (IVCS and MGCS) devote their major efforts to more fundamental and mechanistic studies. The scientific findings, techniques, and concepts developed by these two sections and, of course, the scientific community at large, are utilized by the BES in selected and more applied studies of carcinogenesis and cancer prevention. The laboratory-epidemiology studies in this section require the expertise found in the IVCS and MGCS and in the NCI Epidemiology Program. The Laboratory requires unique and complex resources. For example, collection of viable normal and neoplastic epithelial tissues and cells--well characterized by morphological and biochemical methods from donors with an epidemiological profile--requires continued cooperation among donors and their families, primary care physicians (internists, surgeons, house staff), surgical pathologists, nurses, epidemiologists, and laboratory scientists.

Since its establishment, LHC has been fortunate to have the constructive criticism of a group of colleagues who are recognized experts in molecular biology (Carlo Croce, M.D., Associate Director, Wistar Institute, Philadelphia, PA; Lennart Philipson, M.D., Ph.D., Director, European Molecular Biology Laboratory, Heidelberg, FRG); cell biology (Ted Puck, Ph.D., Director, Eleanor Roosevelt Center for Cancer Research, Denver, CO; David Prescott, Ph.D., Distinguished Professor, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO), and biochemistry (Allan Conney, Ph.D., State of New Jersey Professor of Pharmacology & Chairman, Department of Chemical Biology and Pharmacognosy, College of Pharmacology, Rutgers The State University of New Jersey, Piscataway, NJ). These colleagues visit LHC on an

individual basis at least once a year and review ongoing research projects with LHC staff. The exchange of ideas and their continuing advice have made these visits invaluable.

In addition to the frequent and informal exchanges of information among LHC staff, the Laboratory and each section have monthly scientific and administrative meetings. We also sponsor, with the Laboratory of Cellular Carcinogenesis and Tumor Promotion (LCCTP), a weekly Journal Club. A monthly joint rotating seminar series is presented by LHC, LCCTP, and the Laboratory of Experimental Carcinogenesis. LHC sponsors seminars with extramural speakers at monthly intervals.

LHC also organizes meetings of the Human Studies Collaborative Group, which take place in Bethesda and include participants from the NIH scientific community, extramural experts and collaborators, and staff from LHC resource contracts. These biannual meetings provide a forum for the survey of an ongoing research area, e.g., respiratory carcinogenesis, and for informal discussions.

RESEARCH STRATEGY

A central problem in carcinogenesis research is the extrapolation of data and knowledge of mechanisms from experimental animals to the human population, and within this heterogeneous population, extrapolation among individuals. A subset of this problem is the difficulty associated with extrapolation from one level of biological organization to another, i.e., molecules to macromolecules to organelles to cells to tissues to organs to intact organisms.

The strategy currently employed by LHC is a refinement of the one we formulated a decade ago. Epidemiologic and clinical observations provide clues for generating hypotheses. In many cases, clinical investigations and studies using animal models can be used to test hypotheses. In other cases, in vitro models are more suitable. These models utilize human tissues and cells collected at the time of immediate autopsy (i.e., from organ donors) and surgery. Remarkable progress has been made during the last several years in the development of methodology to culture normal human tissues and their epithelial cells from most major sites of human cancer. Therefore, the mechanisms of action of carcinogens, tumor promoters, growth factors, differentiation inducers, etc., can be investigated at the tissue, cellular, and subcellular levels of biological organization. An integral facet of this strategy is that the same types of tissues and cells from experimental animals can be maintained in identical, controlled in vitro experimental settings so that comparative studies using human and experimental animal material can be conducted.

After developing in vitro models, we are systematically investigating several research areas, i.e., (I) in vitro model development; (II) carcinogen metabolism, DNA damage, and DNA repair; (III) cell and molecular biology of normal and neoplastic cells; (IV) carcinogenesis and related studies (Table 1); and (V) biochemical and molecular epidemiology of human cancer . In recent years, the majority of the research projects have progressively shifted from areas I and II to areas III-V. Although we have developed in vitro models for several types of human tissues, the emphasis has been on lung, esophagus, liver and colon. Summaries of research projects in each of these integrated areas of our research program are listed in the following sections.

TABLE 1. STRATEGY FOR STUDYING HUMAN CARCINOGENESIS

I. In Vitro Model Development

A. Collection of Human Tissues and Cells
B. Explant and Cell Cultures
C. Serum-Free Culture Conditions

II. Carcinogen Metabolism, DNA Damage and DNA Repair

A. Interspecies Comparisons
B. Interindividual Comparisons
C. Intercellular Comparisons
D. Cell-Mediated Responses
E. Others

III. Cellular and Molecular Biology of Normal and Neoplastic Cells

A. Growth Factors
B. Differentiation Factors
C. Cytoskeleton
D. Others

IV. Carcinogenesis and Related Studies

A. Oncogenes and Tumor Suppressor Genes
B. Chemical Carcinogens
C. Physical Carcinogens
D. Tumor Promoters, Aldehydes and Peroxides
E. Others

V. Biochemical and Molecular Epidemiology

A. Lung Cancer
B. AIDS
C. Others

I. IN VITRO MODEL DEVELOPMENT

Remarkable progress has been made during the last few years by this and other laboratories in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. We have developed chemically-defined media for long-term culture of human bronchus, colon, esophagus, and pancreatic duct. Primary cell cultures of human epithelial outgrowths have been obtained from many different types of human tissues.

Defined culture conditions for normal human epithelial cells from the bronchus, esophagus, pleural and liver have been established in LHC. For example, methods have also been developed to culture pleural mesothelial cells obtained from non-cancerous donors. Pure cultures are initiated by pelleting the mesothelial cells from pleural effusion fluid and inoculating the resuspended cells into FN/C/BSA-coated dishes containing LHC basal nutrient medium supplemented with growth factors. Using this protocol, mesothelial cell cultures have been established from more than 200 donors. The cells can be subcultured at clonal density with a colony-forming efficiency of more than 10% and high density cultures can be subcultured four to six times before senescence.

These culture systems are now sufficiently established to permit 1) pathobiologic investigations of normal human bronchial epithelial (NHBE) cells, e.g., regulation of growth and differentiation pathways and their dysregulation during carcinogenesis; 2) short- and long-term asbestos carcinogenesis investigations of mesothelial cells; 3) studies of putative synergistic effects of hepatitis B virus and chemical carcinogens in hepatocellular carcinogenesis.

A. Model Systems for Studying Physical Carcinogens in Normal Human Mesothelial Cells

These studies include the following: (1) develop defined media for replicative mesothelial cell cultures; (2) evaluate cytotoxicity of asbestos fibers and synthetic nonmineral fibers in mesothelial and bronchial epithelial cells; (3) evaluate the effects of asbestos fibers on progression of chromosome rearrangements in mesothelial cells; and (4) evaluate the putative role of oxygen radicals in the mode of action of asbestos-caused carcinogenesis.

Methods to culture human pleural mesothelial (NHM) cells have been defined. Cultures are initiated by pelleting the mesothelial cells from pleural effusion fluid and inoculating the resuspended cells into dishes containing LHC basal nutrient medium supplemented with serum (3%), hydrocortisone (0.5 micromoles), insulin (5 micrograms/ml), epidermal growth factor (EGF) (5 ng/ml), transferrin (10 micrograms/ml), trace elements, and 2% chemically-reduced (factor-free) serum (FFS). Using this protocol, mesothelial cell cultures have been established from more than 200 donors. The cells can be subcultured at clonal density with a colony-forming efficiency of more than 10% and high density cultures can be subcultured four to six times before senescence. We have now established that many factors will induce serum-starved cells to undergo one round of DNA synthesis in the absence of serum. These factors include EGF, transforming growth factor β_1 (TGF-β_1), TGF-β_2, interleuken 1-α (IL-1-α), IL-1-β, transforming growth factor α (TGF-α), human platelet-derived growth factor (hPDGF), porcine PDGF (pPDGF), fibroblast growth factor-acidic (FGF-a), FGF-basic (FGF-b), β-interferon (Inf-β), γ-interferon (Inf-γ), and cholera toxin. However, for sustained growth, the medium must also be supplemented with high density lipids (HDL). Since human mesothelial cells have a peculiarly plastic cytoskeleton, we have characterized the affects of amosite fibers and code 100 glass fibers on the fidelity of division in these cells in an effort to understand the mechanism by which asbestiform fibers induces transformation and mesothelioma. Our results indicate that both amosite and code 100 glass fibers disturb the fidelity of cell division leading to the induction of aneuploid daughter cells. However, the

mechanism of action of these two agents appears to be different. Amosite fibers induce chromosome clustering suggesting an inhibition of mitotic tubulin formation and/or centriole separation. In contrast, code 100 glass fibers cause chromosome dislocation from the spindle at metaphase suggesting an affect on centromere/kinetochore function and/or mitotic spindle function.

B. Growth and SV40 Transformation of Human Hepatocytes

Hepatocellular carcinoma is one of the most frequent worldwide causes of cancer mortality. Hepatitis B virus and certain chemical carcinogens have been implicated as etiological agents. An in vitro model system of replicative normal human hepatocytes is needed to better define the mechanistic roles of these etiological agents. Serum-free medium composed of a modified Ham's F-12 medium was found to support the long-term multiplication of human liver epithelial cells. These epithelial cells were positive for general cytokeratin expression as well as positive for cytokeratin 18 expression through four passages. In addition, human hepatocytes in primary culture transfected with the SV40 large T antigen gene formed foci within 6-8 weeks that were positive for both keratin and large T antigen expression. Conditioned medium from cultures of the transfected human liver cells was shown to cause a 30% increase in DNA synthesis of a malignant human liver cell line (HepG2). These cells should prove to be useful in studying the molecular mechanisms of hepatocellular carcinogenesis.

II. CARCINOGEN METABOLISM, DNA DAMAGE AND DNA REPAIR

The earliest events in the multistage process of chemical carcinogenesis are thought to include (1) exposure to the carcinogen; (2) transport of the carcinogen to the target cell; (3) activation to its ultimate carcinogenic metabolite, if the agent is a procarcinogen; and (4) DNA damage leading to an inherited change. Therefore, one important use of cultured human tissues has been in the investigation of the metabolism of chemical carcinogens because many environmental carcinogens require metabolic activation to exert their oncogenic effects; the metabolic balance between carcinogen activation and deactivation may, in part, determine a person's oncogenic susceptibility. Knowledge of the comparative metabolism of chemical carcinogens among animal species will aid efforts to extrapolate data on carcinogenesis from experimental animals to humans. We and our coworkers have systematically examined the metabolism of procarcinogens of several chemical classes which are considered to be important in the etiology of human cancer. Procarcinogens of several chemical classes can be activated enzymatically to electrophilic reactants that bind covalently to DNA in cultured human tissues. The studies of activation and deactivation of representative procarcinogens have revealed that the metabolic pathways and the predominant adducts formed with DNA are generally similar between humans and experimental animals. Wide quantitative interindividual differences (50- to 150-fold) are found in humans and other outbred animal species. When the metabolic capabilities of specimens from different levels of biological organization are compared, the profile of benzo(a)pyrene metabolites is similar in cultured tissues and cells, but subcellular fractions, e.g., microsomes, produce a qualitative and quantitative aberrant pattern.

Although DNA repair has been studied extensively in human fibroblasts, lymphoid cells, and neoplastic cells, little information is available concerning DNA repair in normal human epithelial cells. Using the methodology to culture human bronchial epithelial and fibroblastic cells developed in our laboratory, we have initiated studies to investigate DNA damage and repair caused by chemical and physical carcinogens as examined by alkaline elution methodology, BND cellulose chromatography, unscheduled DNA synthesis, and high pressure liquid chromatographic analysis of the formation and removal of carcinogen-DNA adducts. As we reported last year, human bronchial epithelial cells repair single-strand breaks in DNA damaged by X-radiation, ultraviolet (UV)-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines at rates similar to bronchial fibroblasts.

A. <u>DNA Damage and DNA Repair</u>

Normal adult human tissues and cultured bronchial epithelial cells and fibroblasts exhibit O^6-alkylguanine-DNA alkyltransferase activity in vitro by catalyzing the repair of the promutagenic alkylation lesion O^6-methylguanine from DNA. Alkyltransferase activity varies in the different human tissues tested in the decreasing order of liver, colon, esophagus, peripheral lung and brain. Formaldehyde inhibits repair of O^6-methylguanine and potentiates the mutagenicity of an alkylating agent, N-methyl-N-nitrosourea, in normal human fibroblasts. In some experimental studies, repeated exposure to alkylating agents has led to an increase in O^6-methylguanine-DNA alkyltransferase activity, i.e., an adaptive response. We have shown that human bronchial epithelial cells do not adapt and increase their DNA repair capability. This finding has important implications in carcinogenesis caused by low doses of N-nitrosamines. The effects of cigarette smoke condensate, catechol and smoke "conditioned" media on the activity of O^6-methylguanine-DNA alkyltransferase (O^6MT) and uracil-DNA glycosylase (UDG) on cultured human bronchial epithelial cells, HUT 292 cells and BEAS-2B cells, is currently under investigation. The activity of these two DNA repair enzymes is also being measured in the alveolar macrophages and peripheral blood lymphocytes of smokers and nonsmokers. Interindividual and intraindividual variation in these activities is up to 100-fold and sixfold, respectively. Preliminary results indicate a significant rise in UDG activity in the macrophages of smokers compared to nonsmokers. In contrast, O^6MT activity is lower in macrophages of smokers which is consistent with the hypothesis that tobacco smoke inhibits repair of alkyl-DNA adducts.

B. <u>The Genotoxicity of Fecapentaene-12</u>

Higher levels of mutagens in the feces of certain populations eating a Western diet have been shown to correlate with an increased risk of colon cancer. Ninety percent of this mutagenicity can be accounted for by a group of compounds called fecapentaenes, which are potent direct-acting mutagens in the Ames <u>Salmonella</u> assay. Although fecapentaenes may play a role in the etiology of human colon carcinoma, their genotoxic effects have not been previously studied in human cells. Fecapentaene-12 (fec-12), a candidate carcinogen in the pathogenesis of colon cancer, is cytotoxic, mutagenic and induces DNA single strand breaks (SSB), sister chromatid exchanges and unscheduled DNA synthesis in normal human fibroblasts. DNA repair-deficient fibroblasts are more sensitive than normal

fibroblasts to the cytotoxic and mutagenic effects, which are dose dependent. Accumulation of SSB as a result of inhibition of the polymerase component of the excision repair mechanism suggests that SSB may be mediated in part by DNA repair mechanisms. These results indicate that fec-12 is genotoxic, mutagenic and causes direct DNA damage in human cells. Further support for the hypothesis that fec-12 is an initiating agent in colon cancer comes from the finding that this compound induces transformation in murine Balb 3T3 cells.

Possible covalent binding of radiolabelled fecapentaene to calf thymus DNA was indicated by cesium chloride density gradient centrifugation. Subsequent enzyme hydrolysis did not confirm the presence of radiolabelled adducts. Preliminary data after acid hydrolysis of fecapentaene-damaged DNA, fast atom bombardment mass spectroscopy (FAB-MS) analysis of a fecapentaene-damaged deoxynucleoside and ^{32}P-postlabelling of fecapentaene- damaged deoxynucleotide suggested that specific adducts may be formed. Acrolein adducts were not detected in fecapentaene-modified DNA by the use of a monoclonal antibody. Further characterization of both specific and non-specific DNA lesions (oxidative damage) is currently in progress.

C. Assessment of Tobacco Smoke Genotoxicity

Puck and coworkers have developed a highly sensitive assay for measuring mutations. The marker genes used in this system are present on a human chromosome that has been transferred to form a Chinese hamster ovary (CHO) hybrid cell. Because the CHO cell does not require the human marker chromosome for normal cell division, mutations that might not be detectable in other systems due to lethality, such as large deletions or rearrangements, will be scored using this system.

Cigarette smoke is carcinogenic in animals, and most of this carcinogenic property is recovered in nonpolar subfractions of the neutral fraction. Although cigarette smoke condensate is mutagenic following activation by metabolic enzymes found in microsomes or the S9 fraction, it has not been reported to act as a direct mutagen in systems used thus far. In addition, the fractions determined to be most carcinogenic have not been found to be the most mutagenic in the Ames assay. We have employed CHO cells containing a human chromosome 11 (termed AL hybrid cells) as a more sensitive detection system for mutagenesis in an effort to better define the direct and indirect mutagenic properties of cigarette smoke condensate and its fractions. The ID_{50} value for MNNG (0.2 µg/ml) in medium containing 8% fetal calf serum (FCS) was similar to previous reported values. The ID_{50} for N-methyl-N-nitro-N-nitrosoguanidine (MNNG) in medium lacking FCS was 0.6 µg/ml in two separate experiments (N=8). At this concentration there was a 300% increase ($p<0.0005$) in survivors following incubation with the a1 antibody and complement. The ID_{50} for cigarette smoke condensate (CSC) in the absence of serum is about 45 µg/ml. CSC is not significantly mutagenic at the ID_{50} concentration, but causes a 60% increase ($p<0.02$) in survivors (presumably mutated) at 60 µg/ml and a 290% increase ($p<0.0005$) at 90 µg/ml. We have conducted dose response experiments with CSC fractions and have determined ID_{50}'s for the acid, neutral and basic fractions of 5, 60 and 250 µg/ml, respectively. The presence of 8% fetal calf serum in the medium reduces the ID_{50} of CSC and of each fraction to 130, 30, 50 and 360 µg/ml, respectively.

III. CELLULAR AND MOLECULAR BIOLOGY OF NORMAL AND NEOPLASTIC CELLS

In vitro models provide an opportunity to investigate the cellular and molecular mechanisms controlling growth and differentiation of normal human epithelial cells. Aberrations in these normal mechanisms can be studied in carcinoma cell lines. The comparison between normal versus neoplastic cells generates hypotheses that can be tested in carcinogenesis studies. In addition, cellular and molecular markers of normal versus neoplastic cells and selective conditions for growth have pragmatic value in carcinogenesis studies and in cancer diagnosis.

A. Growth and Differentiation of Human Bronchial Epithelial Cells

Defined methods to grow replicative cultures of normal human bronchial epithelial (NHBE) cells without serum have been developed. These cells can be subcultured several times, will undergo 35 population doublings, and have expected epithelial cell characteristics of keratin, desmosomes and cell surface antigens. NHBE cells inoculated at clonal density will multiply with an average generation time of 28 hr; the majority of the cells are small and migratory and have few tonofilaments. Adding human whole blood-derived serum (BDS) depresses the clonal growth rate of NHBE cells in a dose-dependent fashion. In contrast, human lung carcinomas either replicate poorly or fail to grow at all when inoculated at clonal density in serum-free medium. Their rates of multiplication increase in direct proportion to the amount of BDS added to the optimized medium. Transforming growth factor (TGF)-β_1 was found to be the primary differentiation-inducing factor in serum for NHBE cells, while TGF-β_1 was not growth inhibitory for malignant cells. These differential effects of TGF-β_1 on normal versus malignant cells are not because of lack of TGF-β_1-specific receptors on malignant cells. Epinephrine antagonized the effect of TGF-β_1 without altering characteristics of TGF-β_1-specific receptors.

On the basis of our culture system for NHBE cells, an Ad12-SV40 transformed cell line (BEAS-2B) has been established which is immortal (>100 passages) and partially retains the ability to be induced by serum to undergo squamous differentiation as normal bronchial epithelial cells. Two subclones of BEAS-2B have been isolated and characterized in respect to squamous differentiation ability. One subclone designated as S.6 is sensitive to serum and TGF-β_1 induced squamous differentiation, and another subclone, R.1, is resistant to these two agents. However, both S.6 and R.1 cells are resistant to 12-O-tetradecanoyl-phorbol-13-acetate (TPA), even though membrane receptors for TGF-β_1 and TPA are not significantly different from that of normal cells at the number and affinity. A density-dependent affect of squamous differentiation induction for normal and transformed cells has been found. At clonal density (25-500/cm^2) cells stop growing and differentiate, but at high cell density (>1000/cm^2) only part of the population differentiates. Time course of morphology change and DNA synthesis inhibition induced by serum treatment suggest that two cell (pseudo) populations arise in the independent cultures that react to serum in different ways. An epidermal growth factor (EGF) requirement for cell growth that is density-

dependent has also been found. EGF is required by clonal growth but is not necessary for high density cell growth (>1 x 104/cm^2). Primary data from S.6 cells' conditioned medium has shown that these cells produce a growth factor which is less than 12,000 molecular weight and heat resistant.

B. Cell Surface Antigen Expression - Normal and Neoplastic Cells

The expression of a variety of cell surface antigens was studied on normal bronchial epithelial cells, small cell lung carcinomas, mesothelial cells, mesotheliomas, normal bronchial epithelial cells, as well as fibroblasts and mesothelial cells transfected with T antigen. The monoclonal antibodies used to study these cell surface antigens defined determinants normally expressed on a variety of different cell types, mainly those cells of hematopoietic and lymphoid origin. Small cell lung carcinoma cell lines and freshly explanted tumor expressed antigens defined by monoclonal antibodies MY4, MY7 and MY9 (myeloid differentiation antigens), as well as certain monoclonal antibodies that detect antigens usually associated with the B cells or, in some cases, epithelial cells. Cultured normal bronchial epithelial (BE) cells did not express these myeloid differentiation antigens (MY4, MY7, MY9), but these antigens are expressed when BE cells were immortalized by transfection with adeno-12, SV40 viral genes. Higher levels of these antigens were also found when these cells were transfected with and expressed *raf* and *myc* oncogenes. In addition, cells expressing both *raf* and *myc* had transient expression of cell surface major histocompatibility complex (MHC) class II antigens. Normal mesothelial cells did not express these myeloid antigens. MY4 and MY7 but not MY9 were expressed when these cells were transfected and immortalized with SV40T.

These findings indicate that bronchial epithelial cells as well as mesothelial cells, both normal and malignant, may express cell surface antigens that are commonly found on other cell types and that expression of certain surface antigens do not necessarily indicate the origin of a malignant cell and appear to reflect markers of differentiation.

C. Cytosine Methylation and Cellular Physiology and Pathology

The role DNA methylation plays in the promoter regions of two separate gene systems has been clarified. Demethylation of at least one Hpa II restriction site upstream from the structural coding sequences of human gamma globin or the hepatitis B core antigen gene is necessary but not sufficient for the initiation of transcription. The final conversion of a quiescent, demethylated gene (gamma globin or hepatitis B core antigen) to an active state requires some endogenous or exogenous inducing agent, which may be highly specific for any given gene or gene complex. Clonal selection is not responsible for observed changes in gene expression, since we have clearly shown that cells remethylate DNA that has been demethylated by 5-azacytidine treatment.

New micro techniques have been developed which enable the analytical quantitation of 5-methylcytosine in less than one microgram of DNA isolated from any source. Thus, the genomic 5-methylcytosine content of normal human bronchial epithelial and pulmonary mesothelial cells has been measured for the first time. These techniques have enabled the determination of changes in the genomic content of 5-

methylcytosine during normal physiological processes. The genomic content of 5-methylcytosine in normal human bronchial epithelial cells and in rodent tissues decreases with increasing in vivo age. Significant decreases in DNA 5-methylcytosine occur concomitantly with the induction of squamous differentiation in normal human bronchial epithelial cell cultures. These techniques have also provided for the demonstration that chemical carcinogens can induce decreases in DNA 5-methylcytosine levels in dividing normal human bronchial epithelial cells.

D. Molecular Analysis of Gene Regulation and Proliferation Control in Human Lung Cells

The goal of this project is to understand, at the molecular level, regulatory interactions of human cells with growth factors, metabolites, and foreign chemicals implicated in carcinogenesis. These substances govern or affect the growth potential and differentiation status of the cells. The topics of present interest are: induction of plasminogen activator inhibitor (PAI-1), interleukin 1 (IL1) and urokinase in normal human bronchial epithelial cells, T antigen transformed normal human bronchial epithelial cells, and lung tumors; development of model systems for the study of differentiation of normal human bronchial epithelial cells and for fiber sensitivity and growth factor regulation in normal human mesothelial cells; and responsiveness to growth factors and auto-production of growth factors by normal human mesothelial cells, T antigen transformed normal human mesothelial cells, and mesotheliomas.

TGF-β_1 induces a burst of PAI-1 production in either normal human bronchial epithelial cells or in SV40 T antigen transformed human bronchial epithelial cells. These cells do not normally produce this gene product. However, tumorigenic, *ras*-transfected, T antigen transformed NHBE cells constitutively produce PAI-1.

Mesothelioma cells, but not normal human mesothelial cells, produce platelet-derived growth factor (PDGF)-A chain protein which is biologically active in receptor binding assays. However, the mesothelioma cell lines as compared to the normal cells do not respond to the mitogenic signals of PDGF. Normal mesothelial cells produce mRNA for basic fibroblast growth factor (FGF). Some mesotheliomas, but no normal mesothelial cells, produce TGF-α while all classes of human mesothelial cells are negative for EGF production. These results indicate the complex interactions that influence growth controls in human lung cells.

E. Proto-oncogene Expression in Growing or Differentiating Human Bronchial Epithelial Cells

TGF-β_1 and epinephrine have opposing effects on NHBE cells. While TGF-β_1 induces squamous differentiation in NHBE cells, epinephrine promotes their growth and neutralizes the effect of TGF-β_1. To investigate mechanisms which might be involved in their antagonism, we examined steady state expression levels of proto-oncogenes c-*myc*, c-*fos*, and c-Ha-*ras* in response to treatment with these two agents. Expression of specific mRNA was detected by Northern blotting and normalized by the constitutive probe glyceraldehyde-3-phosphate-dehydrogenase. Expression of c-*myc* was transiently inhibited by TGF-β_1 up to 40% at 1 hr.

Epinephrine induced the expression of c-*myc* about two- to threefold and neutralized the effect of TGF-β_1 on c-*myc* mRNA level. Expression of c-*fos* was transiently induced by either TGF-β_1 or epinephrine up to 1.5 to two- to threefold at 1 hr, respectively. These two agents synergistically induced c-*fos* up to fivefold. The c-Ha-*ras* mRNA level was not altered by TGF-β_1 or epinephrine treatments. While the initial changes in c-*myc* expression correlated with the proliferative activity of the cells, the steady state level of c-*fos* mRNA did not.

IV. CARCINOGENESIS AND RELATED STUDIES

A central problem of cancer research is the extrapolation of carcinogenesis data and knowledge of carcinogenesis mechanisms from laboratory animals to humans and, within this heterogeneous population, extrapolation among individuals. An aspect of this problem is the difficulty associated with extrapolating from one level of biological organization to another, i.e., from molecules to macromolecules to organelles to cells to tissues to intact organisms. Multiple experimental systems are needed to help investigators find solutions to these and other problems in carcinogenesis research. Animal models are obviously required for experimental in vivo carcinogenesis studies. They are also essential because the integral multisystemic interactions of the organism remain intact and because laboratory animals can be environmentally and genetically controlled. In vitro models using tissues, cells, and subcellular fractions are also useful. This approach can aid in the resolution of the central problem of extrapolation in that one can conduct comparative studies with tissues and cells from experimental animals and humans that are maintained in the same controlled in vitro experimental setting. Carcinogenesis studies using human tissues and cells offer unique opportunities. For example, some rare forms of human cancer reflect inherited, predisposing conditions, and their genetic basis and perhaps common pathways of carcinogenesis may be understood through the study of cells from individuals with these specific types of cancer. In addition, because human cells in vitro are apparently genetically more stable and undergo less "spontaneous" neoplastic transformation than most rodent cells, they may be especially suitable for studying the multistage process of carcinogenesis.

Epithelial cells are of particular interest because most adult human cancers are carcinomas. As noted above, significant progress has been made in the past decade in developing methods for culturing human epithelial tissues and cells. Chemically defined media have been developed for culturing normal human tissues and cells from organs with a high rate of cancer in humans. Serum-free media have several advantages in studies of cultured human cells, including (a) less experimental variability compared to serum-containing media; (b) selective growth conditions of either normal cells of different types (e.g., epithelial versus fibroblastic cells) or normal versus malignant cells; (c) identification of growth factors, inhibitors of growth, and inducers of differentiation; and (d) ease of isolating and analyzing secreted cellular products. Advances in cell biology, including the delineation of biochemical and morphological markers of specific cell types, have also facilitated the identification of cells in vitro (e.g., keratins as markers for epithelial cells and collagen types I and III for identifying fibroblasts).

Carcinogenesis is a multistage process that can be operationally divided into tumor initiation, promotion, conversion, and progression. Genetic changes, perhaps mutations, are considered to be responsible for tumor initiation and malignant conversion. As was noted earlier, metabolism of carcinogens, DNA damage, and DNA repair are considered to be important factors in these stages of carcinogenesis.

A. Characterization and Mode of Action of the raf and myc Families of Oncogenes

An amplification of the c-*myc*, N-*myc* or L-*myc* gene and overexpression of the c-*raf* sequence are common features of small cell lung carcinomas. The major objective of this project is to determine the role of these genes in lung carcinogenesis by a) overexpressing c-*raf* and/or c-*myc* genes in human lung cells, and b) reversion of the malignant phenotype by plasmids capable of anti-sense RNA transcription.

Retroviral recombinants pZip-*raf* and pZip-*myc* were constructed to examine the role of the c-*raf*-1 and c-*myc* proto-oncogene in lung carcinogenesis. Immortalized human bronchial epithelial cells (BEAS-2B) transfected with pZip-*raf* DNA and pZip-*myc* DNA gave rise to undifferentiated carcinomas (*raf*/*myc* tumors) when tested in athymic nude mice, whereas c-*myc* or c-*raf* transfected cells are non-tumorigenic. The *raf*/*myc* tumors expressed markers of small cell lung carcinomas, i.e., neuron-specific enolase and neurosecretory granules. In addition, BEAS-2B cells transformed with the c-*raf* and c-*myc* proto-oncogenes, and derived tumor cell lines acquired HLA class II antigen expression.

The c-*raf*-1 gene has been identified as the predominant transforming gene of three radiation-resistant head and neck cancer cell lines in the NIH 3T3 transfection assay (SQ-20B, JSQ-3, SCC-35). NIH 3T3 cells transformed with SQ-20B DNA also became radiation-resistant, suggesting a correlation between the presence of c-*raf* sequences and the radiation-resistant phenotype. Inhibition of the c-*raf* function by introduction of anti-sense *raf* transcribing plasmids into the SQ-20B cell line reverted not only the tumorigenic phenotype but also reduced the radiation resistance. As a consequence of these experiments, the construction of an inducible promoter system for anti-sense sequences in human cells was undertaken.

B. In Vitro Transformation of Human Lung Cells by SV40, ras and fos Oncogenes

Five human bronchial epithelial (BE) cell lines have been established from normal human BE cells by SV40 early region gene transfer. One additional cell line has been established following transfection of SV40 early region genes into BE cells known to contain an abnormality of the short arm of chromosome 11 from an individual who was cancer-free; the resulting cell line is tumorigenic in athymic nude mice. At least one other cell line has developed weak tumorigenicity. One of these BE lines has been the recipient of *fos* or *ras* oncogene transfer, via retroviral infection or via strontium phosphate coprecipitation of plasmid DNA. Preliminary results indicate that HVV-*fos*, a chimeric construct of human c-*fos* (human)-1 and a viral 3' long terminal repeat (LTR), induces serum resistance and extends the lifespan of NHBE cells. Several different mutant *ras* oncogenes have resulted in malignant transformation. These cell lines, and tumor cell lines

established from the nude mouse tumors, are being utilized to study aspects of multistage carcinogenesis, including chromosomal changes, progressive changes in response to inducers of squamous differentiation and the development of invasiveness.

C. Activities of Promoters/Enhancers in Human Cells

This project is to investigate the relative promoter strengths of various cloned mammalian promoter regions in human cell types of particular interest to the research program of LHC: normal bronchial fibroblasts, mesothelial cells and bronchial epithelial cells, as well as the recently constructed immortalized mesothelial and bronchial epithelial cell lines. The assayed promoter/enhancer regions included those from the Rous sarcoma virus LTR; SV40 virus; Moloney sarcoma virus LTR, and adenovirus major late promoter (MLP) with or without SV40 enhancer sequences, human T-cell leukemia virus (HTLV)-I LTR or human immunovirus (HIV) LTR, with or without their respective trans-activating proteins, metallothionein with or without cadmium, and mouse mammary tumor virus (MMTV) LTR, with and without dexamethasone. The sequences were assayed for their promoter/enhancer activity using the chloramphenicol acetyl transferase assay system.

In the immortalized lines, SV40-enhanced adenovirus MLP, transactivated HTLV-I LTR, and transactivated HIV LTR were highly active. Under the conditions of the assay, the metallothionein promoter was measurably active, but not inducible by cadmium, and MMTV was inducible but showed weak activity. These promoter/enhancer regions are now being tested in normal cells. The very high level of expression by the enhanced adeno-5 MLP and the HTLV-LTR promoters will facilitate construction of vectors for efficient expression of genes in these human cells.

D. Activation of Proto-oncogenes by Ultraviolet Light

We have isolated DNA from three human skin basal and squamous cell carcinomas, a squamous cell carcinoma cell line, and several tumor cell lines carrying known oncogenes. These DNAs were analyzed by two methods. First, genomic DNAs were restriction-digested, electrophoresed, and probed with oligonucleotide probes to detect single-base mismatches in the Ki-*ras* proto-oncogene at the codon 12 region and codon 61 region. Second, these DNAs were amplified by the polymerase chain reaction (PCR) using oligonucleotide amplimers specific for regions containing Ha-*ras* codon 12, Ha-*ras* 61, Ki-*ras* 12, Ki-*ras* 61, N-*ras* 12, N-*ras* 13, and N-*ras* 61. Amplified DNA was spotted onto nylon filters and these dot blots hybridized with oligonucleotide probes under increasingly stringent conditions to examine for single-base mutations. The gel method showed that skin carcinomas 1, 2, and 3 were wild-type for Ki-*ras* 12 and 61 and for Ki-*ras* 61 at positions A and C. The more sensitive PCR/dot blot method enabled rapid screening of each activatable codon of each *ras* proto-oncogene. Skin2 hybridized to probe Ha-*ras* 12B (valine). Skin1, skin3, and SCC13 bound at lower stringency to both Ha-*ras* 12B (val) and Ki-*ras* 12A (cys). Four lung samples bound to Ki-*ras* 12A (cys). No samples bound to any Ha-*ras* 61, Ki-*ras* 61, N-*ras* 12, N-*ras* 13, or N-*ras* 61 probe. The samples appearing to be positive are being confirmed by direct DNA sequencing

of amplified DNA. Alternative detection techniques are also being explored since ultraviolet light, unlike other agents, frequently causes double-base mutations, which would not be detected in the assay for single-base mutations.

E. Detection of ras Mutations in Proto-oncogenes in Human Tissues

We have isolated DNA from 6 human lung tumor cell lines, 20 human lung adenocarcinoma and squamous cell carcinomas, and 5 normal lung tissues corresponding to 5 of the lung carcinomas. These DNAs were analyzed by two methods. First, genomic DNAs were restriction digested, electrophoresed, and probed with oligonucleotide probes to detect single-base mismatches in the Ki-*ras* proto-oncogene at the codon 12 region and codon 61 region. Second, these DNAs were amplified by the polymerase chain reaction (PCR) using oligonucleotide amplimers specific for regions containing Ha-*ras* codon 12, Ha-*ras* 61, Ki-*ras* 12, Ki-*ras* 61, N-*ras* 12, N-*ras* 13, and N-*ras* 61. Amplified DNA was spotted onto nylon filters and these dot blots hybridized with oligonucleotide probes under increasingly stringent conditions to examine them for single-base mutations.

The gel method confirmed previous identification of Ki-*ras* 12A mutations in CaLu-1 and A549 cell lines and Ki-*ras* 12B mutations in A427. It revealed that A549 does not carry a normal Ki-*ras* 12 allele and that cell line SW900 has a mutation at Ki-*ras* 12B. Cell lines A1146 and Hut292 are wild-type for Ki-*ras* 12 and 61 and for Ki-*ras* 61 at positions A and C.

The more sensitive PCR/dot blot method enabled rapid screening of each activatable codon of each *ras* proto-oncogene. Each of the above results obtained with gels was confirmed. In addition, it was found that A1146 did not hybridize to any Ha-*ras* 12 probe, suggesting a deletion or double-base mutation at Ha-*ras* 12. Hut 292 hybridized to probe H12Biii (valine). SW900 bound specifically to probe Ki-*ras* 12Biii (valine, G to T substitution). Four lung samples bound to Ki-*ras* 12Aii (cys). No samples bound to any Ha-*ras* 61, Ki-*ras* 61, N-*ras* 12, N-*ras* 13, or N-*ras* 61 probe. The samples appearing to be positive are being confirmed by direct DNA sequencing of amplified DNA.

F. Multiple Drug Resistance Gene Expression in Mesothelioma

Malignant mesothelioma is a tumor which is extremely recalcitrant to treatment by chemotherapeutic agents. It was of interest to test mesothelioma cell lines for expression of the multiple drug resistance (MDR) gene. If this gene were expressed at a high level in the mesothelioma cells, its action might be expected to be the basis of the resistance of this tumor type to chemotherapy. RNA was prepared and tested from 16 human mesothelioma cell lines, 2 normal human mesothelial cells, and 3 normal human mesothelial cells immortalized by the SV40 T antigen. Of all samples tested only 2 were positive for expression of the MDR gene. We conclude that the MDR gene product is not the protein of importance for the resistance to chemotherapeutic agents shown by human mesothelioma. Therefore, strategies which would specifically inactivate or reverse the action of this gene product are not relevant to the mesothelioma therapeutic dilemma.

G. Tumor Suppression and Somatic Cell Genetics

The specific goal of this project is to determine whether normal human cells contain genes that have the ability to suppress tumorigenicity of human lung carcinoma cell lines. Somatic cell hybrids between a lung carcinoma line, HuT292-DM and BEAS-2B, an immortalized human bronchial epithelial line, have been generated by fusion with polyethylene glycol (PEG), followed by double selection in hypoxanthine/aminopterin/thymidine (HAT) medium containing ouabain. The properties of these hybrid lines are being studied. All lines have near triploid chromosome numbers, whereas the parental lines are near diploid. Serum induces BEAS-2B to terminally differentiate, but stimulates the growth of the carcinoma line. The hybrids vary in their response to serum. Some are unaffected, some are stimulated, but none are inhibited. The tumorigenicity of these lines as compared to their progenitors is now being tested in nude, athymic mice.

H. Tumor Suppression Activities of Specific Human Chromosomes

Genetic changes related to carcinogenesis are being studied using hybrids from fusion of human lung carcinoma cells with normal human bronchial epithelial cells and of microcells of individual marked human chromosomes with human lung tumor cells. Initial studies suggest that a limited population doubling potential (mortality) is the dominant genetic trait in hybrid cells. Other hybrid cell lines have been isolated and are being characterized for doubling potential, karyotype and tumorigenicity in athymic nude mice. When specific human chromosomes have been transferred by microcell methodology into HuT 292 cells, chromosome 11 but not chromosome 13 has altered the tumorigenicity of the HuT 292. The location of the putative tumor suppressor gene on chromosome 11 will be determined.

I. Tumor Suppressor Genes: Isolation of Terminal Differentiation Genes by Subtraction Libraries

The cancer cell is the product of at least two events. First, the cell loses control of its proliferative pathways, resulting in continual cell growth. Second, those signals which normally induce terminal differentiation are not processed correctly by the cancer cell, resulting in cell immortality. Although numerous proto-oncogenes/oncogenes have been implicated in the first event, little is known about the genes involved in the second event. The objective of this project is to identify genes which are essential for the process of (terminal) differentiation of human epithelial cells with the goal of finding and bypassing the defeats in human lung carcinomas.

In order to investigate genes associated with terminal differentiation of epithelial cells, cDNA libraries are being constructed from a variety of epithelial cell types in various stages of differentiation. By using the method of substraction cloning, messages expressed preferentially in one cell type or in a certain stage of differentiation may be examined. Furthermore, effects of inducers of differentiation upon gene expression can be examined as epithelial cells progress from an undifferentiated state to a terminally differentiated state.

NHBE cells can be induced to terminal differentiation in vitro by treatment with a variety of agents such as TGF-β_1, 5-azacytidine, calcium, TPA and fetal bovine serum. To investigate differences in gene expression of undifferentiated NHBE cells and differentiated NHBE, messenger RNA is isolated from both cell types and used to produce two cDNA libraries. From these two libraries, RNA is synthesized in vitro making sense strand RNA from one type and anti-sense strand RNA from the other type. The sense and anti-sense RNAs are then hybridized, and the unique single-stranded sequences are removed from the common double-stranded sequences. By this substraction protocol, unique messages expressed in differentiating cells can be used as probes to investigate the clones from which they were synthesized.

J. Prostatic Carcinogenesis

Cultures of neonatal human prostatic epithelial cells (NP-2S) near the end of their lifespan were transfected by strontium phosphate coprecipitation with a plasmid (pRSV-T) containing the SV40 early region genes. Colonies of transformed cells, isolated from a background of senescing normal cells yielded cell lines with growth potential well beyond that of the normal cells. In all, 28 cell lines have been isolated in 3 separate experiments. Four lines, 267B1, 272E1, 272E4, and 272A9 which have undergone 96.3, 92.3, 30.8, and 64.7 population doublings, respectively, have been studied further and appear to be "immortalized" without having passed through a crisis. These lines contain cytokeratins and SV40 T antigen by immunofluorescence and have ultrastructural features of epithelial cells. All lines consist of mixtures of normal and aneuploid karyotypes with modal chromosome numbers in the near-diploid range (2n=46) resulting from random loss, gain and rearrangement of chromosomes. None gave tumors within one year after s.c. injection in nude mice. The transformed lines, as well as the parental NP-2S, are stimulated by TGF-β_1 in clonal growth assays. These lines should be useful in investigating prostatic carcinogenesis and progression.

V. BIOCHEMICAL AND MOLECULAR EPIDEMIOLOGY

The primary goal of biochemical and molecular epidemiology is to identify individuals at high cancer risk by obtaining pathobiological evidence of (a) high exposure of target cells to carcinogens and/or (b) increased host susceptibility due to inherited or acquired factors. Laboratory methods have been developed recently to be used in combination with analytical epidemiology to identify individuals at high cancer risk. These methods include (1) techniques to assess specific host susceptibility factors; (2) assays that detect carcinogens in human tissues, cells, and fluids; (3) cellular assays to measure pathobiological evidence of exposure to carcinogens; and (4) methods to measure early biochemical and molecular responses to carcinogens.

Currently available techniques exist that would allow the utilization of biochemical and molecular measures to better characterize exposure to carcinogens, to serve as intermediate endpoints on the path to malignancy, to identify measures which halt or reverse this process, and to investigate the mechanisms of human carcinogenesis. Included in these investigations would be the following: (1) efforts to evaluate body burden of chemical carcinogens in studies of occupational and general environmental cancer risk factors; (2) sophisticated

analyses of air, water, and biological specimens for carcinogenic and mutagenic substances in conjunction with specific analytical studies; (3) search for evidence of viral infection including viral segments or oncogenes in the DNA of individuals at high risk of cancers that may be associated with infectious agents or heritable states; (4) evaluation of disturbances in immune function as they may relate to malignancies, particularly those of the hematopoietic system; (5) investigation of the relationship between micronutrients and a variety of epithelial cancers; and (6) determinations of the relationship between macronutrients, including dietary fat and subsequent hormonal changes, to subsequent risk of breast, endometrial, and perhaps colon cancer.

A. Analysis of Hydrocarbon-Macromolecular Adducts in Humans; Relation to Cancer Risk

Classical epidemiology and xenobiochemical studies have led to a better understanding of the genotoxic effects of environmental contaminants, for example, polycyclic aromatic hydrocarbons (PAHs) in humans. Development of assays for carcinogen exposure at the molecular level in humans has therefore been a major focus of research and from the battery of assays that are currently available, both immunological and physico-chemical approaches are being developed. Since each type of assay system clearly has its own advantages and disadvantages, the use of more than one corroborative assay has been of importance. The problems that appear to confront the development of assay systems for human biomonitoring are essentially twofold: the levels of material present in macromolecular complexes challenge the detection limits of conventional assay systems, and complex mixtures of adducted materials confound simple assay systems. Following recognition and definition of cross-reactivity profiles for antibodies raised against benzo(a)pyrene-diol-epoxide-modified DNA, a protocol for PAH-DNA adduct isolation that combines immunoaffinity chromatography, HPLC, fluorescence spectroscopy and GC/MS is being developed. Although this method has been successfully applied to the measurement of benzo(a)pyrene adducts in human placental DNA, studies are in progress that will attempt to apply this approach to the measurement of other classes of carcinogen-DNA adduct, and in other tissues e.g., lung.

B. Genetic Polymorphisms and Human Lung Cancer

There is a clear association between smoking and lung cancer, but it is still not known why some individuals, who are heavily exposed to large concentrations of chemical carcinogens, do not develop tumors, whereas others do. These observations provide circumstantial evidence for the involvement of a genetic factor that predisposes for tumor formation. Recent restriction fragment length polymorphism (RFLP) studies have shown that the loss of normal cellular sequences from chromosome 13 (in the case of retinoblastoma), chromosome 11 (in the case of Wilms' tumor and bladder cancer and breast cancer), chromosome 1 (in the cases of melanoma), chromosome 22 (in the case of acoustic neuroma) and chromosome 3 (in the case of small cell carcinoma of the lung) have been associated with malignancy. It appears that this method might be generally applied to the analysis of inherited susceptibility to cancer and therefore be informative in risk assessment for lung cancer. Tumor and normal tissue from high molecular weight DNA samples have been collected from more than 60 cancer patients for

restriction enzyme digestion and Southern analysis. Initial experiments have centered on examination of genes located on the short-arm of chromosome 11; loss of allelic fragments during tumorigenesis have been detected at the cellular Harvey ras locus, the insulin locus, the calcitonin locus, the β-globin locus, the catalase locus and the Int-2 locus (homologous to the MMTV locus). Loss of allelic heterozygosity was commonly found on chromosomes 3p, 11p and 17p. Experiments that examine additional loci throughout the human genome for these DNA samples are in progress.

C. Detection of Carcinogen-DNA Adducts by ^{32}P-postlabelling

The ability to detect low levels of carcinogen DNA adducts in the tissues of people environmentally exposed to chemical carcinogens is invaluable to epidemiological studies of the incidence of cancer in selective populations. A number of selective methodologies have been developed to quantitate carcinogen DNA adducts. The Randerath ^{32}P-postlabelling technique provides a fingerprint analysis of only polycyclic aromatic hydrocarbon type DNA adducts. However, the basic Randerath methodology can be adapted for sensitive quantitative detection of aryl type DNA adducts in human samples. In addition, the ^{32}P-postlabelling method has been adapted in the present study to enable the detection of small alkylation type carcinogen DNA adducts. The detection and quantitation of O^6-methylguanine (MeGua) adducts in DNA has been shown, by the use of standards, to be accurate as low as one adduct in at least 1,000,000 guanine residues. The N^7-MeGua and the 8-OH Gua adducts also appear to be detectable by this methodology. The presence of unidentified ^{32}P-labelled spots has also been observed from the analysis of DNA from cells treated with acrolein and DNA treated with fecapentaene.

D. Immunology of AIDS and AIDS-related Diseases

Acquired immune deficiency syndrome (AIDS) is characterized by the profound loss of ability to respond to environmental antigens as well as the development of Kaposi's sarcoma. HIV-1 was identified by immunocytochemistry in skin macrophages (Langerhans' cells). Furthermore, cultured Langerhans' cells were shown to express viral particles and to infect HIV-1-negative monocytes. MHC class II antigens were found to be involved in the HIV receptor site on cells expressing these molecules. This involvement appears to be related to the proximity of HLA-DR molecules on the cell surface to the CD4 viral ligand. Monocytes were isolated from peripheral blood lymphocytes and infected with HIV-1. HIV-1 infected monocytes do not appear to be altered in their ability to present antigens (tetanus toxoid) to autologous T cells. When an antigen is presented together with HIV-1, infection of T cells is increased. The presence of cytotoxic lymphocytes to the HIV-transfected and -infected target cells was studied and showed low levels of activity to transfected fibroblast and higher levels to HIV-infected T cell lines.

E. HLA Antigens: Structure, Function, and Disease Association

HLA typing was performed on lymphocytes from patients with a common disease or from families where more than one individual had a common disease type. HLA typing was performed in a cohort of individuals with AIDS, either Kaposi's

sarcoma, or opportunistic infections, or individuals at risk for this disease. A total of 250 individuals have been HLA typed. One hundred of these patients have been followed over a 4-5 year period. The objectives of these studies are to examine possible genetic susceptibility to the development of AIDS or AIDS-related complex that is related to expression of histocompatibility antigens. The HLA-DR1 phenotype is increased in frequency in all AIDS patients compared to HIV-seropositive controls. HLA-DR3 is significantly decreased in the patients with Kaposi's sarcoma. In the HIV-seropositive individuals followed for 65 months, HLA-DR1 appears to be a specific risk factor for disease development. Three families with more than one member with acute lymphocytic leukemia were analyzed for DNA polymorphism in the HLA-DQB genes. Common restriction patterns were found in the leukemic individuals. Sera from 30 hemophiliacs (10 developed AIDS, 10 HIV-seropositive AIDS free and 10 HIV-seronegative) were obtained prior to seroconversion and subsequent yearly samples were tested for antibodies to HLA antigens. Individuals developing AIDS demonstrated increased reactivity to a range of HLA antigens, while losing total $CD4^+$ cells.

F. Immunologic Studies of Human T-Cell Lymphoma Virus

The human T-cell lymphoma virus, HTLV-I, has been found to be associated with patients with adult T-cell leukemia. Studies are underway to understand the mechanism of malignant transformation of cells infected with this virus and the immunologic response of individuals who are infected with this virus and who demonstrate malignancies, or those who are carriers of the virus but have not developed malignancies. Chronic lymphocytic leukemia (CLL) cells were obtained from patients who were HTLV-seropositive; however, their malignant B-cells did not contain the HTLV-I retrovirus. Using hybridoma technology, CLL cells were fused with a B-lymphoblastoid cell line and the immunoglobin was captured. In one instance, the captured immunoglobin reacted with the HTLV-I p24 gag proteins and, in the other instance, reacted with the large envelope protein from HTLV-I. Immunoglobin gene rearrangement present in the B CLL cells was demonstrated in the hybridoma cell line. The results indicate that the CLL cells were antigen-committed cells prior to malignant transformation. Sera from an individual who developed a B cell lymphoma and whose spouse had acute T-cell leukemia (HTLV-I-positive) contained an antibody with reactivity to HTLV-I-positive cells. The B cell lymphoma also produced an antibody reactive with HTLV-I when established in short-term culture.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05192-08 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Repair of Carcinogen-Induced DNA Damage in Human Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Glennwood E. Trivers	Biologist	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Ainsley Weston	Visiting Associate	LHC	NCI

COOPERATING UNITS (if any)
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Biochemical Epidemiology Section

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0.0

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

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

Enzymatic removal of the promutagenic alkylation lesion O6-methylguanine from DNA (a repair mechanism) can be exhibited in vitro in normal adult human tissues and bronchial epithelial cells or fibroblasts from culture. Successful cleavage demonstrates the catalytic activity of O6-alkylguanine-DNA alkyltransferase. Human tissues tested for alkyltransferase activity show highest levels in liver and decreasingly lower amounts in colon, esophagus, peripheral lung and brain. In some in vitro studies, repeated exposure to alkylating agents has induced higher levels in O6-methylguanine-DNA alkyltransferase activity, i.e., an adaptive response. Our previous studies indicate that human bronchial epithelial cells cannot be similarly induced; formaldehyde inhibits O6-methylguanine-DNA methyltransferase (O6-MT) repair and potentiates mutagenicity of the alkylating agent, N-methyl-N-nitrosourea, in normal human fibroblasts. These findings have important implications in carcinogenesis caused by low doses of N-nitrosamines. To resolve the indications of these results we are studying: a) the in vitro effects of cigarette smoke condensate (CSC), catechol and smoke "conditioned" media on the activity of O6-MT and uracil-DNA glycosylase (UDG) in cultured human bronchial epithelial cells (NHBE), HuT 292 cells and BEAS-12 cells; and b) the effects of the polycyclic aromatic hydrocarbons (PAH) in smokers and nonsmokers on the activity of these DNA repair enzymes measured in alveolar macrophages and peripheral blood lymphocytes. Preliminary results show that a) neutral CSC induces 12-O-tetradecanoylphorbol-13-acetate (TPA)-like activity in NHBE cells; and b) interindividual and intraindividual activities in blood cells vary 100-fold and 6-fold, respectively, while, compared to nonsmokers, macrophages of smokers contain significantly higher levels of UDG activity, the levels of which appear related to the detectibility of polycyclic aromatic hydrocarbon-DNA adducts in both cell types studied. The levels of both UDG and O6-MT were related to smoking in macrophages with PAH-DNA adducts.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Glenn Trivers	Biologist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Ainsley Weston	Visiting Associate	LHC	NCI

Objectives:

To study the effects of tobacco smoke and related compounds on the mechanisms of enzymatic repair of DNA damaged by environmental agents, using normal and premalignant human epithelial tissues and cells.

Methods Employed:

Culture of human epithelial and fibroblastic cells and treatment of cultures with carcinogens in vitro. O^6-Alkylguanine-DNA alkyltransferase activity found in extracts from a variety of human cells was characterized and quantitated using the following measurements: (1) the specific loss of labeled O^6-methylguanine (O^6-meGua) from a ^{3}H-methylated DNA substrate; (2) the production of protein containing S-[^{3}H]-methylcysteine during the reaction with the DNA substrate; (3) the formation of [8-^{3}H]-guanine in DNA when the extracts were incubated with a synthetic DNA substrate containing O^6-meGua labeled in the 8-position; and (4) the removal of ^{3}H-deoxyuracil monophosphate from DNA when ^{3}H-dUMP-DNA was incubated with cellular supernatant fractions and isolation of cellular macromolecules.

Collection of macrophages from bronchial lavage and peripheral blood lymphocytes from venus blood of smoking and nonsmoking human volunteers; O^6-alkylguanine-DNA alkyltransferase and uracil-DNA glycosylase measurements (as above) in the supernatant fraction of homogenates of lymphocytes and macrophages, and isolation of cellular macromolecules; and determination of cotinine levels in the serum of each donor to chemically verify the smoking status.

Measure of carcinogen-DNA adducts in macrophages and peripheral blood lymphocytes (PBLs) DNA by immunoassay and/or synchronous scanning fluorescence spectrophotometry, and/or measurement of restriction fragment length polymorphisms (RFLPs).

Major Findings:

We have investigated alkyltransferase activity in various human tissues and in cultured normal human bronchial epithelial cells and fibroblasts. Human colon, esophagus, and lung activities were lower than previously found in human liver, which was higher than human brain. Compared to corresponding rat tissue, human

tissues contained two- to tenfold higher levels of alkyltransferase activity. Both O^6-MT and uracil-DNA glycosylase (UDG) repair of DNA causes mutations. UDG, the most abundant of the glycosylases, hydrolyzes the bond between uracil and deoxyribose.

Formaldehyde (HCHO), a common environmental pollutant found in tobacco smoke and a metabolite of demethylation of drugs and carcinogenic N-nitrosamines, is also a respiratory carcinogen in rats and a potential carcinogenic hazard in humans. Therefore, we previously studied genotoxicity of HCHO in cultured human cells and showed that exposure to HCHO caused DNA-protein cross-links (DPC) and single-strand breaks (SSB) in all cell types. DPC were induced and removed by all cell types, including cells from patients with xeroderma pigmentosum. Excision repair of DNA damage in normal cells generated SSB, also readily repaired. Because HCHO forms in equimolar quantities with methylcarbonium ions during the metabolic activation of N-nitrosodimethylamine, we also examined the effects of HCHO on the repair of the promutagenic lesion O^6-meGua formed following N-nitrosodimethylamine metabolism. HCHO decreased O^6-alkyltransferase activity; inhibited the removal of O^6-meGua; and in low concentrations, synergistically potentiated the cytotoxicity and mutagenicity of N-methyl-N-nitrosourea. These studies indicate the complexity of the HCHO-induced DNA damage and its repair and that HCHO may enhance the cytotoxicity of chemical and physical carcinogens in human cells.

When normal human bronchial epithelial (NHBE) cells were exposed to low single doses of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), the result was a dose-dependent decrease in O^6-methylguanine-DNA methyltransferase (O^6-MT) activity. Further, treatment every 6 hr for 4 to 5 days with nontoxic concentrations of MNNG (0.04-0.12 µg/ml) did not increase O^6-MT activity. These data suggest that human bronchial epithelial cells do not adapt to this repair rate in response to MNNG.

In this period we have studied the effects of cigarette smoke condensate (CSC) and its fractions (neutral, basic, and acidic) on proliferation and differentiation of human cell lines. In NHBE cells, CSC, its neutral (NSC) and acidic (ASC) fractions produced greater proliferation inhibition than the basic (BSC) fraction. While both CSC and NSC caused squamous differentiation, only NSC significantly inhibited specific binding of phorbol dibuterate (PDBU), a TPA-like effect. In human lung cancer (HuT-292) cells, CSC and ASC inhibited PDBU binding; however, compared to NHBE cells, no carcinoma cell line had morphological changes and all were more resistent to proliferation inhibition in the presence of CSC or its fractions. Such differential cytotoxicity in vivo could be the basis for clonal expansion to neoplasia and may reflect differences in DNA repair capabilities between normal and premalignant cell types. As a source of both N-nitrosamines and polycyclic aromatic hydrocarbons (PAHs), CSC is very important to our efforts to understand the role of DNA repair in human carcinogenesis. The cultures from both the normal and the carcinoma cell lines treated with CSC and the fractions are being assayed to determine the effects of these exposures on DNA repair of O^6-meGua.

O^6-MT and UDG were measured in alveolar macrophages (obtained from bronchial lavage) and peripheral blood lymphocytes from 21 smokers and 27 nonsmokers. Both activities were present in all samples tested. The amounts and individual variations observed (inter- and intraindividual, up to 100-fold and 6-fold, respectively) were not unlike previous reports for other cellular sources. There was no correlation between O^6-MT and UDG activities in alveolar macrophages (r = 0.02), but significant correlation in PBLs (r = 0.79). Levels of O^6-MT were lower and UDG higher in smokers than in nonsmokers. These observations were more pronounced in alveolar macrophages, perhaps due to their presence in the lungs. Also, consistent with the potentials of direct exposure to smoke in the lungs, UDG activity was higher ($p<0.05$) in macrophages than in lymphocytes of smokers compared to nonsmokers.

To date, 7 (21%) of 33 (13 smokers, 20 nonsmokers) of the volunteers in the DNA repair study were positive in the ultrasensitive radioimmunoassay (USERIA) for the detectability of polycyclic aromatic hydrocarbon-DNA adducts (PAH-DNA) [positive had a minimum of a) 25% inhibition, and b) 0.1 fm PAH-DNA/µg; 2/2 positive test per sample]. All 7 adduct-positive cases had adducts in macrophages, while only 4 (12%) had adducts in lymphocytes, which is within the range (6%-14%) established for lymphocytes in our previous studies. In the 7 cases, both cell types had similar O^6-MT levels (0.53 vs 0.59, respectively), but macrophages had higher UDG levels (193 +/ 202 vs 44 +/-20) and correspondingly higher adduct levels (0.27 +/- 0.18 vs 0.13 +/ 0.07). For the 4 cases with adduct-positive lymphocytes, both cell types had enzyme levels that were essentially of the same pattern (0.64 vs 0.50 for O6-MT; 120 +/ 26 vs 47 +/ 15 for UDG). However, the adduct levels were not different (0.18 +/ 0,03 vs 0,20 +/ 0.03). Finally, the distribution of this rather small number of cases tended toward smokers (31%) compared to nonsmokers (20%). However, the ages of donors and size of the sample populations will have be exploited to verify these results.

Analysis of ras mutations by the polymerase chain reaction amplification and differential oligonucleotide hybridization will test the hypothesis predicting a higher frequency of ras mutations in smokers versus nonsmokers.

Publications:

Curren RD, Yang LL, Conklin PM, Grafstrom RC, Harris CC. Mutagenesis of xeroderma pigmentosum fibroblasts by acrolein. Mutat Res Let (In Press).

Hsu IC, Harris CC, Lipsky MM, Snyder S, Trump BF. Cell and species differences in metabolic activation of chemical carcinogens. Mutat Res 1987;177:1-7.

Reddel RR, Harris CC. Carcinogenesis. In Roth JA, Ruckdeschel JC, Weisenburger TH, eds. Thoracic oncology, WB Saunders (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05324-06 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

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

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

P.I.:	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (*if any*)
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University of Maryland, Baltimore, MD (E. Gabrielson)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Molecular Genetics and Carcinogenesis Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.1	0.3

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

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

Genetic changes related to carcinogenesis are being studied using hybrids from fusion of human lung carcinoma cells with normal human bronchial epithelial cells and of microcells of individual marked human chromosomes with human lung tumor cells. Initial studies suggest that a limited population doubling potential (mortality) is the dominant genetic trait in hybrid cells. Other hybrid cell lines have been isolated and are being characterized for doubling potential, karyotype and tumorigenicity in athymic nude mice. When specific human chromosomes have been transferred by microcell methodology into HuT 292 cells, chromosome 11 but not chromosome 13 has altered the tumorigenicity of the HuT 292. The location of the putative tumor suppressor gene on chromosome 11 will be determined.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Curtis C. Harris	Chief	LHC	NCI

Objectives:

Somatic cell genetics of mortality, tumorigenicity and other aspects of transformation will be studied using hybrids of human lung carcinoma cell lines with normal human bronchial epithelial cells.

Method Employed:

The methods and media for culturing normal human bronchial epithelial cells have been previously developed in this laboratory. Clones of ouabain-resistant HGPRT-lacking cells from established human lung carcinoma cell lines have been derived for the purpose of selecting hybrids.

Cell-cell fusion is done with polyethylene glycol, and hybrids are selected in a media containing hypoxanthine/aminopterin/thymidine (HAT) and ouabain. This selection media is toxic to both the normal parent (ouabain) and the carcinoma parent (HAT).

Several mouse/human hybrid cell lines containing different segments of human chromosome 11 with a transposed HGPRT locus or chromosome 13 marked with the Neo gene have been introduced into the lab. Microcells from the different cell lines are prepared by exposing the cells to colcimid for 48 hr, and then spinning in the presence of cytochalasin B to strip the micronuclei from the cells. The resuspended pellets are then differentially filtered to remove intact cells. The microcells are then fused and selected by the same methods as for cell-cell hybrids.

Methods for measuring the doubling potential of cell lines have been developed utilizing successive passaging of cells and colony size measurement with the Artec image analyzer. Methods for karyotypic analysis of hybrid cell lines are available and tumorigenicity may be assessed by growth in athymic nude mice.

Major Findings:

Many microcell transfers have been performed using different cell lines as donors and HuT 292 as the recipient line. Selected colonies have been expanded and appear to retain their immortality. Those selected colonies from microcell transfers of chromosome 13 marked with Neo which have been inoculated into nude mice appear not to be different in either latency or tumor growth from the HuT 292 parent line. However, colonies from microcell transfers of two different but overlapping segments of chromosome 11 with a balanced translocation of the X chromosome containing the HPRT locus have a longer latency period in nude mice

and on average to have smaller tumors or no tumors after one month. Histologies and restriction fragment length polymorphism (RFLP) analysis of these tumors are pending.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05325-06 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

DNA Cytosine Methylation, Cellular Physiology, and Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Tohru Masui	Visiting Associate	LHC	NCI

COOPERATING UNITS (if any)

Gerontology Research Center, NIA, Baltimore, MD (R.G. Cutler); Lab. Environmental Carcinogenesis, Copenhagen, Denmark (H. Autrup).

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.7	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The role DNA methylation plays in the promoter regions of two separate gene systems has been clarified. Demethylation of at least one Hpa II restriction site upstream from the structural coding sequences of human gamma globin or the hepatitis B core antigen gene is necessary but not sufficient for the initiation of transcription. The final conversion of a quiescent, demethylated gene (gamma globin or hepatitis B core antigen) to an active state requires some endogenous or exogenous inducing agent, which may be highly specific for any given gene or gene complex. Clonal selection is not responsible for observed changes in gene expression, since we have clearly shown that cells remethylate DNA that has been demethylated by 5-azacytidine treatment.

New micro techniques have been developed which enable the analytical quantitation of 5-methylcytosine in less than one microgram of DNA isolated from any source. Thus, the genomic 5-methylcytosine content of normal human bronchial epithelial and pulmonary mesothelial cells has been measured for the first time. These techniques have enabled the determination of changes in the genomic content of 5-methylcytosine during normal physiological processes. The genomic content of 5-methylcytosine in normal human bronchial epithelial cells and in rodent tissues decreases with increasing in vivo age. Significant decreases in DNA 5-methylcytosine occur concomitantly with the induction of squamous differentiation in normal human bronchial epithelial cell cultures. These techniques have also provided for the demonstration that chemical carcinogens can induce decreases in DNA 5-methylcytosine levels in dividing normal human bronchial epithelial cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Tohru Masui	Visiting Associate	LHC	NCI

Objectives:

To determine the relationship between the changes in 5-methylcytosine patterns in DNA and the carcinogenesis process. It is known that tumor cells contain altered methylation patterns in some genes and DNA sequences as compared to normal tissue. It is not known, however, if these changes in 5-methylcytosine patterns initiate carcinogenesis, occur during carcinogenesis, or are the result of this multistep process. Studies are being directed to determine the ability of chemical carcinogens to inhibit the formation of 5-methylcytosine. These susceptible DNA sequences may be the same areas observed to be undermethylated in tumor cells. The methylation patterns of human tumor DNAs will be probed in the search for demethylated genes and/or DNA sequences that may be specific for the tumor type or tissue of origin.

Methods Employed:

This laboratory has developed and utilized human bronchial tissue and epithelial cell culture as a model for carcinogenesis studies. This system also provides a model for the study of the effects of chemical carcinogenesis on the methylation patterns in the DNA of normal human epithelial cells. DNAs are isolated from carcinogen-treated epithelial cultures; restricted with Hpa II, Msp I, and other enzymes sensitive to cytosine modification; and probed with specific DNA sequences and genes. The genomic levels of 5-methylcytosine will also be monitored in treated cells by a sensitive ^{32}P-postlabeling technique developed in this laboratory. The time course of these effects will also be followed, since previous work has determined that the genomic level of 5-methylcytosine in some mammalian cells is decreased maximally by 48 hr post carcinogenic treatment. Epithelial cell DNA methylation patterns will be compared to those of various carcinoma cell lines and human tumors. High molecular weight DNAs will be isolated from human tumors; subjected to the same enzyme restriction and gel electrophoresis, as above; and probed for alterations in methylation patterns in specific genes and DNA sequences.

Major Findings:

Recent findings have determined not only that methylation patterns in DNA are important to gene expression, but also that changes in these patterns take place during differentiation and in vitro senescence. Thus, the ability of chemical

carcinogens to alter 5-methylcytosine patterns in DNA may provide clues to the carcinogenic action of these agents. Previous studies have determined that the alkylation of DNA by alkylating carcinogens inhibits the enzymatic modifications of cytosine residues. Some aromatic hydrocarbon carcinogens also initiated decreases in genomic 5-methylcytosine levels in BALB/3T3 cells.

Previously, the determination of genomic 5-methylcytosine levels required the labeling of DNA in dividing cells with 6-^{3}H-uridine. Limitations in epithelial cell numbers required toxic levels of tritium in order to sufficiently label the DNA for 5-methylcytosine measurements. We have now developed a new method which is both sensitive and does not require active DNA synthesis and cell division. DNA from any source can be enzymatically digested to nucleotides and labeled with ^{32}P. The labeled nucleotides are then separated by thin layer chromatography and the ratio of 5-methylcytidine to the total cytidine and 5-methylcytidine determined. This highly sensitive modified Randerath ^{32}P-postlabeling method not only enables the above-described chemical carcinogenesis studies to be performed on human epithelial cells but also allows for monitoring of genomic 5-methylcytosine levels in tumors, tissues, and cell types from human and animal sources. Thus, changes in 5-methylcytosine levels during differentiation and during the normal aging process in vivo can now be followed.

Significant decreases in the genomic content of cultured normal human bronchial epithelial cells were initiated by a broad range of chemical carcinogens. These reductions varied from 8% to 31%, depending on the carcinogenic agent and the donor epithelial cells. The reductions in the genomic 5-methylcytosine level were concentration-dependent for the carcinogenic polycyclic aromatic hydrocarbon, benzo(a)pyrene. The weakly carcinogenic and noncarcinogenic agents, benzo(a)pyrene and phenanthrene, did not, however, initiate measurable decreases in DNA 5-methylcytosine levels.

Cultured normal human bronchial epithelial cells were also sensitive to the induction of squamous differentiation by 5-azacytidine, 12-O-tetradecanoylphorbol-13-acetate, or transforming growth factor-beta. The cells were grown in serum-free medium, so that the addition of fetal calf serum also induced this terminal differentiation process. The initiation of squamous differentiation by these agents was accompanied by significant reductions in the genomic 5-methylcytosine content of the epithelial cells. A concentration of only 1.2 pM of transforming growth factor-beta was required to induce these effects, so that the initiation of squamous differentiation and reductions in genomic 5-methylcytosine content were probably mediated by receptor interaction. Thus, genomic changes in DNA methylation patterns may be an important component of normal differentiation processes.

The genomic 5-methylcytosine content of normal human bronchial epithelial cells was also dependent on the age of the donor, decreasing with increasing age. Similar age-dependent decreases in brain, liver, and small intestinal mucosa DNA 5-methylcytosine levels were observed in two rodent species. The rate of loss of DNA methylation sites appears to be proportional to the rate of aging.

Publications:

Wilson VL, Masui T, Smith RA, Harris CC. Genomic 5-methyldeoxycytidine decreases associated with the induction of squamous differentiation in cultured normal human bronchial epithelial cells. Carcinogenesis (In Press).

Wilson VL, Smith RA, Longoria J, Liotta MA, Harper CM, Harris CC. Chemical carcinogen-induced decreases in genomic 5-methyldeoxycytidine content of normal human bronchial epithelial cells. Proc Natl Acad Sci USA 1987;84:3298-301.

Wilson VL, Smith RA, Ma S, Culter RG. Genomic 5-Methyldeoxycytidine decreases with age. J Biol Chem 1987;262:9948-51.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05326-06 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

HLA Antigens: Structure, Function and Disease Association

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Dean L. Mann	Section Chief	LHC	NCI
Others:	William Blattner	Section Chief	EEB	NCI
	James Goedert	AIDS Coordinator	EEB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

HLA typing was performed on lymphocytes from patients with a common disease or from families where more than one individual had a common disease type. HLA typing was performed in a cohort of individuals with acquired immune deficiency syndrome (AIDS), either Kaposi's sarcoma, or opportunistic infections, or individuals at risk for this disease. A total of 250 individuals have been HLA typed. One hundred of these patients have been followed over a 4-5 year period. The objectives of these studies are to examine possible genetic susceptibility to the development of AIDS or AIDS-related complex that is related to expression of histocompatibility antigens. The HLA-DR1 phenotype is increased in frequency in all AIDS patients compared to human immunovirus (HIV)-seropositive controls. HLA-DR3 is significantly decreased in the patients with Kaposi's sarcoma. In the HIV-seropositive individuals followed for 65 months, HLA-DR1 appears to be a specific risk factor for disease development. Three families with more than one member with acute lymphocytic leukemia were analyzed for DNA polymorphism in the HLA-DQB genes. Common restriction patterns were found in the leukemic individuals. Sera from 30 hemophiliacs (10 developed AIDS, 10 HIV-seropositive AIDS free and 10 HIV-seronegative) were obtained prior to seroconversion and subsequent yearly samples were tested for antibodies to HLA antigens. Individuals developing AIDS demonstrated increased reactivity to a range of HLA antigens, while losing total CD4+ cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Section Chief	LHC	NCI
William Blattner	Section Chief	EEB	NCI
James Goedert	AIDS Coordinator	EEB	NCI

Objectives:

To determine function, structure and disease association of major histocompatibility complex genes and/or their products. The studies are directed at the elucidation of genetic associations and potential genetic control of the immune response as it relates to disease process and etiology. Once markers are identified in the disease population, functional and biochemical studies are being performed in order to clearly define the genetic regulation of disease processes as it relates to immunologic response.

Methods Employed:

Standard HLA typing was performed using microcytotoxicity techniques. The technique for HLA-A,B,C has been described by Amos and Poole. The method for typing of the B lymphocytes for HLA-DR determinants was originally described by Mann et al. (Proc Natl Acad Sci USA 1975;72:5103-6). A total of 19 determinants controlled by the HLA-A locus, 26 alloantigens at the B locus, 6 alloantigens at the C locus, 10 alloantigens at the DR locus and 6 MT antigens were tested for in the population study. HLA typing was performed by an NCI support contract. The association of HLA types with disease was examined for significance by statistical methods.

Major Findings:

This project continues to provide significant information relevant to expression of histocompatibility antigens, their genetic control and relationship to disease. HLA typing of the cohort of HIV-1-seropositive homosexuals, some of whom have developed Kaposi's sarcoma (KS) or opportunistic infection in the last five years, revealed HLA associations with the development of the disease. HLA-DR1, DQI was significantly increased in frequency in patients with AIDS compared to HIV-seropositive AIDS-negative individuals. In patients with Kaposi's sarcoma, DR3 was significantly decreased. In a prospective study, individuals with DR1 phenotypes developed AIDS more rapidly than HIV-seropositive individuals with other phenotypes. HLA CW5 was absent in patients with KS. The majority of hemophiliacs (type A) in the United States were exposed to HIV by virtue of virus contamination of therapeutic blood products. Sera obtained from these individuals in late 1970, early 1980 (prior to exposure and seroconversion) and subsequent serum samples were screened for antibody to HLA

antigens. These studies were carried out in order to determine if loss of an immune response to a persistent antigenic challenge occurred with the falling CD4+ lymphocytes accompanying disease progression. Contrary to expectations, the reactivity of the sera to histocompatibility antigens increase, most predominantly in individuals developing AIDS. Three families with two members who had acute lymphocytic leukemia were examined for polymorphism in the HLA-DQB region. The individuals studied serologically were HLA-DQ3; specific patterns of HLA-DQB polymorphism were observed in all affected family members.

Publications:

Mann DL, Murray C, Yarchoan R, Blattner WA, Goedert JJ. HLA antigen frequencies in HIV-seropositive disease free individuals and patients with AIDS. J AIDS (In Press).

Smolen JS, Klippel JH, Penner E, Reichler M, Steinberg AD, Chused TM, Scherak O, Graninger W, Hatter E, Zielinski CC, Wolf A, Davie R, Mann DL, Mayr WR. HLA-DR antigens in systemic lupus erythematosus: Association with specificity of autoantibody responses to nuclear antigens. Ann Rheum Dis 1987;46:457-62.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05328-06 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Immunologic Studies of Human T-Cell Lymphoma Virus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Dean L. Mann	Section Chief	LHC	NCI
Others:	Mikulas Popovic	Medical Officer	LTCB	NCI
	Robert Gallo	Chief	LTCB	NCI
	William Blattner	Section Chief	EEB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Biochemical Epidemiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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

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

The human T-cell leukemia virus, HTLV-I, has been found to be associated with patients with adult T-cell leukemia. Studies are underway to understand the mechanism of malignant transformation of cells infected with this virus and the immunologic response of individuals who are infected with this virus and who demonstrate malignancies, or those who are carriers of the virus but have not developed malignancies. Chronic lymphocytic leukemia (CLL) cells were obtained from patients who were HTLV seropositive; however, their malignant B-cells did not contain the HTLV-I retrovirus. Using hybridoma technology, CLL cells were fused with a B-lymphoblastoid cell line and the immunoglobin captured. In one instance, the captured immunoglobin reacted with the HTLV-I p24 gag proteins and, in the other instance, the large envelope protein from HTLV-I. Immunoglobin gene rearrangement present in the B CLL cells was demonstrated in the hybridoma cell line. The results indicate that the CLL cells were antigen-committed cells prior to malignant transformation. Sera from an individual who developed a B-cell lymphoma and whose spouse had acute T-cell leukemia (ATL) (HTLV-I positive) contained an antibody with reactivity to HTLV-I-positive cells. The B-cell lymphoma also produced an antibody reactive with HTLV-I when established in short-term culture.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Section Chief	LHC	NCI
Mikulas Popovic	Medical Officer	LTCB	NCI
William Blattner	Section Chief	EEB	NCI
Robert Gallo	Chief	LTCB	NCI

Objectives:

These studies were designed to examine the biologic effects of infection with the HTLV-I retrovirus. A number of studies from our laboratory and other laboratories have demonstrated that HTLV-I infection altered the immunologic response effecting the dynamics of help and suppression. We have initiated investigations to study the nature of this suppressive effect using the purified retrovirus and to assess alterations in lymphocyte blastogenesis. HTLV-I infection occurs in individuals with other neoplasms. The retrovirus has been found to be absent in neoplastic cells other than in those individuals who have the T-cell lymphomas. Experiments have been carried out to examine the possibility that HTLV-I infection was indirectly involved in the pathogenesis of malignancies other than the specific transforming event of the HTLV-I infection.

Methods Employed:

Sera from patients with autoimmune diseases were tested for antibody activity to HTLV-I and -II and HIV by standard enzyme linked immunosorbent assay (ELISA) assays. DNA from lymphocytes from this same patient population was probed by the presence of HTLV-I and -II and HIV provirus. CLL cells from HTLV-I-seropositive individuals were fused with a human B-lymphoblastoid cell line and the immunoglobulin tested for antibody activity to HTLV-I proteins by the ELISA assay and Western blot techniques. DNA prepared from the donor cells and the hybrid were digested and probed with IgM and IgK j-region probes using Southern blot techniques.

Major Findings:

Human T-cell leukemia virus (HTLV-I) seropositive individuals have been observed in patients with B-cell chronic lymphocytic leukemia cells from the West Indies, an area endemic for HTLV-I infection. The CLL cells obtained from two patients were fused with the human B-lymphoblastoid cell line resulting in immunoglobin-secreting hybridoma cell lines. The IgM produced by the hybridoma cell lines from one patient reacted specifically with the p24 gag protein from all three types of HTLV with preferential reactivity against the HTLV-I. The hybridoma cell line from patient 2 produced an IgM that reacted with the HTLV-I large envelope protein. The specific immunoglobin gene rearrangement (IgM, k) in the

CLL was demonstrated in the hybridoma cell line. The CLL cells appeared to be a malignant transformation of an antigen-committed B-cell responding to HTLV-I stimulation. Short-term cultures of an explanted B-cell lymphoma from a patient whose husband had an HTLV-I-positive T-cell lymphoma produced an antibody which reacted with HTLV-I-positive B-cells and T-cells. Serum from this patient immunoprecipitated a 66,000 Kd protein from HTLV-I cells, evidence for reactivity with the large envelope glycoprotein of HTLV-I. This data provides further evidence for an indirect mechanism for involvement of retrovirus in the pathogenesis of B-cell tumors.

Publications:

Mann DL, DeSantis P, Mark G, Pfeifer A, Newman M, Popovic M, Gallo R, Clark J, Blattner WA. HTLV-I associated B cell chronic lymphocytic leukemia: Indirect role for retrovirus in leukemogenesis. Science 1987;236:1103-06.

Schneider M, Saal JG, Mann DL, Pawelec G, Schneider J, Schlote W, Wernet P. Respective T and B cell lymphomas in a married couple: In vivo activated T cells lysing both tumor targets and concomitant humoral immune response pointing to a putative novel HLA class I restriction element related to HTLV-I. Int J Cancer (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05341-06 LHC

PERIOD COVERED

October 1, 1987 to September 1988

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

Model Systems for Studying Physical Carcinogens at the Cellular Level

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	John F. Lechner	Section Chief	LHC	NCI
Others:	Angela Somers	Visiting Fellow	LHC	NCI
	Brenda Gerwin	Research Chemist	LHC	NCI
	Helen Reddel	Guest Researcher	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS *(if any)*

Baltimore V.A. Hospital, Baltimore, MD (E. Gabrielson); Institute of Occupational Health, Helsinki, Finland (K. Linnainmaa)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.5	1.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Methods to culture human pleural mesothelial (NHM) cells have been defined. Cultures are initiated by pelleting the mesothelial cells from pleural effusion fluid and inoculating the resuspended cells into dishes containing LHC basal nutrient medium supplemented with serum (3%), hydrocortisone (0.5 micromoles), insulin (5 micrograms/ml), epidermal growth factor (EGF) (5 ng/ml), transferrin (10 micrograms/ml), trace elements, and 2% chemically-reduced (factor-free) serum (FFS). Using this protocol, mesothelial cell cultures have been established from more than 200 donors. The cells can be subcultured at clonal density with a colony-forming efficiency of more than 10% and high density cultures can be subcultured four to six times before senescence. We have now established that many factors <EGF, transforming growth factor beta-1 (TGF-beta-1), TGF-beta-2, interleuken 1 (alpha) (1L-1-alpha), 1L-1-beta, transforming growth factor alpha (TGF-alpha), human platelet derived growth factor (hPDGF), porcine PDGF (pPDGF), fibroblast growth factor-acidic (FGF-a), FGF-basic (FGF-b), beta-interferon (Inf-beta), gamma-interferon (Inf-gamma), and cholera toxinß will induce serum-starved cells to undergo one round of DNA synthesis in the absence of serum. However, for sustained growth, the medium must also be supplemented with high density lipids (HDL). Since human mesothelial cells have a peculiarly plastic cytoskeleton, we have characterized the affects of amosite fibers and code 100 glass fibers on the fidelity of division in these cells in an effort to understand the mechanism by which asbestiform fibers induce transformation and mesothelioma. Our results indicate that both amosite and code 100 glass fibers disturb the fidelity of cell division leading to the induction of aneuploid daughter cells. However, the mechanism of action of these two agents appears to be different. Amosite fibers induce chromosome clustering, suggesting an inhibition of mitotic tubulin formation and/or centriole separation. In contrast, code 100 glass fibers cause chromosome dislocation from the spindle at metaphase, suggesting an affect on centromere/kinetochore function and/or mitotic spindle function.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John F. Lechner	Chief, In Vitro Carcinogenesis Section	LHC	NCI
Angela Somers	Visiting Fellow	LHC	NCI
Brenda Gerwin	Research Chemist	LHC	NCI
Helen Reddel	Guest Researcher	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To study the carcinogenicity and cytopathology of asbestos fibers in human mesothelial in vitro systems. These studies include the following: (1) develop defined media for replicative mesothelial cell cultures, (2) evaluate cytotoxicity of asbestos fibers and synthetic nonmineral fibers in mesothelial cells, (3) evaluate the effects of asbestos fibers on progression of chromosome rearrangements in mesothelial cells.

Methods Employed:

Normal human mesothelial (NHM) cells are obtained from pleural effusions of donors without cancer. The fluid is centrifuged, and the pelleted cells are resuspended and inoculated into 100 mm culture dishes at a ratio of one dish per 150 ml of pleural fluid. The cells are dissociated using trypsin when the cultures attain subconfluency. The cultures are further expanded and either cryopreserved or used according to experimental protocols.

Major Findings:

Growth Control Studies: Serum-starved NHM cells will undergo one round of DNA synthesis if the basal nutrients containing insulin are supplemented with EGF, TGF-β_1 or PDGF. EGF, TGF-β_1 and PDGF are of equal potency alone and further enhancement of mitogenic activity is obtained by using dual combinations of EGF with either PDGF or TGF-β_1. However, these supplements do not support sustained mesothelial cell replication unless high density lipids are also present. In the presence of insulin, transferrin and HDL, cultures of NHM cells developed from different donors respond equally well including EGF, PDGF (both bb homodimer and ab heterodimer forms), TGF-β (both type 1 and type 2), 1L-1 (both α and β forms) 1L-2, β-Inf, γ-Inf, FGF (both acidic and basic forms) and cholera toxin. However, marked interindividual variation among cultures developed from different donors has been noted. Specifically, the mitogenic response among individual cultures for γ-interferon and interleukin-2 has been found to vary from as high as 1000% of control to no significant effect, to highly growth inhibitory. Further, a few NHM cell cultures do not respond to fibroblast growth factor, TGF-β_1 or β interferon.

In addition, no NHM cell culture can respond to all of the above listed factors, but all of the cultures to date are stimulated by EGF. Finally, 75% of the individual cultures exhibit a greater growth rate when incubated in (3%) fetal bovine serum (FBS) medium supplemented with insulin, transferrin, hydrocortisone and EGF, as compared to cells incubated in the same medium, except HDL is substituted for FBS, suggesting that cells require combinations of the above-listed mitogens or perhaps yet unidentified mitogens for maximal growth. Experiments testing dual combinations of factors have resulted in NHM cells growing more rapidly than with either factor alone. The lone case of a combination of growth factors not causing a more rapid growth rate is EGF with FGF. Manuscripts describing these observations have been submitted for publication.

Carcinogenesis Studies with Mesothelial Cells: Two subculturings after amosite asbestos exposure, colonies of phenotypically altered cells were present. The control cultures reached senescence during the fourth to sixth subculture. However, the amosite-exposed cultures have continued to multiply for more than 19 subsequent subculturings (> 50 population doublings). Tumorigenicity was tested by injecting 11th passage post-second amosite exposure cells S.C. into adult athymic nude mice (5 million cells/mouse, 9-20 mice per experiment); no tumors arose within 18 months post-inoculation. Amosite-exposed cells from subsequent experiments have behaved similarly.

Chromosomal Studies of Asbestos-Exposed Mesothelial Cells: After only one exposure to amosite, a significant increase in chromosomal aberrations, including chromosome and chromatid breaks, were observed in mesothelial cell cultures, compared to untreated control cells. The number of aberrant cells was further increased in cultures treated twice with asbestos, the major aberration type being dicentrics. In an approach to delineate the mechanism(s) that underlies the clastogenic action of asbestiform fibers, cells were analyzed by dapi staining (scoring micronuclei), indicative of chromosomal dislocation from the spindle, and direct spindle and chromosome co-staining using brilliant blue R and safranin O to visualize other types of mitotic dysfunctions. What we have determined is that amosite asbestos causes chromosome clustering. Further, the percentage of binucleate cells increases. These observations indicate that the fiber disrupts centriole separation. The effects of glass fibers are different, i.e., micronuclei are formed and dislocated chromosomes are observed. Thus, glass fibers disrupt centromere/kinetochore function.

Publications:

Gabrielson EW, Lechner JF, Gerwin BI, LaVeck MA, Harris CC. Growth factors for mesothelial and mesothelioma cells. Chest (suppl) 1987;91:17-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05403-05 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Analysis of Gene Regulation and Proliferative Control in Human Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Brenda I. Gerwin	Research Chemist	LHC	NCI
Others:	Roger Reddel	Expert	LHC	NCI
	John Lechner	Res. Microbiologist	LHC	NCI
	Tohru Masui	Visiting Associate	LHC	NCI
	Yang Ke	Visiting Fellow	LHC	NCI

COOPERATING UNITS (if any)

Hazelton Labs, Rockville, MD (M. Moore); Inst. of Occupational Health, Helsinki, Finland (K. Linnainmaa); Helsinki Univ., Helsinki, Finland (J. Keski-Oja); Upjohn Labs, Kankakee, IN (D. Carter); Univ. of Uppsala, Sweden (C. Betsholtz)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Transforming growth factor (TGF)-beta-1 but not plasminogen activator inhibitor (PAI)-1 can induce a burst of PAI-1 production in normal human bronchial epithelial (NHBE) cells or in T antigen-transformed normal human bronchial epithelial cells. These cells do not normally produce this gene product. However, tumorigenic, ras-transfected, T antigen transformed NHBE cells constitutively produce PAI-1.

Mesothelioma cells, but not normal human mesothelial cells, produce platelet-derived growth factor (PDGF)-A chain protein which is biologically active in receptor binding assays. However, compared to the normal cells the mesothelioma cell lines do not respond to the mitogenic signals of PDGF. Normal mesothelial cells produce mRNA for basic fibroblast growth factor (FGF). Some mesotheliomas, but no normal mesothelial cells, produce TGF-alpha, while all classes of human mesothelial cells are negative for epidermal growth factor (EGF) production.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Brenda I. Gerwin	Research Chemist	LHC	NCI
Roger Reddel	Expert	LHC	NCI
John Lechner	Res. Microbiologist	LHC	NCI
Tohru Masui	Visiting Associate	LHC	NCI
Yang Ke	Visiting Fellow	LHC	NCI

Objectives:

The goal of this project is to understand, at the molecular level, regulatory interactions of human cells with growth factors, metabolites, and foreign chemicals implicated in carcinogenesis. These substances govern or affect the growth potential and differentiation status of the cells. The topics of present interest are: induction of PAI-1, interleuken-one (IL1), and urokinase (uPA) in NHBE cells, T antigen-transformed NHBE cells, and lung tumors; development of model systems for the study of differentiation of NHBE cells and for fiber sensitivity and growth factor regulation in normal human mesothelial cells; and responsiveness to growth factors and auto-production of growth factors by normal human mesothelial cells, T antigen-transformed normal human mesothelial cells, and mesotheliomas.

Methods Employed:

Normal human bronchial epithelial cells, normal human mesothelial cells, T antigen immortalized lines of each of these cells, and human lung carcinoma and mesothelioma cells are propagated in tissue culture. Whole cell RNA or genomic DNA is prepared from these cells and from fresh or frozen tissue samples. Whole cell lysates labelled with radioactive amino acids are utilized for studies of protein production. Nucleic acids are studied by electrophoresis, transfer, and probing with appropriate gene probes. Proteins are studied by polyacrylamide gel electrophoresis after immune or affinity adsorption.

Major Findings:

Mesothelial Cells: mRNA expression studies indicate that normal human mesothelial cells express mRNA for basic FGF. This mRNA seems to be made to a lesser extent by mesothelioma cell lines. EGF expression is negative in all normal mesothelial cells and mesotheliomas which have been tested, but TGF-alpha is produced by some but not all mesothelioma cell lines. Neither normal mesothelial cells nor mesotheliomas produce detectable quantities of alpha A1 or A2 apolipoprotein mRNA consonant with the requirement for high density lipoproteins shown by this cell type. Immunoprecipitation studies have shown that PDGF A chain is secreted as a 31Kd dimer by human mesotheliomas. Cell lysates contain a 21Kd precursor of the

17Kd A chain subunit. B chain products could not be demonstrated even in cells which produced B chain mRNA. Excellent correlation was found when PDGF receptor binding activity, A chain mRNA production, and A chain immunoprecipitable proteins were compared. However, B chain immunoprecipitable proteins and mitogenicity of conditioned medium for fibroblasts did not correlate with these parameters. Mesothelioma cells, in contrast to normal mesothelial cells and T antigen immortalized cells, do not respond to the mitogenic signals of PDGF.

Bronchial Epithelial Cells: We have shown that plasminogen activator inhibitor 1 (PAI-1) is induced by TGF-beta-1 in normal human bronchial epithelial cells and serum sensitive, T antigen-transformed, normal human bronchial epithelial cells. The mRNA response is maximal at about 4 hr and returns to an essentially negative baseline. 12-O-tetradecanoylphorbol-13-acetate (TPA), which also induces squamous differentiation in these cells does not induce PAI-1 expression. PAI-1 protein was detected in cell lysates, conditioned medium, and extracellular matrix 18 hr after induction. There was no inhibition of PAI-1 induction in high as compared to low density cultures of normal human bronchial epithelial cells or the T antigen-transformed cell line. A cell line produced from a *ras*-transfected T antigen-transformed human bronchial epithelial cell culture produced PAI-1 constitutively. With respect to growth factor effects on human bronchial epithelial cells, it has been shown that EGF inhibits cell growth at high density but that this inhibition is lessened by treatment with IL1. Furthermore, it has been shown that the TPA induction of IL1 can be inhibited by H7, an inhibitor of protein kinase C, indicating that this molecule may be induced by second messenger pathways involving protein kinase C.

Publications:

Gabrielson EW, Gerwin BI, Harris CC, Roberts AB, Sporn MB, Lechner JF. Stimulation of DNA synthesis in cultured primary human mesothelial cells by specific growth factors. FASEB J (In Press).

Gerwin BI, Lechner JF, Reddel RR, Roberts AB, Robbins KC, Gabrielson EW, Harris CC. Comparison of production of transforming growth factor-beta and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res 1987;47:6180-4.

Harris CC, Gerwin B, Ke Y, Masui T, Miyashita M, Pfeifer A, Reddel R, Wilson VL, Lechner JF. Growth, differentiation, and neoplastic transformation of human bronchial epithelial cells. In: Moses HL, Lengyel P, Stiles CC, eds. Growth factors and their receptors. New York: Alan R Liss, (In Press).

Ke Y, Reddel RR, Gerwin BI, Miyashita M, McMenamin M, Lechner JF, Harris CC. Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. Differentiation (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05409-05 LHC

PERIOD COVERED
October I, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)
Control of Growth and Differentiation of Human Bronchial Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	John F. Lechner	Section Chief	LHC	NCI
Others:	Yang Ke	Visiting Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Brenda I. Gerwin	Res. Chemist	LHC	NCI

COOPERATING UNITS (if any)
Univ. of MD School of Medicine, Balt., MD (B.F. Trump); Georgetown Univ. School of Medicine, Washington, DC (H. Yeager); VA Hospital, Washington, DC (P. Schafer)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2.2	1.3

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

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

On the basis of our culture system for normal human bronchial epithelial (NHBE) cells, an Ad12-SV40-transformed cell line (BEAS-2B) has been established which is immortal (>100 passages) and partially retains the ability to be induced by serum to undergo squamous differentiation as normal bronchial epithelial cells. Two subclones of BEAS-2B have been isolated and characterized with regard to squamous differentiation ability. One subclone designated as S.6 is sensitive to serum and transforming growth factor (TGF)-beta-1 induced squamous differentiation, and another subclone, R.1, is resistant to these two agents. However, both S.6 and R.1 cells are resistant to 12-O-tetradecanoylphorbol-13-acetate (TPA), even though membrane receptors for TGF-beta-1 and TPA are not significantly different from that of normal cells at the number and affinity. A density-dependent affect of squamous differentiation induction for normal and transformed cells has been found. At clonal density (25-500/cm2) cells stop growing and differentiate, but at high cell density (>1000/cm2) only part of the population differentiates. Time course of morphology change and DNA synthesis inhibition induced by serum treatment suggest that two cell (pseudo) populations arise in the independent cultures that react to serum in different ways. An epidermal growth factor (EGF) requirement for cell growth that is density-dependent has also been found. EGF is required by clonal growth but is not necessary for high density cell growth (>1 x 104/cm2). Primary data from S.6 cells' conditioned medium has shown that these cells produce a growth factor which is less than 12,000 molecular weight and heat resistant.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John F. Lechner	Section Chief	LHC	NCI
Yang Ke	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Brenda I. Gerwin	Res. Chemist	LHC	NCI

Objectives:

To develop systems to study mechanisms involved during malignant transformation of human epithelial cells. These studies include the following: (1) develop efficient assays to quantify the various squamous differentiation-inducing factors; (2) identify and characterize an autocrine growth factor; (3) identify and characterize an autocrine squamous differentiation-inducing factor; (4) elucidate the pathways of squamous differentiation and determine aberrations that cause human lung carcinoma cells not to respond to these squamous differentiation-inducing factors.

Methods Employed:

Human bronchial tissues are obtained from a medical examiner and "immediate" autopsy donors. Bronchial tissues are dissected from surrounding stroma, cut into 0.5 cm square pieces and used to establish explant cultures. Replicative cultures of normal bronchial epithelial cells are developed from explant culture outgrowths. Upon transfer of the explants to new dishes, the outgrowth cultures remaining in the original dishes are incubated in defined serum-free medium to expand the population and are then subcultured. These normal human bronchial epithelial (NHBE) cells are used in growth and differentiation studies or are cryopreserved for future use. Mitogenicity is quantified by measuring the clonal growth rate, rate of incorporation of tritiated-thymidine into acid precipitable material, and the labeling index by autoradiography. Squamous differentiation is determined by colony forming efficiency (CFE) and DNA synthesis, extracellular plasminogen activator activity and Ca ionophore-induced cross-linked envelope formation. Since epinephrine antagonizes the effect of TGF-β_1, laboratory of human carcinogenesis (LHC)-8 (LHC-9 medium without epinephrine and retinoic acid) is mainly used. Transformation of the cells is described in another project (Z01CP05505-04).

Major Findings:

Ad12-SV40 hybrid virus-transformed HBE-BEAS-2B cells have been cultured for more than two years. When first established, the cells were very sensitive to serum induction of squamous differentiation and the CFE in 8% serum was zero. To assess the stability of the serum response with cell passage, CFE was measured at various passages through 58. Beyond passage 34, the phenotype became relatively

stable at 20% of controls' level for CFE. Two subclones from BEAS-2B cells have been isolated; one is serum sensitive (S.6), one is resistant (R.1). Characteristic analysis shows that although both cell lines have the same marker chromosomes as BEAS-2B, they have different morphologies, with S.6 being more epithelial and R.1 more fusiform. However, both are keratin-positive. In the absence of serum, the CFE was 35.2% for S.6 and it progressively decreased with increasing serum supplementation. In contrast, the R.1 cells formed few colonies without serum (0.2% CFE); it reached to 35% with 4% serum. Serum also caused a dose-dependent increase in CLEs by BEAS-2B and S.6, while R.1 cells formed few CLEs and no serum-dose relationship was noted. TGF-β_1, an important differentiation inducer in the serum, markedly inhibited the CFE of BEAS-2B and S.6 but not R.1 cells. In contrast, TPA, an exogenous squamous differentiation inducer for NHBE cells did not significantly alter the growth of any of the cells above. For TGF-β_1 receptors, the results are reported as binding capacity relative to the value for A549 lung carcinoma cells (10,400 binding sites/cell). The values for BEAS-2B, S.6 and R.1 cells are 0.2, 0.34 and 1.1, respectively. Further, all three cell lines have 1.0 to 1.2 x 10^6 TPA receptors per cell with Kd from 43 to 50 nM. Thus, the TPA binding activities are not significantly different from NHBE cells.

Cell density has been found to affect the differentiation results of NHBE and S.6 cells; that is, at low cell density (25-500 cell/cm^2), the cells cannot grow in 8% serum of TGF-β_1 for normal and S.6 cells, but at high cell densities (>1 x $10^4/cm^2$) they still grow. A difference is that DNA synthesis is inhibited for low density cells in serum irreversibly, while it recovers within 48 hr at high cell density. This DNA synthesis "recovery response" of high density cells is not because of an autocrine anti-differentiation factor, since changing medium cannot influence the response. We propose that different subpopulations exist in the bronchial epithelial cultures which respond to serum in different ways and the morphologies of serum-treated cells support this hypothesis.

EGF is the major growth factor NHBE cells need for clonal growth. Surprisingly, at cell densities above 1 x $10^4/cm^2$, cells grow better without EGF. The data on S.6 cells demonstrated that the conditioned medium from high density EGF-free cultures stimulate cell growth at low density. We plan to isolate and characterize this putative mitogen.

Publications:

Harris CC, Brash DE, Lechner JF, Mark G. Aberrations of growth and differentiation pathways during neoplastic transformation of human epithelial cells. IARC Sci Publ (In Press).

Harris CC, Reddel RR, Ke Y, Pfeifer A, Mark G, Masui T, Yoakum G, Gerwin BI, Lechner JF. Cellular and molecular studies of growth, differentiation, and neoplastic transformation. In: Feo F, ed. Models and mechanisms in chemical carcinogenesis. New York: Plenum Press, (In Press).

LaVeck MA, Lechner JF. Isolation and culture of normal human bronchial epithelial cells from autopsy tissue. In: Freshney RE, ed. Culture of animal cells, 2nd Edition. New York: Alan R Liss, (In Press).

Lechner JF. Replicative cultures of human prostatic epithelial cells. In: Coffee DS, Bruchovski N, Gardner WA Jr, Resnick MI, Karr PJ, eds. Assessment of current concepts and approaches to the study of prostate carcinoma. New York: Alan R Liss, 1987;497-509.

Lechner JF, Masui T, Miyashita M, Willey JC, Reddel R, LaVeck MA, Ke Y, Yoakum GH, Amstad P, Gerwin BI, Harris CC. Human lung cells: In vitro models for studying carcinogens. In: Langenbach R, Elmore E, Barrett JC, eds. Tumor promoters: biological approaches for mechanistic studies and assay systems. New York: Plenum Press, (In Press).

Lechner JF, Stoner GD, Haugen A, Autrup H, LaVeck MA, Trump BF, Harris CC. In vitro human bronchial model systems for carcinogenesis studies. In: Webber MM, Sekely L, eds. In vitro models for cancer research. Boca Raton: CRC Press, (In Press).

Masui T, Lechner JF, Mark GE III, Pfeifer AMA, Miyashita M, Yoakum GH, Willey JC, Mann DL, Harris CC. Growth and differentiation programs of normal and transformed human bronchial epithelial cells. UCLA Symp Mol Cell Biol Vol. 32, New York: Alan R Liss, Inc, 1987;191-202.

Wakefield LM, Smith DM, Masui T, Harris CC, Sporn MB. Distribution and modulation of the cellular receptor for transforming growth factor-β_1. J Cell Biol 1987;105:965-75.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05426-04 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Characterization and Mode of Action of the raf Subfamily of Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Andrea Pfeifer	Visiting Associate	LHC	NCI
Others:	Dean L. Mann	Section Chief	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Louise Malan-Shibley	Chemist	LHC	NCI

COOPERATING UNITS (if any)

Dept. of Radiation Medicine, Georgetown Univ. School of Medicine (U. Kasid)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Retroviral recombinants pZip-raf and pZip-myc were constructed to examine the role of the c-raf-1 and c-myc proto-oncogene in lung carcinogenesis. Immortalized human bronchial epithelial cells (BEAS-2B) transfected with pZip-raf DNA and pZip-myc DNA gave rise to undifferentiated carcinomas (raf/myc tumors) when tested in athymic nude mice, whereas c-myc or c-raf transfected cells are non-tumorigenic. The raf/myc tumors expressed markers of small cell lung carcinomas, i.e., neuron-specific enolase and neurosecretory granules. In addition, BEAS-2B cells transformed with the c-raf and c-myc proto-oncogenes, as well as derived tumor cell lines acquired HLA class II antigen expression.

The c-raf-1 gene has been identified as the predominant transforming gene of three radiation-resistant head and neck cancer cell lines in the NIH 3T3 transfection assay (SQ-20B, JSQ-3, SCC-35). NIH 3T3 cells transformed with SQ-20B DNA also became radiation-resistant, suggesting a correlation between the presence of c-raf sequences and the radiation-resistant phenotype. Inhibition of the c-raf function by introduction of anti-sense raf transcribing plasmids into the SQ-20B cell line reverted not only the tumorigenic phenotype but also reduced the radiation resistance. As a consequence of these experiments, the construction of an inducible promoter system for anti-sense sequences in human cells was undertaken.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on This Project:

Andrea Pfeifer	Visiting Associate	LHC	NCI
Louise Malan-Shibley	Chemist	LHC	NCI
Dean L. Mann	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

An amplification of the c-*myc*, N-*myc* or L-*myc* gene and overexpression of the c-*raf* sequence are common features of small cell lung carcinomas. The major objective of this project is to determine the role of these genes in lung carcinogenesis by a) overexpressing c-*raf* and c-*myc* genes in normal human lung cells, and b) reversion of the malignant phenotype by plasmids capable of antisense RNA transcription.

In several human and animal tumors the c-*raf* gene was identified as the predominant transforming gene in the NIH 3T3 transfection/transformation assay. NIH 3T3 cells transfected with DNA from the radiation-resistant head and neck tumor cell line, SQ-20B, were tumorigenic and revealed an activated c-*raf*-1 locus. The second objective is to analyze the linkage between the malignant and radiation-resistant phenotype of SQ-20B and the activation of the c-*raf*-1 gene.

Methods Employed:

DNAs were transfected into recipient mammalian cells employing calcium or strontium phosphate co-precipitation. Epitropic and amphotropic retroviruses containing proto-oncogenes/oncogene sequences were obtained using the psi 2 or psi AM packaging systems, respectively, and pZipSV(X) neo or related vector systems. RNAs and DNAs were isolated from cells in culture by the guanidinium isothiocyanate-CsCl method and tested by Northern and Southern blot analyses. To increase the sensitivity of Northern blotting, riboprobe (RNA) probes were employed in addition to nick-translated DNA probes. DNA sequences were obtained by the dideoxy-method according to Sanger. Proteins were isolated from the cell lines according to a protocol specific for nuclear proteins, run on denaturing polyacrylamide gels and analyzed in a Western blot system using immunoperoxidase detection.

Major Findings:

To determine the role of c-*raf*-1 and c-*myc* proto-oncogene in lung carcinogenesis, the human c-*raf*-1 cDNA and the murine c-*myc* cDNA were cloned into Mulligan's pZipneoSV(X) and pLJ vectors. Although the neo-expression obtained with the Zip-vector was less than in pLJ, the Zip-system was preferentially used as an effect of the polioma sequence in the pLJ vector could not be excluded.

pZip-raf, pZip-myc or the combination of the two proto-oncogenes were introduced into immortalized human bronchial epithelial cells (BEAS-2B) and populations of G418-resistant cells were screened in irradiated, athymic nude mice for tumorigenicity. BEAS-2B cells containing both pZip-raf and pZip-myc DNA were tumorigenic in nude mice (5/8) with a latency of four weeks. c-raf-1 or c-myc alone is nontumorigenic. Isoenzyme pattern, karyotypic and morphological markers identified BEAS-2B as the progenitor cell line of the tumors. The tumors induced by the c-raf and c-myc genes revealed some markers commonly found in small cell lung carcinomas (SCLC), i.e., neuron-specific enolase and neurosecretory granules. L-dopa decarboxylase was not detectable.

In addition, BEAS-2B cells transfected with pZip-neo, pZip-raf, pZip-myc and the combination of both and tumor cell lines established from pZip-raf + pZip-myc tumors were screened for specific surface antigens. The monomyelocytic markers, MY4 and MY9, have been found in SCLC. MY4 and MY7 (early monocytic markers) were present in all of the cell lines and the expression of these markers may be related to the presence of the SV40 T antigen. MY9 was elevated in the transfected lines, including the Zip-neo-carrying BEAS-2B cells. The most remarkable result was the expression of HLA class II antigens in BEAS-2B cells transfected with both proto-oncogenes and in the corresponding tumor cell lines.

The transfected cell lines and the tumor cell lines have been screened for expression of the introduced murine c-myc sequence. However, antibodies specific for the murine (Eisenman) or human (Evans) myc protein showed cross-hybridization on Western blots and require immunoprecipitation for higher species specificity.

Three head and neck cancer cell lines (SQ-20B, SCC-35, JSQ-3) were screened by the NIH 3T3 transfection assay for transforming oncogenes. Only the c-raf-1 oncogene was found in transformed NIH 3T3 clones. The c-raf-1 sequence was activated in these clones by truncation of the amino half of the gene and the breakpoint was determined between exon 8 and 9 based on Southern blot analysis. 4.2kb and 2.6kb transcripts were identified in the tumorigenic NIH 3T3 clones. One non-tumorigenic clone was lacking the larger transcript and showed on the DNA level deletions in the kinase domain of the c-raf-1 locus.

To investigate the correlation between the presence of the c-raf-1 gene and the radiation-resistant phenotype of these tumor cell lines, anti-sense transcribing constructs have been made using vectors with strong promoters in human cells, i.e., adenovirus major late promoter (pADMLP) and the enhanced versions of pADMLP (pD5). Fragments of the c-raf sequence encoding the whole gene or the 3' end were cloned into pADMLP and pD5. The SQ-20B cell line transfected with the anti-sense construct showed a decrease in tumorigenicity, as well as in the radiation resistance, suggesting (a) an essential role of c-raf-1 in head and neck cancer and (b) a correlation between c-raf-1 and the radiation-resistant phenotype.

The anti-sense experiments with SQ-20B cells also demonstrated the requirement for an inducible promoter system since SQ-20B cells expressing the anti-sense message of c-raf-1 have a growth disadvantage. Only a minor portion of these cells survive the selection with G418. Therefore, genes in sense and anti-sense orientation were cloned into CD23 plasmid which contains an HIV 3 promoter inducible by the tat III gene. The CD23 constructs were transfected into BEAS-2B cells and selected for G418 resistance. To induce gene expression, an amphotropic Zip-tat III virus was generated using the psi 2/psi AM cell systems.

Publications:

Kasid U, Pfeifer A, Weichselbaum RR, Dritschilo A, Mark GE. The raf oncogene is associated with a radiation resistant human laryngeal cancer. Science 1987;237:1039-41.

Mark GE, Pfeifer AMA, Berman R, Pert CB. Raf proto-oncogene expression in neural and immune tissues. In: Bridge TP, ed. Psychological, neuropsychiatric and substance abuse aspects of AIDS. New York: Raven Press, 1988;45-55.

Pfeifer AMA, Lechner JF, Masui T, Reddel RR, Mark GE, Harris CC. Control of growth and squamous differentiation in normal human bronchial epithelial cells by chemical and biological modifiers and transferred genes, Environ Health Perspect (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05432-04 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

The Biological Activity of Fecapentaene-12 in Human Tissues and Cells

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Curtis C. Harris	Chief	LHC	NCI
Others:	Andrew Povey	Visiting Fellow	LHC	NCI
	Dean L. Mann	Section Chief	LHC	NCI
	Vincent Wilson	Senior Staff Fellow	LHC	NCI
	Peter Roller	Section Chief	LEC	NCI

COOPERATING UNITS (*if any*)

Karolinska Inst., Sweden (R. Grafstrom); Microbiological Assoc., Bethesda, MD (R. Curren, L.L. Yang); IARC, France (I.K. O'Neill); Am. Health Fdn., Valhalla, NY (P. Foiles); Francis Scott Key Med. Ctr., Baltimore, MD (E. Gabrielson, E. Rosen)

LAB/BRANCH

Laboratory of Human Carcinogenesis

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Biochemical Epidemiology Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Fecapentaenes, candidate carcinogens in the pathogenesis of colon cancer, are cytotoxic, mutagenic and induce DNA single strand breaks, sister chromatid exchange and unscheduled DNA synthesis in normal human fibroblasts. This compound also induces transformation in murine Balb 3T3 cells. Possible covalent binding of radiolabelled fecapentaene to calf thymus DNA was indicated by cesium chloride density gradient centrifugation. Subsequent enzyme hydrolysis did not confirm the presence of radiolabelled adducts. Preliminary data after acid hydrolysis of fecapentaene damaged DNA, fast atom bombardment mass spectroscopy (FAB-MS) analysis of a fecapentaene-damaged deoxynucleoside and 32P-postlabelling of fecapentaene-damaged deoxynucleotide suggested that specific adducts may be formed. Acrolein adducts were not detected in fecapentaene-modified DNA by the use of a monoclonal antibody. Further characterization of both specific and non-specific DNA lesions (oxidative damage) is currently in progress. Formation of free-radicals is under study by trapping techniques coupled with electron paramagnetic resonance (EPR) spectroscopy. Fecapentaene is degraded in aqueous solution to a large number of ultraviolet (UV) absorbing compounds, including, at least initially, aldehydes. Characterization by nuclear magnetic resonance (NMR) spectroscopy is currently in progress.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Curtis C. Harris	Chief	LHC	NCI
Andrew Povey	Visiting Fellow	LHC	NCI
Dean L. Mann	Section Chief	LHC	NCI
Vincent Wilson	Senior Staff Fellow	LHC	NCI
Peter Roller	Section Chief	LEC	NCI

Objectives:

To assess the possible importance of fecapentaenes in the etiology of human colon cancer. Studies are directed to investigate the molecular mechanisms of DNA damage. Both specific (adducts) and non-specific (oxidative damage) lesions caused by fecapentaenes are being sought and will be used to assess fecapentaene-induced DNA damage in DNA isolated from human colon epithelium in populations with different risk from colon cancer.

Methods Employed:

Radiolabelled [^{3}H]-fecapentaene has been used to study the binding of fecapentaene to DNA and the possible formation of fecapentaene-DNA adducts. Such binding was further studied by the use of fecapentaenes containing the radiolabel at different sites in the molecule. Adduct formation was investigated by enzymatic (and/or acid) hydrolysis of the DNA followed by high performance liquid chromatography (HPLC) separation of the constituent deoxynucleotides or nucleosides.

Unlabelled fecapentaene has also been used to study induced DNA damage. ^{32}P-postlabelling techniques have been used in conjunction with HPLC separation to investigate the formation of (1) both small and bulky adducts and (2) oxidative damage products. Deoxynucleotides and nucleosides have been used as model compounds to characterize fecapentaene-induced DNA damage by the use of ^{32}P-postlabelling and FAB-MS. Monoclonal antibodies against acrolein-modified DNA were used in an assay for fecapentaene-induced DNA damage.

Formation of oxidized fecapentaene breakdown products was studied by the use of HPLC and NMR spectroscopy. Free-radical trapping techniques coupled with EPR spectroscopy was used to study free-radical formation during fecapentaene oxidation.

Major Findings:

Initial studies with radiolabelled fecapentaene-12 showed that the radiolabel was strongly associated with DNA; subsequent enzymatic hydrolysis did not reveal the presence of any adducts. Acid hydrolysis, however, suggested the formation of an unstable radiolabelled adduct. Preliminary data obtained from FAB-MS of a nucleoside-fecapentaene reaction mixture also suggests that specific adducts may have been formed. ^{32}P-postlabelling of HPLC fractions from a fecapentaene-damaged deoxynucleotide resulted in the formation of a small number of as yet unidentified spots which may be fecapentaene-specific adducts or lesions resulting from oxidative damage.

Incubation of fecapentaene at neutral pH results in the time-dependent formation of a large number (20-30) of UV absorbing products. Initially, NMR spectroscopy showed that aldehydes were present.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CP05434-04 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)
Immunology of AIDS and AIDS-Related Diseases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Dean L. Mann	Section Chief	LHC	NCI
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	J. J. Goedert	AIDS Coordinator	EEB	NCI
	R. J. Bigger	Medical Officer	EEB	NCI
	Stanley H. Weiss	Medical Staff Fellow	EEB	NCI
	R. C. Gallo	Chief	LTCB	NCI
	Mikulas Popovic	Medical Officer	LTCB	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Human Carcinogenesis

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Biochemical Epidemiology Section

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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

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

Acquired immune deficiency syndrome (AIDS) is characterized by the profound loss of ability to respond to environmental antigens as well as the development of Kaposi's sarcoma. Human immunovirus (HIV)-1 was identified by immunocytochemistry in skin macrophages (Langerhans' cells). Furthermore, cultured Langerhans' cells were shown to express viral particles and to infected HIV-1 negative monocytes. Major histocompatibility complex (MHC) class II antigens were found to be involved in the HIV receptor site on cells expressing these molecules. This involvement appears to be related to the proximity of HLA-DR molecules on the cell surface to the CD4 viral ligand. Monocytes were isolated from peripheral blood lymphocytes and infected with HIV-1. HIV-1 infected monocytes do not appear to be altered in their ability to present antigens (tetanus toxoid) to autologous T-cells. When an antigen is presented together with HIV-1, infection of T-cells is increased. The presence of cytotoxic lymphocytes to the HIV-transfected and -infected target cells was studied and showed low levels of activity to transfected fibroblast and higher levels to HIV-infected T-cell lines.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Section Chief	LHC	NCI
William Blattner	Section Chief	EEB	NCI
J. J. Goedert	AIDS Coordinator	EEB	NCI
Robert Gallo	Chief	LTCB	NCI
Robert Bigger	Medical Officer	EEB	NCI
Stanley H. Weiss	Medical Staff Fellow	EEB	NCI
Mikulas Popovic	Medical Officer	LTCB	NCI

Objectives:

The objective of this investigation is to study the immunology of AIDS and AIDS-related disease. These studies include investigation of the action on cell depletion, i.e., cell death, the specific receptor site for the attachment of the HIV and the mechanism of destruction of these helper cells. The studies are also directed at understanding the nature of the determinant on the retroviral envelope which attaches to the OKT4 receptor and on an investigation of the immune response of individuals who are infected with this retrovirus who do not develop AIDS or AIDS-related diseases.

Methods Employed:

Monoclonal antibodies detecting the epitopes on the T4 molecule, as well as the T8 molecules, were used to study lymphocytes from patients with AIDS and male homosexuals who are at risk for the development of AIDS, and who are either HIV seropositive or seronegative. Peripheral blood lymphocytes were obtained from these populations of individuals and cryopreserved until used. The cells were thawed and reacted with monoclonal antibodies and fluorescence-conjugated rabbit anti-mouse immunoglobulin. After appropriate periods of incubation, the cells were analyzed with the fluorescence-activated cell sorter (FACS). The percentages of total populations reacting with monoclonal antibodies were determined and analyzed in relationship to epidemiologic parameters. Four (4) mm skin punch biopsies were obtained from the buttocks of individuals who were HIV seropositive and without apparent disease, individuals with AIDS-related complex, and individuals with either opportunistic infection or Kaposi's sarcoma. The biopsies were sectioned and stained by immunohistochemical techniques for T4 and T6 cell surface antigens to determine the presence of Langerhans' cells. The presence of HIV infection was determined using monoclonal antibodies detecting the P17 HIV gag protein. Additional biopsies were fixed in the appropriate fashion and scanned by electron microscopy for the presence of virus particles and/or virus budding from cells in the biopsies. Normal peripheral blood lymphocytes were obtained and monocytes isolated by the adherence technique.

These monocytes were cultured and infected with the HIV. HIV-infected and non-infected monocytes were assessed for function of antigen presentation and induction allogeneic lymphocyte response using the standard ^{3}H thymidine technique. The cell surface antigen expression was determined on these cells with and without infection. The binding of the HIV retrovirus to T-cells was studied in the following manner: The uninfected H9 T-cell line was incubated with HIV retrovirus from several different sources (e.g., different strains) for periods of 15, 30, 60, and 120 min. The cells were washed and fixed and the cell surface antigens determined using the fluorescence-activated cell sorter. Antibodies used in these studies were directed against different epitopes on the T4 molecule as well as antibodies detecting HLA-DR, DQ, and DP.

Major Findings:

In the skin biopsies from patients with AIDS, or at risk for AIDS, and normal individuals, Langerhans' cells were easily identified using immunohistochemical techniques. These cells are characterized by the presence of the T6 marker as well as antibodies identifying with the T4 marker. HIV infection was identified in 7 of the 40 individuals who were HIV seropositive. Five positive biopsies were found in individuals who had Kaposi's sarcoma and two who had AIDS-related complex. Electron microscopy examination of these biopsies demonstrated the presence of budding virus as well as viral particles characteristic of the HIV retrovirus. Langerhans' cells were isolated from explanted tissue sections and used as a source of HIV which was captured in monocyte cultures. These studies identify a new reservoir for HIV and are of particular interest in that positive biopsies were found in AIDS patients with Kaposi's sarcoma, a malignant disease that is often present in the skin.

Studies of binding of the HIV retrovirus to human T-cells were carried out to determine the specific receptor site for the binding of HIV. Within 15 min of virus exposure, there is binding of the retrovirus to the T4 molecule as demonstrated by the absence of binding antibodies detecting the T4A epitope. In parallel with this decrease in the ability to detect T4A antigen, we also found that HLA-DR was also decreased on these cells within 15 to 30 min of viral attachment, while other products of two other loci in the HLA-D region, HLA-DP and DQ, increased or remained unchanged. Monoclonal antibodies and alloantibodies were also capable of inhibiting HIV attachment to phytohemoglutinin (PHA) stimulated T cells. While there is no evidence that a specific viral protein interacts with HLA-DR, the close proximity of the CD4 and HLA-DR molecules on the cell surface could explain the inhibition observed. Peripheral blood monocytes were studied for the expression of cell surface antigens before and after HIV infection. It was found that peripheral blood monocytes express the T4A antigenic determinants, which decreases with HIV infection. Monocytes infected with HIV were able to stimulate allogeneic T cells but were slightly less efficient than uninfected cells. Infected monocytes were also able to present tetanus toxoid to autologous T-cells like their uninfected counterparts. We have

also investigated the ability of lymphocytes from HIV-infected and -noninfected individuals to affect cellular cytotoxicity to HIV-infected T-cell lines and fibroblasts transfected a vector expressing HIV large envelope protein. Low levels of cytotoxicity were observed in the other cells when HLA class I identity was present. Higher levels of cytotoxicity was observed in HIV-infected T-cell lives which appear to be restricted by MHC class II molecules.

Publications:

Mann DL, Gartner S, LeSane F, Blattner WA, Popovic M. Cell surface major histocompatibility complex class II antigen expression is altered by HIV binding and infection. In: Bolognesi D, ed. Human retroviruses. Cancer and AIDS, approaches to prevention and therapy. New York: Alan R Liss, 1988;123-32.

Rappersberger K, Gartner S, Schenk P, Stingl G, Groh V, Tschachler E, Mann DL, Wolff K, Konrad K, Popovic M. Langerhans' cells are an actual site of HIV-1 replication. Intervirology (In Press).

Shepp DH, Daguillard F, Mann D, Quinnan GV. Human Class I MHC restricted cytotoxic T lymphocytes specific for human immunodeficiency virus envelope antigens. AIDS (In Press).

Tschachler E, Groh V, Popovic M, Mann DL, Konrad K, Safai B, Eron L, Veronese F, Wolff K, Stingl G. Epidermal Langerhans cells: A target for HTLV-III/LAV infection. J Invest Dermatol 1987;88:233-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05435-04 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Analysis of Hydrocarbon-Macromolecular Adducts in Humans and Cancer Risk

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Ainsley Weston	Visiting Associate	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Dean L. Mann	Section Chief	LHC	NCI
	Glennwood E. Trivers	Research Biologist	LHC	NCI
	Miriam C. Poirer	Research Chemist	CCTP	NCI

COOPERATING UNITS (if any)
Louisiana State Univ., Baton Rouge, LA (MJ Newman); M.R.C., Carshalton, England (P Farmer); Children's Hospital, Denver, CO (DK Manchester); M.I.T., Boston, MA (S Tannenbaum); Columbia Univ., New York, NY (RM Santella)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Biochemical Epidemiology Section

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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☐ (a1) Minors
☐ (a2) Interviews

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

Classical epidemiology and xenobiochemical studies have led to a better understanding of the genotoxic effects of environmental contaminants, for example, polycyclic aromatic hydrocarbons (PAHs) in humans. Development of assays for carcinogen exposure at the molecular level in humans has therefore been a major focus of research and from the battery of assays that are currently available, both immunological and physico-chemical approaches are being developed. Since each type of assay system clearly has its own advantages and disadvantages, the use of more than one corroborative assay has been of importance. The problems that appear to confront the development of assay systems for human biomonitoring are essentially twofold: the levels of material present in macromolecular complexes challenge the detection limits of conventional assay systems, and complex mixtures of adducted materials confound simple assay systems. Following recognition and definition of cross-reactivity profiles for antibodies raised against benzo(a)pyrene-diol-epoxide (BPDE)-modified DNA, a protocol for PAH-DNA adduct isolation that combines immunoaffinity chromatography, high performance liquid chromatography (HPLC), fluorescence spectroscopy and gas chromatography/mass spectroscopy (GC/MS) is being developed. Although this method has been successfully applied to the measurement of benzo(a)pyrene adducts in human placental DNA, studies are in progress that will attempt to apply this approach to the measurement of other classes of carcinogen-DNA adduct and in other tissues e.g., lung.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ainsley Weston	Visiting Associate	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Dean L. Mann	Section Chief	LHC	NCI
Glennwood E. Trivers	Research Biologist	LHC	NCI
Miriam C. Poirer	Research Chemist	CCTP	NCI

Objectives:

To identify and characterize PAH-DNA and PAH-protein adducts that may be detected in humans as a result of exposure to PAH contamination in the environment. To utilize the methods that have been developed for the detection of benzo(a)pyrene (BP)-hemoglobin adducts for the analysis of human peripheral blood samples. To examine the cross-reactive nature of the rabbit anti-BPDE-DNA antibody and its use in the isolation of PAH-DNA adducts by immunoaffinity chromatography and to test human sera for the presence of anti-PAH-DNA antibodies by enzyme-linked immunosorbent assay (ELISA).

Methods Employed:

Formation of PAH diol-epoxide DNA adducts was performed by dissolving the reactive hydrocarbon in tetrahydrofuran (THF):ethanol (1:24) and mixing with an aqueous solution of DNA in the ratio 1:10 (w/w). Fluorescence characterization of five PAH-DNA adducts and products of acid hydrolysis (NHCl, 90°C, 3 hr) was performed using a Perkin Elmer MFP-66 spectrophotofluorimeter interfaced with a Perkin Elmer series 7000 data station.

Competitive enzyme immunoassays, ultra-sensitive-radioimmuno assay (USERIA) and ELISA, were performed on test DNA using rabbit anti-BPDE-DNA antibody. Polyvinyl 96 well microtiter plates were coated with unmodified (control) DNA and BPDE-modified DNA (0.2 ng/well for USERIA and 1 ng/well for ELISA). Standard competitive inhibition curves were obtained by mixing serial dilutions of known standard BPDE-DNA with rabbit antisera. Percentage inhibition of the test samples was determined from the standard curves. All tests and assays were performed in triplicate, and the standard deviation was less than 10%.

Immunoaffinity columns were prepared with rabbit polyclonal anti-BPDE-DNA antibodies. To avoid potential contamination with standard materials, one column was used with ^{3}H-BPDE-DNA for calibration of the batch and the other columns were used for human DNA samples only. DNA samples were partially digested with DNAse I in order to generate oligonucleotide fragments that could be recognized by the immobilized antibodies. Up to 2 mg of digested DNA was applied to a single column at one time, and each DNA sample was loaded onto the columns with ten void

volumes of phosphate buffered saline (PBS) and eluted with ten void volumes of alkali (NaOH, 50 mM). It was necessary to repeat this cycle five times for each sample loading in order to recover all of the DNA that was applied. Columns were washed extensively with PBS between applications of DNA to achieve neutral pH. DNA fragments that were bound by the antibodies and eluted with alkali were pooled so that milligram quantities of DNA were available for solvent extraction. Putative-diol-epoxide-DNA adducts were acid hydrolyzed to the corresponding tetrahydrotetrols, extracted into isoamyl alcohol, dried in vacuo, redissolved in water and subjected to HPLC as described below.

HPLC, using reverse-phase C-18 octadecasilane (ODS) columns (4.6 x 250 mm) that were eluted with linear gradients (30-100%) of methanol in water, was used to separate the major products that are formed when mixtures of adducts are hydrolyzed with HCl (0.1 M, 90°C, 3 hr).

Synchronous fluorescence spectra (SFS) were acquired by driving the excitation and emission monochromators of a fluorescence spectrophotometer (Perkin-Elmer Corp., Rockville, MD 20850) simultaneously with a fixed wavelength difference of 34 nm. The Savitsky-Golay algorithm was used to convert zeroth order spectral data to second derivative spectra.

DNA extracts that had been subjected to HPLC/SFS, either with or without immunoaffinity chromatography, were analyzed by GC/MS under the following conditions. Samples were derivatized to tetratrimethylsilylates and analyzed by selected ion monitoring (SIM) for the presence of the molecular ion m/z 608^+, a base peak m/z 404^+ or a smaller fragment ion m/z 191^+ using splitless injection.

Major Findings:

Highly sensitive and specific methods are required to detect and quantitate carcinogen-macromolecular adducts in humans who are exposed to complex mixtures of chemical carcinogens. High performance liquid chromatography and fluorescence spectroscopy have been used successfully to detect and identify residues of benzo(a)pyrene-7,10/8,9-tetrahydrotetrol (BP-7,10/8,9-tetrol) that were released upon mild acid hydrolysis of human DNA of hemoglobin. Among a cohort of 13 coke-oven workers, levels of benz(a)pyrene-diol-epoxide-DNA (BPDE-DNA) adducts as high as 1.54 fmol BPDE/μg DNA were detected (1 adduct in 5 million nucleotides) in peripheral blood lymphocytes; these data were subsequently corroborated by gas chromatography/mass spectroscopy (GC/MS) single ion monitoring analysis (m/z 404^+). In a group of 5 lung cancer patients, 5 samples of tumor DNA were found to be negative and 1 of 4 samples of corresponding lung tissue was found to be positive.

High performance liquid chromatography in combination with synchronous fluorescence spectroscopy was used to examine 28 placentas for the presence of benzo(a)pyrene-diol-epoxide-DNA adducts, and 10 of these were found to be positive. DNA samples from these placentas were subsequently pooled and subjected to partial enzymatic digestion to oligonucleotide fragments.

Concentration of those DNA fragments containing benzo(a)pyrene-diol-epoxide-DNA adducts was achieved by immunoaffinity chromatography with polyclonal antibodies raised against these adducts. Column eluates were hydrolyzed under mild acid conditions and extracted with an organic solvent. The presence of benzo(a)-pyrene-7,10/8,9-tetrahydrotetrol residues in the extracts was determined by high performance liquid chromatography and synchronous fluorescence spectroscopy and confirmed by gas chromatography/mass spectrometry. These results unequivocally confirm bioactivation and formation of DNA adducts from benzo(a)pyrene in human placenta in vivo and establish a methodological approach to direct measurement of carcinogen-DNA adducts in humans.

Publications:

Harris CC, Weston A, Willey JC, Trivers GE, Mann DL. Biochemical and molecular epidemiology of human cancer: indicators of carcinogen exposure, DNA damage, and genetic predisposition. Environ Health Perspect 1987;75:109-19.

Haugen A, Becher G, Benestad C, Vahakangas K, Trivers GE, Newman MJ, Harris CC. Biomonitoring of individuals exposed to high levels of PAH in the work environment. In: Cooke WM, Dennis AG, eds. Polynuclear aromatic hydrocarbons. Columbus, Ohio: Battelle Press, 1988;377-92.

Newman MJ, Light BA, Weston A, Tollerude D, Clark JL, Mann DL, Harris CC. Detection and characterization of human serum antibodies to polycyclic aromatic hydrocarbon diol-epoxide DNA adducts. J Clin Invest (In Press).

Vahakangas K, Pelkonen O, Harris CC. Synchronous spectrophotometry of benzo(a)pyrene-diol-epoxide-DNA adducts - a tool for detection of in vitro and in vivo developed DNA damage by benzo(a)pyrene exposure. IARC Sci Publ (In Press).

Weston A, Newman M, Trivers GE, Vahakangas K, Rowe M, Mann DL, Harris CC. Measurement of carcinogen-macromolecular adducts and serum antibodies recognizing DNA-adducts in biological specimens from people exposed to chemical carcinogens. In: Sandhu S, DeMarini D, Mass M, Moore M, Mumford J, eds. Short-term bioassays in the analysis of complex environmental mixtures V. New York: Plenum Press, 1987;91-102.

Weston A, Rowe M, Poirier M, Trivers G, Vahakangas K, Newman M, Haugen A, Manchester D, Mann D, Harris CC. The application of immunoassays and fluorometry to the detection of polycyclic hydrocarbon-macromolecular adducts and anti-adduct antibodies in humans. Int Arch Occup Environ Health (In Press).

Weston A, Willey JC, Manchester DK, Wilson VL, Brooks BR, Choi JS, Poirer MC, Trivers GE, Newman MJ, Mann DL, Harris CC. Dosimeters of human exposure to carcinogens: polycyclic aromatic hydrocarbon-macromolecular adducts. IARC Sci Publ 1988;181-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05477-03 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Ultraviolet Light Activated Proto-Oncogenes in Human Skin Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Douglas E. Brash Senior Staff Fellow LHC NCI

COOPERATING UNITS (if any)

Department of Dermatology, Massachusetts General Hospital, Boston, MA (H. Baden)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

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☐ (a1) Minors

☐ (a2) Interviews

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

We have isolated DNA from three human skin basal and squamous cell carcinomas, a squamous cell carcinoma cell line, and several tumor cell lines carrying known oncogenes. These DNAs were analyzed by two methods. First, genomic DNAs were restriction-digested, electrophoresed, and probed with oligonucleotide probes to detect single-base mismatches in the Ki-*ras* proto-oncogene at the codon 12 region and codon 61 region. Second, these DNAs were amplified by the polymerase chain reaction (PCR) using oligonucleotide amplimers specific for regions containing Ha-*ras* codon 12, Ha-*ras* 61, Ki-*ras* 12, Ki-*ras* 61, N-*ras* 12, N-*ras* 13, and N-*ras* 61. Amplified DNA was spotted onto nylon filters and these dot blots hybridized with oligonucleotide probes under increasingly stringent conditions to examine for single-base mutations. The gel method showed that skin carcinomas 1, 2, and 3 were wild-type for Ki-*ras* 12 and 61 and for Ki-*ras* 61 at positions A and C. The more sensitive PCR/dot blot method enabled rapid screening of each activatable codon of each *ras* proto-oncogene. Skin2 hybridized to probe Ha-*ras* 12B (valine). Skin1, skin3, and SCC13 bound at lower stringency to both Ha-*ras* 12B (val) and Ki-*ras* 12A (cys). Four lung samples bound to Ki-*ras* 12A (cys). No samples bound to any Ha-*ras* 61, Ki-*ras* 61, N-*ras* 12, N-*ras* 13, or N-*ras* 61 probe. The samples appearing to be positive are being confirmed by direct DNA sequencing of PCR'd DNA. Alternative detection techniques are also being explored since ultraviolet light, unlike other agents, frequently causes double-base mutations, which would not be detected in the assay for single-base mutations.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Douglas E. Brash	Sr. Staff Fellow	LHC	NCI

Objectives:

To identify oncogenes activated in human skin tumors having a sunlight etiology and to compare the sites and types of mutations to those induced by ultraviolet (UV) light.

Methods Employed:

Genomic DNA is isolated from human tumors by crushing in liquid nitrogen, homogenizing, digesting with proteinase K plus sodium dodecyl sulfate (SDS), centrifuging on cesium chloride (CsCl) gradients, and dialyzing. The DNA is digested with EcoRI and electrophoresed on agarose gels. These gels are dried and directly hybridized with oligonucleotide probes (20 nucleotides in length) radiolabeled by kinasing. After hybridization, gels are washed at a stringent temperature chosen to dissociate the probe if there is a single-base mismatch. The gel is then autoradiographed. Alternatively, isolated DNA is amplified by the polymerase chain reaction in which the genomic DNA is incubated with 20 nt oligonucleotide amplimers, deoxynucleotide triphosphates (dNTPs), and Taq DNA polymerase; heated to denature the DNA strands, cooled to anneal the oligonucleotide amplimer primers, and incubated to allow the polymerase to extend the primers to copy the DNA. This procedure is repeated 30 times, resulting in a 10 million-fold amplification of the specific region of the DNA bounded by the two amplimers. An aliquot of this DNA is spotted onto a nylon filter, denatured, fixed to the filter by UV irradiation, and hybridized to oligonucleotide probes specific for various mutations in *ras* proto-oncogenes under conditions in which single-base mutations prevent hybridization.

Major findings:

All three basal and squamous cell skin carcinomas and the squamous cell carcinoma cell line appear to hybridize to Ha-*ras* or Ki-*ras* codon 12 probes. These preliminary results are being confirmed by direct DNA sequencing.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05479-03 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Detection of Carcinogen DNA Adducts by 32P Postlabeling

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Vincent L. Wilson	Sr. Staff Fellow	LHC	NCI
Others:	Andy Povey	Visiting Fellow	LHC	NCI
	Robert Metcalf	Medical Staff Fellow	LHC	NCI
	Dean Mann	Section Chief	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Department of Chemistry, Massachusetts Institute of Technology, Boston, MA (J.M. Essigman); The Children's Hospital, Denver, CO (D.K. Manchester); American Health Foundation, Valhalla, NY (S.S. Hecht and P. Foiles).

LAB/BRANCH

Laboratory of Human Carcinogenesis

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Biochemical Epidemiology Section

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

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

☐ (a1) Minors

☐ (a2) Interviews

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

The ability to detect low levels of carcinogen DNA adducts in the tissues of people environmentally exposed to chemical carcinogens is invaluable to epidemiological studies of the incidence of cancer in selective populations. A number of selective methodologies have been developed to quantitate carcinogen DNA adducts. The Randerath 32P-postlabelling technique provides a fingerprint analysis of only polycyclic aromatic hydrocarbon type DNA adducts. However, the basic Randerath methodology can be adapted for sensitive quantitative detection of aryl type DNA adducts in human samples. In addition, the 32P-postlabelling method has been adapted in the present study to enable the detection of small alkylation type carcinogen DNA adducts. The detection and quantitation of O6-methyl guanine (MeGua) adducts in DNA has been shown, by the use of standards, to be accurate as low as one adduct in at least 1,000,000 guanine (gua) residues. The N7-MeGua and the 8-OH Gua adducts also appear to be detectable by this methodology. The presence of unidentified 32P-labeled spots has also been observed from the analysis of DNA from cells treated with acrolein and DNA treated with fecapentaene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Andrew Povey	Visiting Fellow	LHC	NCI
Robert Metcalf	Medical Staff Fellow	LHC	NCI
Dean Mann	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To provide a sensitive physical technique for the quantitation of small DNA adducts and selective forms of base damage in DNA from people environmentally exposed to carcinogens. To establish the usefulness of the analysis of O6-MeGua and other selective adducts to the study of the relationship of carcinogen exposure to human cancer.

Methods Employed:

DNA treated with N-methyl-N-nitrosourea (MNU) or other selective direct-acting carcinogens, liver DNA from N-nitrosodimethylamine-treated rodents, and human tissue DNA from unknown environmental exposure to methylating carcinogens, will be enzymatically digested to 3'-monophosphate nucleotides. The normal and adducted nucleotides will be separated by high performance liquid chromatography (HPLC) and then labeled and quantitated by the newly adapted ^{32}P-postlabeling method (Wilson et al., Anal. Biochem. 152:275-284, 1986). The 3'-monophosphate adducted nucleotide standards must be synthesized for each alkylation product quantitated.

Major Findings:

The successful adaptation of the Randerath ^{32}P-postlabeling methodology to the study of the normal and small molecule alkylated nucleotides in nucleic acids has enabled the physical detection of O^6-alkylguanine residues in DNA. Quantitation of standard mixtures of O^6-methylguanine (O^6-meGua) and guanine by HPLC and subsequent ^{32}P-postlabeling has demonstrated the accuracy of the methodology to one adduct in 1,000,000 guanine residues. The determination of O^6-meGua levels in liver DNAs from rats treated with [^{14}C-Me]N-nitrosodimethylamine by ^{32}P-postlabeling provided results similar to those of both 14C quantitation and fluorescence methods. O^6-ethylguanine was detectable by ^{32}P-postlabeling in spiked samples to about the same limit of sensitivity as O^6-methylguanine.

The usefulness of this methodology for the identification of other alkylation (and DNA damage) products of exposure to environmentally important contaminants is under study. 8-Hydroxydeoxyguanosine 3'-monophosphate can be postlabelled and

the detection and quantitation of this damage, and N^7-alkylguanine adducts, may be performable by these techniques. DNA isolated from acrolein-treated human fibroblasts contains several unidentified adducted or damaged nucleotides not seen previously by other techniques. Fecapentaene-treated DNA contains at least four unknown products which may be separated and possibly identified by subsequent liquid chromatography/mass spectroscopy (LC/MS) analysis.

Publications:

Wilson VL, Basu AK, Essigmann JM, Smith RA, Harris CC. O^6-alkyldeoxyguanosine detection by ^{32}P-postlabelling and nucleotide chromatographic analysis. Cancer Res 1988;48:2156-61.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05480-03 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic Polymorphisms and Human Lung Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Ainsley Weston	Visiting Associate	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	James C. Willey	Biotechnology Fellow	LHC	NCI
	Dean L. Mann	Section Chief	LHC	NCI
	Haruhiko Sugimura	Visiting Fellow	LHC	NCI

COOPERATING UNITS *(if any)*
New England Medical Center, Boston, MA (T. Krontiris); Children's Hospital of LA, Los Angeles, CA (W. Benedict); National Institute of Occupational Health, Oslo, Norway (A. Haugen); University of Maryland, Baltimore, MD (B. F. Trump)

LAB/BRANCH
Laboratory of Human Carcinogenesis

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Biochemical Epidemiology Section

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☐ (a1) Minors
☐ (a2) Interviews

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

There is a clear association between smoking and lung cancer, but it is still not known why some individuals, who are heavily exposed to large concentrations of chemical carcinogens, do not develop tumors, whereas others do. These observations provide circumstantial evidence for the involvement of a genetic factor that predisposes for tumor formation. Recent restriction fragment length polymorphism (RFLP) studies have shown that the loss of normal cellular sequences from chromosome 13 (in the case of retinoblastoma), chromosome 11 (in the case of Wilms' tumor and bladder cancer and breast cancer), chromosome 1 (in the case of melanoma), chromosome 22 (in the case of acoustic neuroma) and chromosome 3 (in the case of small cell carcinoma of the lung) have been associated with malignancy. It appears that this method might be generally applied to the analysis of inherited susceptibility to cancer and may therefore be informative in risk assessment for lung cancer. Tumor and normal tissue from high molecular weight DNA samples have been collected from more than 60 cancer patients for restriction enzyme digestion and Southern analysis. Initial experiments have centered on examination of genes located on the short-arm of chromosome 11; loss of allelic fragments during tumorigenesis have been detected at the cellular Harvey ras locus, the insulin locus, the calcitonin locus, the beta-globin locus, the catalase locus and the Int-2 locus (homologous to the MMTV locus). Experiments that examine additional loci throughout the human genome for these DNA samples are in progress.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ainsley Weston	Visiting Associate	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
James C. Willey	Biotechnology Fellow	LHC	NCI
Dean L. Mann	Section Chief	LHC	NCI
Haruhiko Sugimura	Visiting Fellow	LHC	NCI

Objectives:

To examine the DNA restriction patterns of normal and lung tumor tissues with human genomic and cDNA probes. Initial studies have focused on genetic loci assigned to the short (p) arm of chromosome 11, since deletions in this region of the genome have been shown to be associated with a variety of malignant conditions. Known genetic loci throughout the human genome will also be examined to determine whether specific genetic polymorphisms are associated with carcinogenesis per se or whether deletion of genetic loci in carcinogenesis is part of a more general mechanism.

Methods Employed:

High molecular weight DNA was extracted from fresh or frozen tissues by gentle mechanical disruption of the tissue; enzyme digestion to degrade protein and RNA; phenol, phenol:chloroform extractions; alcohol precipitation and spooling high molecular weight materials.

Restriction analysis was performed by the method of Southern blotting. DNA was digested to completion with appropriate restriction enzymes, phenol extracted and subjected to agarose gel electrophoresis. The DNA was transferred (by Southern blotting) to nitrocellulose filters that were baked to immobilize the DNA. The filters were hybridized to cloned human genomic or cDNA fragments of known genetic loci, which were previously radiolabeled to high specific activity with phosphorous 32 according to the random primer method or Nick-translation. X-ray films were exposed to the filters in light-proof cassettes at -70°C for periods of between 4 hrs and 6 days.

Major Findings:

Initial studies on 70 lung cancer cases identified a high frequency of rare allelic types of the c-Ha-*ras* gene when compared to Krontiris' original control data-base. However, when non-cancer controls from the Baltimore area were assayed for the presence of rare c-Ha-*ras* alleles, it was found that although the frequency was lower, there was no statistically significant difference between

cases and controls. It has been shown now, however, that one of the rare allelomorphs (previously not described) segregates with race. A second phase case-control study is now in progress.

Constitutional loss of heterozygosity in lung cancer has been observed at every polymorphic locus studied in at least one case from a panel of more than 50 lung tumors. Analysis of polymorphic genes located on chromosomes other than 11 (3, 13, 17, 18 and 20) showed that 4/4 17p heterozygous squamous cell carcinomas but 0/8 17p heterozygous adenocarcinomas were found to have lost heterozygosity. An additional 13 tumors (either squamous of adenocarcinomas) have been added to these studies in order to confirm this striking difference. Although some loss of heterozygosity has been detected at the pH_3H_2 (3p) gene locus (Kok et al., Nature, 1987;330:585), reduction to homozygosity at this site appears to be no more prevalent than from loci on chromosome 11.

Publications:

Weston A, Willey JC, Newman MJ, Trivers GE, Haugen A, Manchester DK, Choi JS, Krontiris T, Light B, Mann DL, Harris CC. Application of biochemical and molecular techniques to the epidemiology of human lung cancer. In: Cheney J, ed. Microsomes and drug oxidations, Philadelphia: Taylor and Francis, (In Press).

Willey JC, Weston A, Haugen A, Krontiris T, Resau J, McDowell E, Trump B, Harris CC. DNA restriction fragment length polymorphism analysis of human bronchogenic carcinoma. IARC Sci Publ 1988;89:439-50.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05494-01 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Prostatic Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	M. Edward Kaighn	Expert	LHC	NCI
Others:	Roger R. Reddel	Expert	LHC	NCI
	John F. Lechner	Section Chief	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Department of Urology, Stanford Medical Center, Stanford, CA (D. M. Peehl); Department of Urology, Northwestern University Medical School, Chicago, IL (J. Kozlowski)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.6	0.35	0.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Cultures of neonatal human prostatic epithelial cells (NP-2S) near the end of their life span were transfected by strontium phosphate coprecipitation with a plasmid (pRSV-T) containing the SV40 early region genes. Colonies of transformed cells isolated from a background of senescing normal cells yielded cell lines with growth potential well beyond that of the normal cells. In all, 28 cell lines have been isolated in three separate experiments. Four lines, 267B1, 272E1, 272E4, and 272A9 which have undergone 96.3, 92.3, 30.8, and 64.7 population doublings, respectively, have been studied further and appear to be "immortalized" without having passed through a crisis. These lines contain cytokeratins and SV40 T antigen by immunofluorescence and have ultrastructural features of epithelial cells. All lines consist of mixtures of normal and aneuploid karyotypes with modal chromosome numbers in the near-diploid range (2n=46) resulting from random loss, gain and rearrangement of chromosomes. None gave tumors within one year after s.c. injection in nude mice. The transformed lines, as well as the parental NP-2S, are stimulated by TGF-beta-1 in clonal growth assays. These lines should be useful in investigating prostatic carcinogenesis and progression.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

M. Edward Kaighn	Expert	LHC	NCI
Roger R. Reddel	Expert	LHC	NCI
John F. Lechner	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Despite the increasing importance of prostatic cancer in an aging population, little is known about either the etiology or development of this disease. We, therefore, developed a culture model suitable for investigating the role of possible prostatic carcinogens and for defining molecular and genetic changes in human prostate cells during multistage carcinogenesis. The initial goal was to significantly extend the culture life span of normal epithelial cells to facilitate such studies.

Methods Employed:

Serum-free media for the culture of both normal and neoplastic prostatic epithelial cells were previously developed. Transfection of the pRSV-T plasmid by coprecipitation with strontium phosphate has been described. No selectable marker was needed since the normal cells were nearly senescent when transfected. Isolated transfectant lines were characterized by standard procedures including morphology by electron microscopy, immunofluorescence for SV40 T antigen and cytokeratins, and Giemsa banding for karyotyping. Growth was measured by standard cell counts and a clonal assay was used to assess response to growth factors. Acquisition of tumorigenic potential was monitored by injection of cells into nude mice.

Major Findings:

Work on this collaborative project, reported by the laboratory of experimental pathology (LEP) in last year's Annual Report (Z01CP05494-02 LEP) resulted in the isolation and initial characterization of the transfected lines. During this report period, it was found that (1) normal prostate epithelial cells could be immortalized by transfection with SV40 T antigen early region genes without undergoing a crisis in serum-free medium; (2) the immortalized lines, although aneuploid, have near-diploid chromosome numbers and have no consistent marker chromosomes; (3) growth of the transfected lines, as well as the parental line, is stimulated by TGF-β_1; (4) an unknown factor(s) present in bovine pituitary extract is required for growth of these lines.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05505-04 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

In Vitro Transformation of Human Bronchial Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Roger R. Reddel	Expert	LHC	NCI
Others:	Jesse Quintero	Biologist	LHC	NCI
	Louise Malan-Shibley	Chemist	LHC	NCI
	Ke Yang	Visiting Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Brenda I. Gerwin	Research Chemist	LHC	NCI
	Douglas Brash	Senior Staff Fellow	LHC	NCI

COOPERATING UNITS (if any)

Fox Chase Cancer Center, Philadelphia, PA (A. Klein-Szanto)
Medical College of Ohio, Toledo, OH (G. Stoner)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	0.75	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

Five human bronchial epithelial (BE) cell lines have been established from normal human BE cells by SV40 early region gene transfer. One additional cell line has been established following transfection of SV40 early region genes into BE cells known to contain an abnormality of the short arm of chromosome 11 from an individual who was cancer-free; the resulting cell line is tumorigenic in athymic nude mice. At least one other cell line has developed weak tumorigenicity. One of these BE lines has been the recipient of ras oncogene transfer, via retroviral infection or via strontium phosphate coprecipitation of plasmid DNA. Several different mutant ras oncogenes have resulted in malignant transformation. These cell lines, and tumor cell lines established from the nude mouse tumors, are being utilized to study aspects of multistage carcinogenesis, including chromosomal changes, progressive changes in response to inducers of squamous differentiation and the development of invasiveness.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Roger R. Reddel	Expert	LHC	NCI
Ke Yang	Visiting Fellow	LHC	NCI
Jesse Quintero	Biologist	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Louise Malan-Shibley	Chemist	LHC	NCI
Douglas Brash	Senior Staff Fellow	LHC	NCI

Objectives:

To establish cell culture models of the development of human lung cancer. In particular, the objective of this ongoing project was to develop nontumorigenic human bronchial epithelial (BE) cell lines for the systematic investigation of the relationship between differentiation and carcinogenesis and of the effects of chemical carcinogens, cloned oncogenes, and tumor oncogenes. During the past year the emphasis has been on transfer of cloned ras oncogenes into these cells, and analysis of the properties of the cells thus modified.

Methods Employed:

This project utilizes techniques developed in this laboratory, including culture of human BE cells in serum-free media and transfection of these cells with plasmid DNA via strontium phosphate coprecipitation. In addition, these cells have been infected with a recombinant retrovirus containing v-Ha-ras constructed in this laboratory by Dr. P. Amstad. Cell lines have been characterized by Southern analysis, Northern analysis, indirect immunofluorescence, Western blotting, light and electron microscopy, and isoenzyme and karyotype analysis. In vivo growth potential has been tested by subcutaneous injection into athymic nude mice and an assay of ability to re-epithelialize rat tracheas implanted in nude mice. Colony forming efficiency has been examined in the presence of agents that induce squamous differentiation in normal human BE cells.

Major Findings:

Six human BE cell lines with apparently unlimited in vitro proliferative potential have been established. One cell line, HB56B/5T, developed by transfection of SV40 early region genes into the BE cells of a cancer-free individual, was tumorigenic in nude mice. The cell population was noted to have an abnormality of chromosome 11p before transfection, and this has persisted in

the established cell line. At least one other cell line, BEAS-2B, is weakly tumorigenic, forming highly cystic tumors after a long latency period in a minority of injected animals; this cell line contains a high SV40 copy number. The relationship of tumorigenicity with chromosomal abnormalities is being studied in these cell lines.

Several mutant ras oncogenes have been transferred into BEAS-2B cells, including v-Ha-ras in a recombinant retroviral vector, v-Ki-ras both by transfection of plasmid DNA and by infection with Kirsten sarcoma virus, and the mutant c-Ha-ras from the human carcinoma cell line, EJ. These oncogenes result in the development of highly tumorigenic BE cells that are resistant to inducers of squamous differentiation, that are positive in in vitro assays of invasiveness, and form rapidly growing tumors in nude mice.

Publications:

Brash DE, Reddel RR, Quanrud M, Farrell M, Harris CC. Strontium phosphate transfection of human cells in primary culture. Cell Mol Biol 1987;7:2031-4.

Reddel RR, Ke Y, Gerwin BI, McMenamin MG, Lechner JF, Su RT, Brash DE, Park JB, Rhim JS, Harris CC. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing the SV40 early region genes. Cancer Res 1988;48:1904-9.

Patent:

Reddel RR, Ke Y, Rhim JS, Brash DE, Su RT, Lechner JF, Gerwin BI, Harris CC, Amstad P. US Patent 07/114,508: Immortalized Human Cell Lines, October 30, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05507-04 LHC

PERIOD COVERED
October 1, 1987 - September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cell Surface Antigens on Human Lung Carcinomas

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Dean L. Mann	Section Chief	LHC	NCI
Others:	George Mark	Expert	LHC	NCI
	Roger Reddel	Expert	LHC	NCI
	John Lechner	Section Chief	LHC	NCI
	Curtis Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Biochemical Epidemiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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

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

The expression of a variety of cell surface antigens was studied on normal bronchial epithelial cells, small cell lung carcinomas, mesothelial cells, mesotheliomas, normal bronchial epithelial cells, as well as fibroblasts and mesothelial cells transfected with T antigen. The monoclonal antibodies used to study these cell surface antigens defined determinants normally expressed on a variety of different cell types, mainly those cells of hematopoietic and lymphoid origin. Small cell lung carcinoma cell lines and freshly explanted tumor-expressed antigens defined by monoclonal antibodies MY4, MY7 and MY9 (myeloid differentiation antigens), as well as certain monoclonal antibodies that detect antigens usually associated with the B cells or, in some cases, epithelial cells. Cultured normal bronchial epithelial (BE) cells did not express these myeloid differentiation antigens (MY4, MY7, MY9) but these antigens are expressed when BE cells were immortalized by transfection with adeno-12, SV40 viral genes. Higher levels of these antigens were also found when these cells were transfected with and expressed raf and myc oncogenes. In addition, cells expressing both raf and myc had transient expression of cell surface MHC class II antigens. Normal mesothelial cells did not express these myeloid antigens. MY4 and MY7 but not MY9 were expressed when these cells were transfected and immortalized with SV40T.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Section Chief	LHC	NCI
Curtis Harris	Chief	LHC	NCI
John Lechner	Section Chief	LHC	NCI
Roger Reddel	Expert	LHC	NCI
George Mark	Expert	LHC	NCI

Objectives:

The objectives of these studies are to define cell surface antigens on normal and malignant cells of the lung. These studies may potentially identify a specific phenotype associated with the malignant transformation of cells from various histologic constituents of the lung that, in turn, may identify properties of malignant transformation. The function of many of these cell surface antigens detected on these cell lines is not known. They may represent receptors for growth factors or be a reflection of cell activation. In addition, these cell surface antigens may be used to select certain phenotypes which would allow a more definitive study of events involved in transformation.

Methods Employed:

Cell surface markers were examined using the fluorescence-activated cell sorter and monoclonal antibody directed against cell surface determinants. These monoclonal antibodies define specific antigens associated with particular cell types. In addition, monoclonal antibodies detecting major histocompatibility complex class I and class II antigens were used in these studies. Cell lines were maintained in tissue culture and were removed to achieve single cell suspension prior to labeling and analysis. The small cell lung carcinoma cell lines were maintained in tissue culture as suspension cells.

Major Findings:

Some antigens present on normal or leukemic cells from a B lymphocyte and/or myeloid monocytic lineages are expressed on small cell lung carcinoma cell lines. These results have led some investigators to conclude that small cell lung carcinomas and malignancies are of hematopoietic origin. We have investigated the presence of these cell surface antigens on both the small cell lung carcinoma cell lines, normal bronchial epithelial cells and other tumors of the lung. The following monoclonal antibodies were used in this study: antibodies detecting major histocompatibility complex (MHC) class I and class II antigens; monoclonal antibodies that detect antigens that are associated with B lymphocytes and epithelial cells; and antibodies that define antigens associated with monocyte and/or granulocytes.

These latter monoclonal antibodies are defined as MY4 which reacts with mature cells, and MY9 and MY7 which react with immature myeloid cells. Changes in expression of these antigens were observed where normal cells from bronchial epithelium or mesothelium were immortalized and/or transfected with oncogenes. Normal bronchial epithelial cells and mesothelia did not express these myeloid differentiation antigens. Cells immortalized by SV40 adeno 12 transfection expressed MY4, MY7 and MY9 as did these same cell lines transfected with and expressing the raf and myc oncogenes. MHC class II antigens were only expressed in the cells expressing both myc and raf; however, the expression appears to be transient depending on culture conditions. Mesothelial cells immortalized by SV40 transfection expressed only MY4 and MY7. Tumor cell lines of mesothelioma origin showed variable expression of all three antigens. The relationship of expression of these antigens to immortalization is under investigation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05508-04 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Isolation of Tumor Suppressor Genes by Subtraction Libraries

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Curtis C. Harris	Chief	LHC	NCI
Others:	Teresa A. Lehman	IRTA	LHC	NCI
	Andrea Pfeifer	Visiting Associate	LHC	NCI
	Chad Giri	Microbiologist	LHC	NCI
	John Lechner	Section Chief	LHC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Genes controlling terminal differentiation are one class of putative tumor suppressor genes. In order to investigate genes associated with terminal differentiation of epithelial cells, cDNA libraries are being constructed from a variety of epithelial cell types in various stages of differentiation. By using the method of subtraction cloning, messages expressed preferentially in one cell type or in a certain stage of differentiation may be examined. Furthermore, effects of inducers of differentiation upon gene expression can be examined as epithelial cells progress from an undifferentiated state to a terminally differentiated state.

Normal human bronchial epithelial cells (NHBE) can be induced to terminal differentiation in vitro by treatment with a variety of agents such as transforming growth factor (TGF)-beta-1, calcium, 12-0-tetradecanoylphorbol-13-acetate (TPA) and fetal bovine serum. To investigate differences in gene expression of undifferentiated NHBE cells and differentiated NHBE, messenger RNA is isolated from both cell types and used to produce two cDNA libraries. From these two libraries, RNA is synthesized in vitro making sense strand RNA from one type and anti-sense strand RNA from the other type. The sense and anti-sense RNAs are then hybridized, and the unique single-stranded sequences are removed from the common double-stranded sequences. By this subtraction protocol, unique messages expressed in differentiating cells can be used as probes to investigate the clones from which they were synthesized.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

Teresa A. Lehman	IRTA	LHC	NCI
Andrea Pfeifer	Visiting Associate	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
John Lechner	Section Chief	LHC	NCI

Objectives:

Terminal differentiation of cells is the mechanism involved in the senescence of cells. Certain genes are believed to be responsible for this differentiation, although their structure and function are not well defined. The purpose of this study is to identify and investigate genes which initiate and maintain the progression of cells to a state of terminal differentiation.

Methods Employed:

In order to investigate genes associated with terminal differentiation of epithelial cells, cDNA libraries are being constructed from a variety of epithelial cell types in various stages of differentiation. By using the method of subtraction cloning, messages expressed preferentially in one cell type or in a certain stage of differentiation may be examined. Furthermore, effects of inducers of differentiation upon gene expression can be examined as epithelial cells progress from an undifferentiated state to a terminally differentiated state.

Normal human bronchial epithelial cells (NHBE) can be induced to terminal differentiation in vitro by treatment with a variety of agents such as TGF-β_1, calcium, TPA and fetal bovine serum. To investigate differences in gene expression of undifferentiated NHBE cells and differentiated NHBE, messenger RNA is isolated from both cell types and used to produce two cDNA libraries. From these two libraries, RNA is synthesized in vitro making sense strand RNA from one type and anti-sense strand RNA from the other type. The sense and anti-sense RNAs are then hybridized, and the unique single-stranded sequences are removed from the common double-stranded sequences. By this subtraction protocol, unique messages expressed in differentiating cells can be used as probes to investigate the clones from which they were synthesized.

Major Findings:

In order to obtain sufficient quantities of near full-length RNA for the subtraction protocol, we developed an in vitro transcription system to produce mRNA from the libraries created from various cell types. The cloning vector used

to create the libraries was constructed by vector-primed cDNA synthesis based on the Okayama-Berg protocol. The basic cloning vehicle is a modified pGEM 4 plasmid which contains both an SP6 RNA polymerase promoter and a T7 RNA polymerase promoter oriented in opposite directions and separated by a multiple cloning site for insertion of cDNA. Vector-primer and linker molecules were constructed according to the Okayama-Berg protocol, and mRNA isolated from each cell type was used to create the cDNA library in the modified pGEM 4 plasmid. Two of the restriction sites within the multiple clone site were converted to 8-base rare-cutting restriction sites to ensure minimal cutting of internal sites of the inserted sequences during in vitro synthesis. To obtain in vitro synthesized RNA from the new constructs, the plasmid is linearized using the rare-cutter enzyme and the appropriate promoter site is utilized to make a full length RNA from the cDNA. The opposite orientation of the promoters allows synthesis of sense RNA from one promoter and cell type and anti-sense RNA from the other promoter and cell type. The sense and anti-sense strands can be distinguished from one another either by radioactive labelling or by incorporation of biotinylated uridine triphosphate (UTP) in one set of transcripts. Following hybridization of the sense and anti-sense RNAs to a high R_0t, the unique single-stranded RNA can be recovered either by column chromatography using cellulose for radioactive RNA or by affinity chromatography of biotin for streptavidin agarose. This unique RNA can be used to screen the initial libraries and allow further investigation of genes involved in terminal differentiation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05509-04 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Detection of Proto-Oncogene Mutations in Tumors

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Douglas E. Brash	Senior Staff Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (*if any*)
Shanghai Cancer Institute, People's Republic of China (Dr. Gu Jian-Ren)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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

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

We have isolated DNA from 6 human lung tumor cell lines, 20 human lung adenocarcinoma and squamous cell carcinomas, and 5 normal lung tissues corresponding to 5 of the lung carcinomas. These DNAs were analyzed by two methods. First, genomic DNAs were restriction-digested, electrophoresed, and probed with oligonucleotide probes to detect single-base mismatches in the Ki-*ras* proto-oncogene at the codon 12 region and codon 61 region. Second, these DNAs were amplified by the polymerase chain reaction (PCR) using oligonucleotide amplimers specific for regions containing Ha-*ras* codon 12, Ha-*ras* 61, Ki-*ras* 12, Ki-*ras* 61, N-*ras* 12, N-*ras* 13, and N-*ras* 61. Amplified DNA was spotted onto nylon filters and these dot blots hybridized with oligonucleotide probes under increasingly stringent conditions to examine them for single-base mutations.

The gel method confirmed previous identification of Ki-*ras* 12A mutations in CaLu-1 and A549 cell lines and Ki-*ras* 12B mutations in A427. It revealed that A549 does not carry a normal Ki-*ras* 12 allele and that cell line SW900 has a mutation at Ki-*ras* 12B. Cell lines A1146 and HuT 292 are wild-type for Ki-*ras* 12 and 61 and for Ki-*ras* 61 at positions A and C.

The more sensitive PCR/dot blot method enabled rapid screening of each activatable codon of each *ras* proto-oncogene. Each of the above results obtained with gels was confirmed. In addition, it was found that A1146 did not hybridize to any Ha-*ras* 12 probe, suggesting a deletion or double-base mutation at Ha-*ras* 12. HuT 292 hybridized to probe H12Biii (valine). SW900 bound specifically to probe Ki-*ras* 12Biii (valine, G to T substitution). Four lung samples bound to Ki-*ras* 12Aii (cys). No samples bound to any Ha-*ras* 61, Ki-*ras* 61, N-*ras* 12, N-*ras* 13, or N-*ras* 61 probe. The samples appearing to be positive are being confirmed by direct DNA sequencing of PCR'd DNA.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Douglas E. Brash	Sr. Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To identify oncogenes activated in human lung tumors.

Methods Employed:

Genomic DNA is isolated from human tumors by crushing in liquid nitrogen, homogenizing, digesting with proteinase K plus sodium dodecyl sulfate (SDS), centrifuging on cesium chloride (CsCl) gradients, and dialyzing. The DNA is digested with EcoRI and electrophoresed on agarose gels. These gels are dried and directly hybridized with oligonucleotide probes (20 nucleotides in length) radiolabeled by kinasing. After hybridization, gels are washed at a stringent temperature chosen to dissociate the probe if there is a single-base mismatch. The gel is then autoradiographed. Alternatively, isolated DNA is amplified by the polymerase chain reaction in which the genomic DNA is incubated with 20 nt oligonucleotide amplimers, deoxynucleotide triphosphates (dNTPs), and Taq DNA polymerase; heated to denature the DNA strands, cooled to anneal the oligonucleotide amplimer primers, and incubated to allow the polymerase to extend the primers to copy the DNA. This procedure is repeated 30 times, resulting in a 10 million-fold amplification of the specific region of the DNA bounded by the two amplimers. An aliquot of this DNA is spotted onto a nylon filter, denatured, fixed to the filter by ultraviolet (UV) irradiation, and hybridized to oligonucleotide probes specific for various mutations in ras proto-oncogenes under conditions in which single-base mutations prevent hybridization.

Major findings:

SW900 carries a Ki-ras 12B mutation (valine, G to T substitution).

A1146 carries a Ha-ras 12 deletion or double base mutation.

Several lung tumors appear to carry Ki-ras 12A (cys) mutations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05540-01 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Multiple Drug Resistance Gene Expression in Mesothelioma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Brenda I. Gerwin	Research Chemist	LHC	NCI
Others:	Michael Gottesman	Section Chief	LMB	NCI
	John Lechner	Section Chief	LHC	NCI

COOPERATING UNITS *(if any)*

Institute of Occupational Health, Helsinki, Finland (K. Linnainmaa)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.15	0.15

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Malignant mesothelioma is a tumor which is extremely recalcitrant to treatment by chemotherapeutic agents. It was of interest to test mesothelioma cell lines for expression of the multiple drug resistance (MDR) gene. If this gene were expressed at a high level in the mesothelioma cells, its action might be expected to be at the basis of the resistance of this tumor type to chemotherapy. RNA was prepared and tested from 16 human mesothelioma cell lines, 2 normal human mesothelial cells, and 3 normal human mesothelial cells immortalized by the SV40 T antigen. Of all samples tested only 2 were positive for expression of the MDR gene. We conclude that the MDR gene product is not the protein of importance for the resistance to chemotherapeutic agents shown by human mesothelioma. Therefore, strategies which would specifically inactivate or reverse the action of this gene product are not relevant to the mesothelioma therapeutic dilemma.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Brenda I. Gerwin	Research Chemist	LHC	NCI
Michael Gottesman	Section Chief	LMB	NCI
John F. Lechner	Section Chief	LHC	NCI

Objectives:

The goal of this project is to determine whether the resistance of human mesothelioma to chemotherapy is mediated by the action of the MDR gene product.

Methods Employed:

Normal mesothelial cells, T antigen-immortalized normal human mesothelial cells and malignant mesothelioma cell lines are grown in tissue culture. Whole cell RNA is prepared from these cells and analyzed for gene expression by Northern blotting and hybridization to specific probes. Slot blotting against standard cell lines which express high, low or negative levels of the MDR gene mRNA is utilized for quantitation of the MDR signal against a constitutive probe which serves as an internal standard for the amount of RNA used.

Major Findings:

RNA was prepared from 16 human mesothelioma cell lines, 3 SV40 T antigen transformed normal human mesothelial cells, and 2 primary normal human mesothelial cells. These samples were compared, in quantitative slot blot assays to an established negative control cell (KB31) and a cell which is induced to express high levels of MDR mRNA (KB85). The negative control level was subtracted from that of other samples to provide a non-specific hybridization control. Of the cells tested, most mesothelioma cell lines (15/16) showed a very low level of MDR expression as did the normal human mesothelial cells and 2 of the 3 T antigen-transformed normal human mesothelial cells. These results indicate that the MDR gene product is not responsible for the resistance of human mesotheliomas to chemotherapeutic protocols. Furthermore, these results suggest that therapies which seek to specifically inactivate or repress the MDR gene and its product are not likely to succeed in the treatment of human malignant mesothelioma.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05541-01 LHC

PERIOD COVERED

October 1, 1987 to September 1988

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

Growth of Human Hepatocytes

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

P.I.:	John F. Lechner	Section Chief	LHC	NCI
Others:	Katherine E. Cole	Special Volunteer	LHC	NCI
	Roger Reddel	Expert	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (*if any*)

Collaborative Research, Bedford, MA (R. DiPaolo)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.25	0.75

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Serum-free medium composed of a modified Ham's F-12 medium was found to support the long-term multiplication of human epithelial liver cells. These epithelial cells were positive for general cytokeratin expression as well as positive for cytokeratin 18 expression through four passages. In addition, human hepatocytes in primary culture transfected with the SV40 large T antigen gene formed foci within 6-8 weeks that were positive for both keratin and large T antigen expression. Conditioned medium from cultures of the transfected human liver cells was shown to cause a 30% increase in DNA synthesis of a malignant human liver cell line (HepG2).

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Katherine E. Cole	Special Volunteer	LHC	NCI
John F. Lechner	Section Chief	LHC	NCI
Roger Reddel	Expert	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To establish a replicative culture of human hepatocytes through the development of optimal medium formulations. To characterize the proliferating cells for specific markers of differentiated hepatocytes. Additionally, studies are directed towards the development and subsequent characterization of a transformed human liver epithelial cell line through transfection of human hepatocytes in primary culture with SV40 T antigen DNA. The normal and transformed cell lines will be used to study metabolism and possible sensitivity to known carcinogens.

Methods Employed:

In order to optimize factors responsible for human liver cell proliferation, studies using a malignant human liver cell line (HepG2) as a model have been employed. Known growth factors, as well as unknown growth factors, contained in conditioned medium from normal and transfected human liver cells are evaluated using an assay for DNA synthesis of the HcpG2 cells. Morphology of the replicating human liver cells is evaluated by light and electron microscopy. Markers for epithelial cells are identified using fluorescent staining techniques. Markers specific for differentiated hepatocytes are examined using both fluorescent staining and avidin-biotin-alkaline phosphatase immunocytological staining. Human hepatocytes in primary culture are transfected with recombinant plasmids containing the SV40 large T antigen gene linked to the Rous sarcoma virus (RSV) long terminal repeat (LTR) pRSV-T using strontium chloride in serum-free medium. The loci formed following transfection are evaluated for keratin and T antigen expression using a double-labelled fluorescence technique; specific hepatocyte markers are found using fluorescence or avidin-biotin-alkaline phosphatase immunocytochemical staining. Secretion of hepatocyte-specific antigens are evaluated for normal and transfected liver cells using a modification of the RBC-lysis plaque assay technique.

Major Findings:

This project has led to two new findings: (1) Development of a serum-free medium that supports the long-term multiplication of human liver epithelial cells. This medium is a modification of Ham's F-12 medium supplemented with fatty acids,

insulin, epidermal growth factor (EGF), hydrocortisone, cholera toxin, 0.25% aqueous pituitary abstract and 10% chemically denatured serum. The human liver cells cultured with this medium have been shown to contain general cytokeratins, as well as cytokeratin 18, through four passages; (2) Development of transfected human liver epithelial cells shown to express keratin and large T antigen. During the past year we have obtained knowledge concerning the factors necessary to induce normal human hepatocytes to divide in vitro. These proliferating liver cells contain keratin and display an epithelial-appearing morphology. Additionally, human hepatocytes transfected with SV40 large T antigen DNA have grown very rapidly in medium similar to that used for the normal liver epithelial cells. However, medium conditioned by these transfected human liver cells has been shown to increase DNA synthesis of malignant liver cells (HepG2), while conditioned medium from the normally dividing liver cells does not.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05542-01 LHC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Assessment of Tobacco Smoke Genotoxicity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Curtis C. Harris	Chief	LHC	NCI
Others:	James C. Willey	Biotech. Fellow	LHC	NCI
	Norio Matsukura	Special Volunteer	LHC	NCI

COOPERATING UNITS (if any)

Eleanor Roosevelt Research Institute, Denver, CO (C. Waldren and T. Puck); Nippon Medical School, Tokyo, Japan (M. Miyashita)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Cigarette smoke is carcinogenic in animals, and most of this carcinogenic property is recovered in non-polar subfractions of the neutral fraction. Although cigarette smoke condensate is mutagenic following activation by metabolic enzymes found in microsomes or the S9 fraction, it has not been reported to act as a direct mutagen in systems used thus far. In addition, the fractions determined to be most carcinogenic have not been found to be the most mutagenic. We have employed Chinese hamster ovary (CHO) cells containing a human chromosome 11 (termed AL hybrid cells) as a more sensitive detection system for mutagenesis in an effort to better define the direct and indirect mutagenic properties of cigarette smoke condensate and its fractions. The inhibitory dose (ID)50 value for N-methyl-N-nitro-N-nitrosoguanidine (MNNG) (0.2 ug/ml) in medium containing 8% fetal calf serum (FCS) the known direct mutagen was similar to previous reported values. The ID50 for MNNG in medium lacking FCS was 0.6 ug/ml in two separate experiments (N=8). At this concentration, there was a 300% increase ($p<0.0005$) in survivors following incubation with the a1 antibody and complement. The ID50 for cigarette smoke condensate (CSC) in the absence of serum is about 45 ug/ml. CSC is not significantly mutagenic at the ID50 concentration, but causes a 60% increase ($p<0.02$) in survivors (presumably mutated) at 60 ug/ml and a 290% increase ($p<0.0005$) at 90 ug/ml. We have conducted dose response experiments with CSC fractions and have determined ID50s for the acid, neutral and basic fractions of 5, 60 and 250 ug/ml, respectively. The presence of 8% fetal calf serum in the medium reduces the ID50 of CSC and of each fraction to 130, 30, 50 and 360 ug/ml, respectively.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Curtis C. Harris	Chief	LHC	NCI
James C. Willey	Biotechnology Fellow	LHC	NCI
Norio Matsukura	Special Volunteer	LHC	NCI

Objectives:

To study effects of cigarette smoke and cigarette smoke fractions on human chromosomal genes. The marker genes used in this system are present on human chromosome 11, even though the chromosome 11 resides in a CHO hybrid cell. Because the CHO cell does not require the human marker chromosome for normal cell division, mutations that might not be detectable in other systems due to lethality, such as large deletions or rearrangements, will be scored using this system.

To evaluate the significance of direct mutagenicity resulting from CSC. Due to the increased sensitivity of this assay, it may be possible to detect direct mutagenicity that is not detectable by other assays.

To determine what types of mutations are occurring most commonly in response to CSC, i.e., deletions, rearrangements, or point mutations.

To investigate the lack of correlation between mutagenicity (tested by the Ames Salmonella assay) and carcinogenicity (animal studies) caused by cigarette smoke condensate and its fractions. For instance, while the neutral fraction of CSC is the most carcinogenic, it is poorly mutagenic, even after activation; and while the weakly acidic fraction is the most mutagenic, it is weakly carcinogenic.

Methods Employed:

The mutagenesis assay employed has been described by Puck et al. It involves Chinese hamster ovary cells that have acquired a human chromosome 11 through somatic cell hybridization. There are three surface antigen genes on chromosome 11, termed a1, a2, and a3. Cells that acquire a mutation in one of these genes looses the corresponding cell surface marker. If a population of wild-type and mutated cells is then exposed to antibodies to the surface marker in the presence of complement, only those cells that possess a mutated gene and are unable to produce the surface antigen will survive. The hybrid cells are routinely cultured in medium F-12 with 8 % FCS. Cells are seeded at 10^5 cells/60 mm dish; the next day cells are incubated with medium containing the test compound with or

without 8% FCS for 3 hr. When the cells are pre-confluent, they are dissociated and seeded at 5 x 10^4 cells/dish. Cells are then incubated in medium containing antibody and complement. Six to seven days later the cells are fixed and stained.

Major Findings:

The ID_{50} for MNNG in the presence of serum (0.2 µg/ml) was similar to values reported previously using this system. The ID_{50} for MNNG in the absence of serum was 0.6 µg/ml and at this concentration there was a 300% increase in surviving cells.

CSC contains materials that lead to direct mutagenesis in A_L cells.

The cytotoxicity of CSC fractions varies, with the acidic fraction being the most cytotoxic (ID_{50} = 4 µg/ml)

The cytotoxicity and mutagenicity of CSC and fractions is decreased in the presence of 8% fetal calf serum.

Publications:

Gabrielson EW, Rosen GM, Grafstrom RC, Strauss KE, Miyashita M, Harris CC. Role of oxygen radicals in 12-O-tetradecanoylphorbol-13-acetate-induced squamous differentiation of cultured normal human bronchial epithelial cells. Cancer Res 1988;48:822-5.

Grafstrom RC, Dypbukt JM, Willey JC, Sundqvist K, Edman C, Atzori L, Harris CC. Pathobiological effects of acrolein in cultured human bronchial epithelial cells. Cancer Res 1988;48:1717-21.

Grafstrom RC, Sundqvist K, Dypbukt JM, Harris CC. Pathobiological effects of aldehydes in cultured human bronchial cells. In: Bartsch H, O'Neill I, Schulte-Hermann R, eds. Relevance of N-nitroso compounds to human cancer, UK: Oxford University Press, 1987;443-5.

Harris CC. Biochemical and molecular effects of N-nitroso compounds in cultured human cells: an overview. IARC Sci Publ 1987;20-5.

Harris CC, Trump BF. Laboratory and epidemiological studies of human carcinogenesis. In: Rand MJ, Raper, C, eds. Pharmacology, Amsterdam: Elsevier Biomedical, 1987;845-52.

Willey JC, Grafstrom RC, Moser CE Jr, Ozanne C, Sundqvist K, Harris CC. Biochemical and morphological effects of cigarette smoke condensate and its fractions on normal human bronchial epithelial cells in vitro. Cancer Res 1987;47:2045-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05543-01 LHC

PERIOD COVERED

October 1, 1987 to December 31, 1987

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

Tumor Suppression and Somatic Cell Genetics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Edward Kaighn	Expert	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Francis Scott Key Medical Center, Baltimore, MD (E. Gabrielson)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.75	0.75

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The specific goal of this project is to determine whether normal human cells contain genes that have the ability to suppress tumorigenicity of human lung carcinoma cell lines. Somatic cell hybrids between a lung carcinoma line, HuT292-DM, and BEAS-2B, an immortalized, but non-tumorigenic human bronchial epithelial line, have been generated by fusion with polyethylene glycol (PEG), followed by double selection in hypoxanthine/aminopterin/thymidine (HAT) medium containing ouabain. The properties of these hybrid lines are being studied. All lines have near triploid chromosome numbers, whereas the parental lines are near diploid. Serum induces BEAS-2B to terminally differentiate, but stimulates the growth of the carcinoma line. The hybrids vary in their response to serum. Some are unaffected, some are stimulated, but none are inhibited. The tumorigenicity of these lines as compared to their progenitors is now being tested in nude, athymic mice.

PHS 6040 (Rev. 1/84) GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

M. Edward Kaighn	Expert	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The overall goal is to identify, isolate and study the mechanism of action of putative tumor suppressor sequences present in normal bronchial epithelial cells but absent from tumor cells. Specifically, somatic cell hybrids are generated by fusion of lung tumor lines with normal or immortalized bronchial epithelial lines. Tumorigenicity of the resultant hybrids in nude mice is assessed to determine whether the suppressor activity is expressed. Other characteristics of the hybrids, e.g., morphology, growth behavior, response to growth factors and growth inhibitors, and karyotype are studied with a view to establishing correlates with tumorigenicity.

Methods Employed:

Media and methodology for the culture of both normal and neoplastic bronchial epithelial cell lines were previously developed in this laboratory. Initial fusions have been limited to crosses between (1) lung carcinoma (HuT292-DM, HPRT-, ouabain resistant) and BEAS-2B, a non-tumorigenic, immortalized bronchial epithelial line; and (2) B39-TL, a mouse tumor line resulting from injection of late passage BEAS-2B. Fusions are carried out both in monolayer and in suspension by treatment with polyethylene glycol. One day later, selective medium is added (LHC-8 w\o adenine + HAT + ouabain). After four weeks, non-selective medium (LHC-8) is used. Hybrid lines are isolated, expanded and stored frozen. Hybrid lines are characterized by standard procedures. Giemsa banding is used for karyotyping. Growth is measured by standard cell counts. A clonal assay is used to assess response to growth factors. Acquisition of tumorigenic potential is monitored by injection of cells into nude mice.

Major Findings:

Hybrid lines have been isolated from both crosses, 11 from HuT292-DM x BEAS-2B and 6 from HuT292-DM x B39-TL. Preliminary chromosome counts on 7 of these lines show that all are near-triploid, whereas the parental lines are near-diploid.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05544-01 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Proto-oncogene Expression in Normal Human Bronchial Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	Tohru Masui	Visiting Associate	LHC	NCI
Others:	Brenda I. Gerwin	Research Chemist	LHC	NCI
	John F. Lechner	Section Chief	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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 (Use standard unreduced type. Do not exceed the space provided.)

Transforming growth factor beta-1 (TGF-beta-1) and epinephrine have opposing effects on normal human bronchial epithelial (NHBE) cells. While TGF-beta-1 induces squamous differentiation in NHBE cells, epinephrine promotes their growth and neutralizes the effect of TGF-beta-1. To investigate mechanisms which might be involved in their antagonism, we examined steady state expression levels of proto-oncogenes c-*myc*, c-*fos*, and c-Ha-*ras* in response to treatment with these two agents. Expression of specific mRNA was detected by Northern blotting and normalized by the constitutive probe, glyceraldehyde-3-phosphate-dehydrogenase. Expression of c-*myc* was transiently inhibited by TGF-beta-1 up to 40% at 1 hr. Epinephrine induced the expression of c-myc about two- to threefold and neutralized the effect of TGF-beta-1 on c-*myc* mRNA level. Expression of c-*fos* was transiently induced by either TGF-beta-1 or epinephrine up to 1.5 or two- to threefold at 1 hr, respectively. These two agents synergistically induced c-*fos* up to 5 fold. The c-Ha-*ras* mRNA level was not altered by TGF-beta-1 or epinephrine treatments. While the initial changes in c-*myc* expression correlated with the proliferative activity of the cells, the steady state level of c-*fos* mRNA did not.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Tohru Masui	Visiting Associate	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
John F. Lechner	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To study molecular mechanisms involved in differentiation induction by TGF-β_1 and prevention by epinephrine on NHBE cells in vitro through measuring changes in proto-oncogene steady state levels, because proto-oncogenes are implicated to be responsible for growth and differentiation of cells.

Methods Employed:

NHBE cells were prepared from the outgrowths of normal human tissues and cultures were maintained in the serum-free medium developed in our laboratory. Total RNA from the cells was prepared by a combination of a guanidine thiocyanate lysis and a cesium density gradient. Steady-state levels of mRNA expression were analyzed by probing Northern blots with radiolabelled c-*fos*, c-*myc*, and c-Ha-*ras* DNA probes. A series of measurements were done on the same membrane after stripping the previous probes and data were normalized by using expression levels of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as an internal control. Quantitation was achieved with a Shimadzu CS930 densitometer and the data were expressed as a percent of the control value at each time point.

Major Findings:

The pursuit of this project has led to major new findings: (1) TGF-β_1 and epinephrine increased steady state levels of c-*fos* mRNA and these two agents synergized in the induction of c-*fos* mRNA levels; (2) c-*myc* mRNA steady state levels were decreased transiently by exposure to TGF-β_1 in a dose-dependent manner. Though TGF-β_1 inhibited growth of NHBE cells irreversibly, c-myc mRNA levels returned to control levels after 9 hr of exposure to TGF-β_1; (3) epinephrine by itself enhanced growth of NHBE cells and induced high levels of c-*myc* mRNA for at least 24 hr. Epinephrine antagonized the negative effect of TGF-β_1 on growth of NHBE cells and also blocked the early decrease in c-*myc* mRNA levels; (4) the steady state levels of c-Ha-*ras* mRNA were not influenced by TGF-β_1 and/or epinephrine.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05545-01 LHC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Activated Proto-oncogenes on Human Bronchial Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Tohru Masui	Visiting Associate	LHC	NCI
Others:	M. Edward Kaighn	Expert	LHC	NCI
	Rossella Rolfini	Special Volunteer	LHC	NCI
	John F. Lechner	Section Chief	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

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

Because of the known association of activated oncogenes with human lung cancers, the expected roles of these genes in the multiple steps leading to metastatic cancer and the possible linkage between phenotypic alterations and oncogene activation in carcinomas, it is important to define these relationships. The role of transfected oncogenes on normal human bronchial epithelial cells (NHBE) is being investigated using assays that measure transient alterations of expression of the differentiated phenotype. Cloned activated oncogenes are introduced into NHBE cells by strontium phosphate transfection. "Immortalization" or escape from senescence is evidenced by the appearance of transformed colonies. Since this process is lengthy, direct effects of exogenous genes may be difficult to detect. Therefore, the transient assay approach is used to assess direct effects of oncogenes in the short term. Preliminary results indicate that HVV-*fos*, a chimeric construct of human c-*fos*(human)-1 and a viral 3' long terminal repeat (LTR), induces serum resistance and extends the life span of NHBE cells. Use of the short-term assay will facilitate study of the action and interaction of activated oncogenes during multistage lung carcinogenesis and progression.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Tohru Masui	Visiting Associate	LHC	NCI
M. Edward Kaighn	Expert	LHC	NCI
Rossella Rolfini	Special Volunteer	LHC	NCI
John F. Lechner	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The major goal of this project is to assess the role of oncogene activation in human lung carcinogenesis. Transfection of activated oncogenes provides a convenient means of investigating their direct effects in the NHBE phenotype. Both short- and long-term effects are being investigated. The sensitivity of transfected cultures to known inducers of differentiation is measured by transient effects on incorporation of tritiated thymidine. Long-term effects by integrated oncogenes are easily seen by the development of transformed colonies that have escaped from senescence.

Methods Employed:

NHBE cells were prepared from outgrowths of normal human bronchus cultured in the serum-free media developed in this laboratory. Selected cloned oncogenes under the control of various promoters are introduced into NHBE cells by transfection with strontium phosphate. The influence of differentiation inducers on DNA synthesis is measured by pulse labelling with tritiated thymidine at successive times after transfection (2-4 days). Longer term effects are measured by isolating, expanding and assaying colonies that escape from senescence. Both clonal growth assays and DNA synthesis are used for this purpose.

Major Findings:

Preliminary work on this project has led to new findings: (1) HVV-*fos*, a chimeric construct of c-*fos*(human)-1 and a viral 3' fragment with a LTR, extended the culture lifespan of NHBE cells for more than two passages (10-12 population doublings) beyond that of control NHBE cells; (2) HVV-*fos* induced serum resistance in NHBE cells; (3) the control pBR322 plasmid did not induce resistance to serum as assayed by thymidine pulse labelling in the short term.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05192-08 LHC, Z01CP05324-06 LHC, Z01CP05325-06 LHC, Z01CP05341-06 LHC, Z01CP05403-05 LHC, Z01CP05409-05 LHC, Z01CP05426-04 LHC, Z01CP05432-04 LHC, Z01CP05435-04 LHC, Z01CP05477-03 LHC, Z01CP05479-03 LHC, Z01CP05480-03 LHC, Z01CP05507-04 LHC, Z01CP05540-01 LHC, Z01CP05541-01 LHC, Z01CP05544-01 LHC and Z01CP05545-01 LHC

UNIVERSITY OF MARYLAND (N01-CP-51000)

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Current Annual Level: $491,667

Man Years: 6.65

Objectives: To provide a resource to the LHC for the procurement, transport, and characterization of normal, preneoplastic, and neoplastic human bronchus, pancreatic duct, colon, and liver from patients with an epidemiological profile.

Major Contributions:

1. Collection of Tissues

Tissue specimens were collected from a total of 254 cases including surgery patients (134) with and without cancer, cardiothoracic (60) and colonic (74) patients, and at time of autopsy (120). Autopsy specimens are collected from noncancer patients subjected to either immediate autopsy (8) (i.e. within 60 min after death) or routine autopsy (112) (i.e., between 2 and 12 hr after death). Tissues received at the NIH are residuals of materials taken for regular diagnostic and corrective purposes and not for research per se.

A. Surgical Specimens

A total of 120 surgeries resulted in tissue donations.

Bronchus: Tumor and noninvolved tissues were collected from 60 cases of lung carcinoma. Bronchial specimens uninvolved with tumor were provided from all (60) of these cases and transported to the NIH. The tumors were defined and classified as described below.

Colon: Tumor tissues were collected from 112 cases of colon carcinoma. Colonic tissue uninvolved with tumor was available from most of these. All of the tumors were defined and classified as described below.

B. Autopsy Specimens

Immediate Autopsy: There were eight immediate autopsies. The specimens collected are shown below.

Organ	Number of Specimens
Colon	8
Bronchus with lung attached	8
Pancreas	4
Liver	4 (8 for cell collections)

Tissues required for pathological examination and assessment of viability were retained by the contractor. The major portions of the specimens were received at the NIH. The 8 liver cell collections had viability of 40%-80% and 89 vials of liver cells are now stored at -70°C in the contractor's facility; 25 vials of liver cells isolated in 1986 and 10 vials frozen from an immediate autopsy (IA) in August were received by the In Vitro Carcinogenesis Section in ice-chilled L-15 medium.

Routine Autopsy: The numbers of specimens collected from 78 routine autopsies and other procedures (pleural fluids are a lucrative source of mesothelial cells for asbestos studies) were as listed below:

Organ	Number of Specimens
Bronchus	79
Pleural Fluids	242
Pulmonary arteries	105

2. Viability Evaluation

Bronchus: Small pieces of nontumorous bronchial epithelium were successfully grown in explant cultures from 45 of the 79 cases from intermediate autopsy. All of the cases were viable, based upon morphology, LDH enzyme release, and cellular outgrowth measurements. No IA cases were tested in this manner.

Liver: Cellular preparations from an immediate autopsy in August were implanted into four nude mice. The transplanted cells produced no observable growth and no cells were found when the animals were autopsied. Cells were stored by the contractor and are available upon request for shipment to the NIH.

Pancreas: Pancreatic epithelia were tested in explant organ cultures as described for bronchus. The observed growth was slower and produced much lower densities of outgrowth layers.

3. Definition and Classification of Nonneoplastic and Neoplastic Tissue

Epidemiologic data are provided to allow the study of relationships between tumor type, selected risk factors and the amount of benzo(a)pyrene (BP) or aflatoxin B_1 (AFB), respectively (e.g., in lung cancer and hepatoma), bound to DNA by the same patient's noncancerous epithelium.

Bronchus: Morphological and histochemical characterization of human primary lung carcinomas are routine. The characterization with immunocytochemistry is by the peroxidase-antiperoxidase method to demonstrate various antigens. Tissues are examined for beta human chorionic gonadotropin (HCG-beta), calcitonin, adrenocorticotropic hormone (ACTH), serotonin, alphafetoprotein (AFP), keratin, somatostatin, neuron-specific enolase (NSE), calmodulin, and tubulin.

Normal and abnormal (but non-neoplastic) adult bronchi contain only muco-substances, keratin, calmodulin, and tubulin. Keratin is seen in normal bronchial epithelium. Calmodulin increases at cell borders.

Lung tumors were examined for a series of immunochemically defined markers. Each was found occasionally in tumors (HCG and keratin in 80% and 75% of non-small cell tumors; somatostatin in keratinizing tissues and a small number of adeno-carcinomas; NSE and serotonin only in endocrine tumors with dense-cored granules). Less common markers (ACTH, somatostatin, calcitonin, HCG, AFP) rarely occur in types of lung tumors.

Colon: Morphological and histochemical examinations of all tissues were routine: Apical vesicles in ascending segments and the rectum were examined by electron microscopy (EM) which differentiates them, and by light microscopy (LM) with histochemical stains which act upon the highly acid mucous in all segments. Other stains (HID-AB) reveal the large amounts of sulphomucin present in all four regions of this organ. The cross-reactivity of antibody to carcinoembryonic antigen (CEA) with nonmalignant colon tissue was studied and shown to be suppressed by fixation in 4% formaldehyde-1% gluteral- dehyde. Following this step, the antibody could be used as a tumor-specific marker, a very important accomplishment for the characterization of colon tissues collected for LHC studies. The specificity of peanut agglutinin (PNA) for the disaccharide B-D-Gal-(1-3)-D-Gal-NAc (T antigen), expressed in malignant and premalignant colonic tissue, is also now being used to characterize the colon delivered to the NIH.

Pancreas: Eight pancreatic tissues from immediated autopsy were examined by morphological techniques, histochemistry, and immunohistochemistry. They were maintained by organ explant and cell culture techniques. One tissue culture was shown to have bacterial contamination. Routinely excluded from the collection procedure were known drug overdose victims, intravenous drug users, and human T-cell leukemia virus (HTLV)-III profiled individuals. Pancreatic exocrine tumors are still being used for marker studies (alphafetoprotein, alcian-blue periodic acid shift [PAS] and keratin). Cells from these tumor are passaged and stored at -135°C.

Liver: Samples are stored at -70°C. Comparison of methods for the primary culture of human and rat hepatocytes continued using different substrates and media. In this period, the contractor has investigated lysosomal proteolysis after isolation and culturing within one hr of collection from autopsy. Under these conditions, hepatocytes degrade cell proteins at 1-7% per hr when stimulated with Dibutyryl cAMP, which can be inhibited by microtubule poisoning (VBL). This observation predicts recovery of viable cells after prolonged postmortem intervals, a difficult problem in the ongoing effort to provide liver cells for study at the NIH. Specimens quick-frozen in liquid nitrogen,are used in metabolic studies at the NCI.

Oncogene Proteins: Using specific antibodies to gene products and ABC immunohistochemistry, selected colon tissues were assayed for the presence of *ras* p21 (wild-type and position 12 mutants), and/or *ras* p21-s (wild-type specific) which are most frequently detected in human tumors, including precancer and cancer of the colon:

3 colonic mucosa were negative for normal and codon 12 mutants of *ras* p21;

3 adenocarcinomas showed moderate expression of the wild-type and mutant p21;

2 cases of Crohn's disease had weak to intense expression of both forms of p21 products;

1 adenocarcinoma showed no expression of either *ras* product.

4. Epidemiological Profile Construction and Storage

Abstracting medical records, compiling donor histories, and computerizing these data are essential requirements of this project. In this period, 243 medical records were abstracted for surgery patients (173 for thoracic and 70 for colonic); donor histories were compiled for 75 patients (37 bronchus and 38 colon) via interviews using the standard questionnaire (developed by LHC and the contractor); and in data processing, a total of 220 (65 colonic and 155 thoracic) records (medical and epidemiological) were coded for computer storage and analysis.

The total number of cases with these data collected from the beginning of the contract (from the seven participating hospitals) are listed below:

	Univ. Hosp.	LRVA	Un. Mem.	BCG
Bronchus	350(25)	162(13)	54(16)	1(0)
Colon	373(37)	121(14)	30(20)	2(0)

	St. Ag.	South Baltimore General Hospital	Washington Veteran's Administration Hospital	Sinai	Medical	Total
Bronchus	83(10)	10(1)	7(0)	9(5)	421(104)	1097(174)
Colon	0	0	51(5)	13(0)	0	591(90)

To date, the number of completions in the efforts to provide epidemiological profiles for donors of tissues delivered in this period are as follows:

				Refused Interview			
	Med. Rec.	Interviewed	Coded	Patient/Doctor/UMH			Med. Ex.
Bronchus	862(163)	461(70)	862(186)	34(3)	6(1)	38(6)	421(104)
Colon	506(70)	412(38)	497(65)	26(2)	6(1)	30(20)	0
Total	1541(243)	910(75)	1514(220)	61(3)	12(1)	84(36)	421(104)

( ) = number accomplished in this report period.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05432-04, Z01CP05479-03 and Z01CP05480-03 LHC

UNIVERSITY OF MARYLAND (N01-CP-71012)

Title: Resource for Human Esophageal Tissues and Cells from Donors with Epidemiological Profiles

Current Annual Level: $91,234

Man Years: 1.1

Objectives: To provide tissue specimens and cells of human esophagus from epidemiologically defined donors for the Laboratory of Human Carcinogenesis; to provide fresh, well-characterized, and viable esophageal tissue for primary organ culture at the NIH; to create, characterize, and store monolayer cultures from esophageal tissues for delivery on request to the NIH.

Major Contributions: Fifty-five specimens were collected and characterized from 55 cases. The contractor obtained 1 surgery case and a total of 54 from autopsies. An epidemiological profile of the donors has been provided whenever possible. Morphological, cytochemical, and immunocytochemical characteristics were determined for each tissue collected and are available to the NCI upon request. Each case was routinely sampled for histologic criteria of cell injury, viability, and histology. In addition, assays are ongoing to determine specific biochemical markers (HCG_B, AFP, CEA, etc.) occurring in normal, premalignant, and malignant human esophageal epithelium. Fixed tissues from each case collected are also preserved in bouins, ethanol, and/or aldehydes for additional characterization if required.

Monolayer cultures from organ explants are developed according to a modification of the methods of DeBuysscher et al. (1984). Explants from "normal" uninvolved immediate autopsies and malignant human esophageal mucosa were cultured in this period. Plating efficiency was 100% for cells from tissues obtained less than 4 hr postmortem. Small pieces of each type of epithelium are maintained in explant organ culture or cell culture to further assess the biologic viability of these tissues.

The cultures of cells from normal and malignant esophageal mucosa are frozen and thawed as viable cell stocks. The tissue culture cell bank of esophageal cells and tissue explants is maintained in a Queue -135° freezer in the contractor's facility (centralized tissue culture lab, MSTF 7-60). Currently, there are approximately 561(-706) vials of frozen stock, including 403 vials from 54 intermediate autopsy cases; 120 vials from 12 immediate autopsy cases; 38 vials from 4 surgical cases (one with 6 passage lines, one with 2); and 19 vials from 3 xenotransplanted esophageal tumors in nude mice. One case of cultured human esophageal tumor (#228) and one of xenotransplanted human esophageal tumor (#233) were received at LHC in this period for use in our gene polymorphism studies. Tumor and uninvolved cells from one case of esophageal cancer (#665), cultured for 30 days, was subjected to a kinetics of freezing protocol to be tested in experiments in the LHC.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05192-08 LHC, Z01CP05324-06 LHC, Z01CP05341-06 LHC, Z01CP05432-04 LHC, Z01CP05435-04 LHC, Z01CP05480-03 LHC, Z01CP05540-01 LHC, Z01CP05544-01 LHC and Z01CP05545-01 LHC

GEORGETOWN UNIVERSITY (N01-CP-85606, REPLACES N01-CP-31007)

Title: Collection and Evaluation of Human Tissues and Cells from Donors with an Epidemiological Profile

Current Annual Level: $82,972

Man Years: 0.96

Objectives: To provide the NCI with (1) a source of human lung and bronchial tissues taken at surgery, (2) pleural fluid from patients with benign and malignant lung disease, (3) human bronchial alveolar cells from bronchial lavage of normal, smoking and nonsmoking volunteers, and (4) completed epidemiological questionnaires for medical and environmental histories.

Major Contributions: In this first period of the new contract, the contractor provided 18 specimens (10 benign, 8 malignant) from selected bronchi with peripheral lung, 2 specimens of pleural fluid for mesothelial cells from patients with malignancies, and 44 specimens of colon from 16 patients with malignancies (mostly adenocarcinomas) and 11 without. In addition, we received 16 sets of matching peripheral blood lymphocytes and alveolar macrophages (from 5 smokers and 11 nonsmokers), twelve with matching serum samples. Epidemiological (medical and environmental history) profiles were completed for all participating patients and normal volunteers. These records were filed in the contractor's facility for future use by the NCI.

Tissues collected by this contractor were used in ongoing studies in the In Vitro Carcinogenesis and Biochemical Epidemiology Sections of LHC: human bronchial and peripheral lung tissues in studies of chemical carcinogenesis in human lung cancer; cultured mesothelial cells (from pleural fluids) to examine in vitro effects of asbestos and other environmental agents potentially involved in the induction of malignant mesothelioma; lung and colon tissues for studying carcinogen DNA adducts, DNA repair, and genetic restriction fragment length polymorphisms (RFLP).

This contract is unique among LHC resources. In addition to providing surgically-derived tissues, and pleural effusions, it is the sole provider of alveolar macrophages (i.e., bronchial lavage) and peripheral blood lymphocytes (PBLs) from normal, non-hospitalized, smoking and nonsmoking adult volunteers. Blood cells from healthy, adult populations serve as a source of macromolecules with which to establish a data base of normal levels for frequency and distribution of humans positive for certain carcinogen-induced damage to genes and gene products, chemical markers thought of as potential indices for individuals at risk for chemical carcinogenesis. Currently, studies are ongoing to detect carcinogen-DNA and carcinogen-hemoglobin adducts, and to associate these phenomena with DNA repair in normal and endemically exposed populations.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05324-06 LHC, Z01CP05341-06 LHC, Z01CP05432-04 LHC, Z01CP05435-04 LHC, Z01CP05480-03 LHC and Z01CP05507-04 LHC

VETERANS ADMINISTRATION HOSPITAL (Y01-CP-70501; formerly CP-30255)

Title: Resource for Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: $61,447

Man Years: 1.6

Objectives: This interagency agreement provides (1) specimens of normal, premalignant, and malignant human lung and colon tissues (taken at the time of surgery) for the study of human epithelial responses to carcinogens in cell and organ cultures and as xenotransplants in immunodeficient mice; (2) morphologic and pathologic characterization by light and electron microscopy and histochemistry of normal, premalignant, and malignant epithelium for each tissue; (3) an epidemiological profile (including preoperative medical and environmental histories) for each donor.

Major Contributions: From cardiothoracic and colonic surgeries, the contractor delivered a total of 113 specimens: 47 lung (19 normal, 18 malignant); 51 colon (27 normal, 24 malignant); 7 bronchus; 19 pleura; and 1 malignant esophagus. Of the 53 patients who participated, 43 (81%) had a malignancy: 8 (15%) had squamous cell carcinomas and 5 (9.4%) had adenocarcinomas of the lung; 24 (45%) had adenocarcinoma of the colon; 3 (5.6%) had broncho-alveolar carcinoma; and one each (2%) had leukemia, myeloma and mesothelioma. Eight patients (15%) did not harbor a malignancy, but had a number of other conditions including granuloma and plasmacytosis myeloma and mycetoma.

Thus, from a variety of surgical procedures (including pneumonectomy, lobectomy, colectomy, esophagectomy, local excision and biopsy) performed in this period, the contractor has provided numbers and types of tissues that contribute to the LHC program. Of the 45 malignancies diagnosed, 18 (40%) were in lung; 24 (53%) in colon and 1 (2%) in mesothelium. Cooperating donors have epidemiological profiles completed and filed in the contractors facility.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05324-06 LHC, Z01CP05341-06 LHC, Z01CP05432-04 LHC, Z01CP05435-04 LHC, Z01CP05480-03 LHC,Z01CP05507-04 LHC, Z01CP05540-01 LHC, Z01CP05544-01 LHC and Z01CP05545-01 LHC

WALTER REED ARMY MEDICAL CENTER (Y01-CP-70500; formerly CP 30504)

Title: Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: $23,287

Man Years: 1.0

Objectives: This interagency agreement (1) provides pathologically characterized specimens of malignant and noninvolved bronchus, lung, pleural effusions, and colonic epithelium (obtained at time of surgery for cancer or for benign lesions); and (2) epidemiological profiles (medical and environmental histories) for each donor to the NIH for the study of carcinogen metabolism in human tissues.

Major Contributions: The new technician (March 1987) is now well trained to function between Walter Reed and the NCI and has performed commendably in the collection and delivery of tissues. In this period, from cardiothoracic and colonic surgeries, the agency has provided a total of 152 specimens from 57 surgeries (32 cardiothoracic; 25 colonic) to the NIH, including 90 specimen of lung and bronchial epithelium (48 normal; 48 malignant) and 59 specimens of colon mucosa (30 normal; 29 malignant). Providing the pleural fluids required as a source of mesothelial cells has been a problem (only one received). The agency organization apparently creates difficulty for inter-departmental efforts to achieve this type of collection. Therefore, their good potential for improving the supply of these valuable cells is not yet under way. We hope to resolve the problem soon. In addition, the smooth acquisition of histological and pathological reports is only now being tested.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05324-06 LHC, Z01CP05341-06 LHC, Z01CP05403-05 LHC, Z01CP05409-05 LHC, Z01CP05494-01 LHC and Z01CP05543-01 LHC

HAZELTON LABORATORIES, INC. (N01-CP-51004)

Title: Resource for Xenotransplantation Studies of Carcinogenesis of Human Tissues in Athymic Nude Mice

Current Annual Level: $349,406

Man Years: 2.70

Objectives: To provide an immunodeficient animal model, the athymic nude mouse, for (1) long-term survival of human tissue xenografts; (2) for long-term xenotransplantation, proliferation, and tumorigenicity studies of normal, premalignant, and malignant human tissues; and (3) for study of the in vivo development of preneoplastic and neoplastic transformation in human tissues experimentally induced in vitro and in vivo by selected chemical agents, cellular manipulations, and genetic transfections.

Major Contributions: Human bronchus, pancreatic duct, colon, breast, prostate, and esophagus are maintained for 16 months and beyond as xenografts, as evidenced by viable-appearing epithelium with normal histology, detection of radiolabeled precursors into epithelial cells of the grafts, and positive test for human isozymes.

In the breeding stock, 757 Swiss litters contained 5,368 pups (7/litter), including 2,618 nu/nu pups. Forty-four percent (44%) of the nu/nu newborns survived, giving a total of 1,160 nudes or 1.5 nudes/litter. During the year, the contractor maintained a monthly average colony population of 1,272 mice: 170(+50) breeders, 218 newborns, 96 weanlings for new experiments, and 788 (+418 above previous period) mice in experimental protocols.

As indicated for the breeder and experimental animal numbers above, important changes in colony procedures were instituted in this period, which affected an increasing rate of experimental starts and the population levels for the ongoing capacity of the experimental effort in the colony. Experiments using 1,139(+583) irradiated nude mice were initiated in this period. They included:

1) Experiments (totaling approximately 731 mice with about 517 ongoing) to study growth rates, morphology and tumorigenicity of xenotransplanted bronchial epithelial (BE) cell lines transformed by oncogene transfection and viral infection in irradiated nudes, e.g.: a) BEAS-2B Zip-v-Ha-*ras* control BE cells; b) BEAS-2B cells of different passages; c) BES-1A1 cells; BEAS-2B cells; d) B39-TL tumor cells from BEAST-12 cells grown in nude mice; BZR-T33 cells for tumor suppression pilot experiments; 12 different BEAS-2B/*ras* transfected constructs in nudes of increasing age-- to list a few.

2) Experiments (totaling approximately 283 mice with some 223 ongoing) with mesothelioma cells and mesothelial cell lines to study growth, morphology and transplantability by different routes with and without irradiation, and exposure to Amosite asbestos.

3) Experiments (totaling approximately 125 mice, 114 ongoing) performed for various miscellaneous objects of a pilot and/or study initiation or finalization design. These include studies with pancreatic duct, kidney, esophagus, and tissue culture cells transfected with viral vectors (PLJ).

The contractor maintained a monthly average of 45 ongoing experiments requiring 788 mice, 681 of which survived the period. In brief, the increased level of colony performance relates directly to our accumulated experience in the use of experimental designs optimized to accomplish in vivo characterization of effects induced in vitro. All experiments are designed with a predetermined shelf time (6 weeks, mostly, to 52 weeks as the maximum) and all experiments of a certain design are routinely terminated by the contractor with proper notification to the individual investigators. Thus, the turnover is more rapid and there is more space available for new experiments. Consequently, the production rate of mice for experimental use has been increased and more studies are now being accomplished.

Malignant transformation from tissue treated chemically in vitro continues to elude observation in xenografts. Chemically induced in vitro abnormalities do not maintain for sufficiently extended periods as xenografts. Squamous metaplasia occurs in grafts given carcinogens in vivo but have not become malignant.

However, the successful xenotransplantation of H-ras-transfected human bronchial epithelial (HBE) cells and zip-ras adeno 12 SV40 (hybrid virus)-transfected cell lines continue to produce tumors in nude mice. Other inducers are being tested for this property. Among these are the SV40 T antigen, and the raf, PDGF or c-raf modified PLJ retroviral vector. Several cell lines treated with these substances are now in long-term studies to define their tumorigenic properties.

All animals found dead or sacrificed at the request of the investigators are processed by the contractor for histological and pathological diagnosis by high resolution and/or EM, provided by an assigned veterinarian pathologist from the contractor's staff.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05326-06 LHC, Z01CP05328-06 LHC, Z01CP05434-04 LHC

BRATON BIOTECH, INC. (N01-CP-51086)

Title: Immunologic Studies of High Risk Groups

Current Annual Level: $189,000

Man Years: 0.75

Objectives: The contractor provides the following capabilities: HLA typing, analysis of cell-surface components by flow microfluorometry.

Major Contributions: Analysis of cell-surface antigens on lymphocytes from acquired immune deficiency syndrome (AIDS) patients or at-risk populations have been studied during the past year. These studies are in collaboration with the Environmental Epidemiology Branch, NCI. Cell-surface antigen profiles have also been determined on freshly explanted tumor cells from patients with small cell lung carcinoma as well as small cell lung carcinoma cell culture. HLA typing has been performed on the AIDS patients and at-risk populations. Serum samples from hemophiliac patients were analyzed for cytotoxic reaction to histocompatibility antigens. A variety of cell lines infected with human T-cell leukemia virus (HTLV)-I and II, or human immunovirus (HIV) are being carried out in the appropriate P3 facilities. These cell lines are being used to study the immunobiology of HTLV-1 and II, and HIV.

ANNUAL REPORT OF

LABORATORY OF MOLECULAR CARCINOGENESIS
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 to September 30, 1988

The Laboratory of Molecular Carcinogenesis (LMC) plans, develops, and conducts a research program designed to (1) clarify the molecular biology of chemical and physical 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 genetic, environmental, and endogenous factors which relate to and modify the carcinogenic process; and (4) clarify the genetic and environmental roles in the metabolic activation of chemical carcinogens and the detoxification and activation of xenobiotics such as drugs and environmental chemicals.

The goal of the Laboratory of Molecular Carcinogenesis is to understand the molecular basis of carcinogenesis with the view toward identifying susceptible populations and preventing human cancer. The research program is designed to understand the molecular basis by which carcinogenic agents cause malignant transformation, and to identify and characterize those exogenous and endogenous factors involved in carcinogenesis. The Laboratory seeks to clarify the metabolic interaction of exogenous and endogenous agents in the living organism at the molecular, cellular, and organism levels, and seeks to understand the consequences of these interactions in terms of cell regulation and carcinogenesis. Emphasis is made on the role of carcinogen and drug activation and the genetics of the human population related to sensitivity to carcinogens, drugs, and environmental chemicals.

The Laboratory research program utilizes advanced techniques of molecular biology and immunology, as well as classical enzymology and protein chemistry. The staff are highly experienced in DNA recombinant and related molecular biology techniques, protein chemistry, and hybridoma technology. The power and precision of these technologies have promoted considerable progress in many of the projects of the Laboratory.

<u>Office of the Chief</u> - Studies (1) the nature of human genetic predisposition to cancer; and (2) the interaction of chemical and physical carcinogens with DNA, the repair of resulting damage and its relationship to human cancer formation.

Newly developed shuttle plasmids were used as sensitive probes to measure DNA repair, DNA ligation and mutagenesis at the molecular level in human cells. These methods open an entirely new potential for understanding the nature of DNA damage and repair processes. Using the newly developed methodology, the molecular, cellular, and clinical abnormalities in patients with xeroderma pigmentosum (XP), with the dysplastic nevus syndrome (DNS) of familial cutaneous melanoma, and with Bloom's syndrome (BS) are being studied. A shuttle vector plasmid, pZ189, was used to determine that there is a restricted spectrum of mutations induced in ultraviolet (UV)-treated DNA replicating in XP cells of complementation groups A and D. The generation of plasmids with multiple base

substitution mutations was related to an error-prone polymerase activity acting on nicked DNA which may be relevant to a generation of immunoglobulin diversity. DNS cells introduced more mutations into UV-treated pZ189 than normal cells but had fewer tandem mutations. The major UV photo-product, the T-T cyclobutane dimer, was found to be only weakly mutagenic in XP, DNS and normal lines. Photoproduct frequency was not the major determinant of UV mutation frequency in plasmids replicated in human cells. BS cell lines were reported by others to have diminished ligase activity in vitro. Utilizing a linearized replicating plasmid we demonstrated reduced ability of BS cells to ligate plasmids in vivo. The BS cells also introduced more deletion mutations than normal cells into the recircularized plasmids. A Registry of XP patients has been established. Utilizing an assay of G2 phase chromosomal hypersensitivity, collaborative studies have, for the first time, detected XP heterozygotes.

A 3-year clinical trial of cancer chemoprevention demonstrated that high dose (2 mg/kg/da) oral 13-*cis* retinoic acid (Accutane) is effective in preventing formation of new skin cancers in patients with XP.

Metabolic Control Section - Studies (1) the metabolic activation and detoxification of the polycyclic hydrocarbons (PCH) and other carcinogens and drugs and the relationship of this metabolism to individual sensitivity and susceptibility to carcinogenesis; (2) the immunochemistry of cytochrome P-450 with the use of monoclonal antibodies for the detection, purification, and identification of forms of cytochrome P-450 responsible for different carcinogen and drug metabolism; (3) the protein structure and membrane topology of cytochrome P-450 with the goal of understanding structure-function relationships; and (4) use of expression vectors to produce pure P-450 for determination of functional specificity.

This section studies the molecular events in the activation of carcinogens by cytochrome P-450s. The aim is to understand the enzymatic conversion of carcinogens to either detoxified forms or to active carcinogenic forms. Higher organisms have systems for the detoxification and elimination of foreign chemical compounds, including carcinogens. These systems primarily involve microsomal cytochrome P-450 mixed-function oxygenases, but also include epoxide hydratase and conjugating enzymes. The vast majority of foreign compounds are processed by these enzyme systems. The mixed-function oxygenases are influenced by a variety of environmental factors such as drugs, pesticides, and carcinogens; and by nutritional and hormonal states of the animal as well as by the age, sex, and genetic makeup. Work in this Laboratory provided the key studies which showed that this enzyme system is responsible for the activation of PCH procarcinogens to their ultimate carcinogenic forms. A primary goal is to define the enzymatic mechanism by which carcinogens are activated, either to carcinogenic forms or to detoxified products. As these enzymes are characterized and as sensitive molecular biological and immunochemical techniques are developed, 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 carcinogen-induced carcinogenesis.

The approach is to identify and fully characterize the enzymes responsible for carcinogen activation and metabolism. In addition, we seek to understand the molecular biology and regulation of this system, both at the genetic and epigenetic levels. We plan to assess the types and amounts of these enzymes in

human populations, using molecular, biological, immunological, and metabolic approaches. We will carry out multilevel investigations of the carcinogen-metabolizing enzyme systems, continuing our use of high pressure liquid chromatography (HPLC) to study carcinogen metabolites, using monoclonal antibodies (MAbs) to study the properties of the enzymes.

Monoclonal antibodies were successfully prepared to a number of different cytochrome P-450s. These include a constitutive form that metabolizes steroid hormones, a form inducible by ethanol and involved in the metabolism of nitrosamines, a compound suspected of being a human carcinogen, and a form of P-450 induced by pregnenolone 16 alpha-carbonitrile. Monoclonal antibodies (MAbs) to various forms of rat liver cytochrome P-450 have been used as specific probes for the cytochrome P-450s in human liver. Western blot analysis with MAbs to rat ethanol-induced P-450, a form with high nitrosamine metabolizing activity, detected a related P-450 in human liver microsomes. Individual variation in the level of this P-450 was observed. A radioimmunoassay (RIA) to this P-450 was developed with MAb 1-98-1. Using this assay we were able to detect and quantitate the MAb-specific cytochrome P-450 in human liver microsomes. This human P-450 was immunopurified and analyzed by peptide maps and amino acid sequencing.

Using a MAb to a nitrosamine-metabolizing ethanol-inducible rat liver P-450, a P-450 has been immunopurified from both rat and human liver. These differed in primary structure as evidenced by different amino terminal sequences and peptide maps. The membrane organization of P-450s was probed by a cross-linking study which revealed specific association among certain forms of P-450. Such interactions may influence the disposition of P-450 substrates in secondary metabolism. The induction of rat liver microsomal and nuclear envelope P-450s by 2-acetyl-aminofluorene (AAF) was immunochemically probed with MAbs to P-450c and P-450d. These P-450s were induced in both microsomes and envelopes, although AAF decreased total P-450 in envelopes but not microsomes.

Human individuals differ in their capability to metabolize and detoxify drugs and xenobiotics. MAbs to P-450 were prepared and used for identification and phenotyping of P-450 in tissues and organs of mice, rats and humans by RIA, Western blotting, histochemical staining and inhibitory effect on catalytic activity. When C57BL/6 mice were treated with 3-methylcholanthrene (MC), MC-inducible P-450s (MC-P-450s) were identified in liver, lung and kidney but not in those organs of DBA/2 mice. When neonatal rats were treated with the carcinogenic compound 3-nitro-fluoranthene, a nitrated product of environmental fluoranthene, MC-P-450s were found to be the predominant induced forms. In untreated rats, the MC-P-450s were increased during the first month after birth. The MC-P-450s were also identified in human fetal and adult livers. The inducibility of MC-P-450 was modulated by diet and increased by feeding rats a high cholesterol diet. These results indicate that epitope specific MAbs are very useful probes for identification of P-450s in various tissues and for the study of P-450 changes during development and under different environmental conditions.

Previously cloned cDNAs of two P-450s were used to construct two cDNAs into two recombinant viruses, infectious vaccinia and retrovirus. These vectors express the incorporated cDNA into single P-450s when infected into host cells. In both the vaccinia and retrovirus systems, proteins were expressed with the appropriate molecular weights and exhibit enzyme activity typical of each P-450. Studies are in progress to determine the specificity of each expressed protein in the 1) stereochemistry of polycyclic aromatic hydrocarbon metabolism, (2) aflatoxin

metabolism, and (3) the metabolism of aminopyrine, antipyrine and theophylline. The results from the above studies indicate a variability of specificity depending on the substrate; a high degree of specificity for one form of P-450 with some substrates and cross reactivity for both P-450s with other substrates. In a different project various expression systems are being developed with a large variety of cDNAs and different vectors. Success in this effort will enable us to define the contribution of each of these enzymes to carcinogen and drug metabolism and to mutagenesis and cell transformation mediated by chemical carcinogens.

Nucleic Acids Section - Studies (1) the structure and evolutionary relationships of human and rodent cytochrome P-450 genes; (2) the mechanisms by which P-450s are induced by endogenous and xenobiotic substances; (3) the mechanisms by which P-450 genes are developmentally activated; (4) the enzymatic specificity of P-450s through the use of yeast and higher eukaryote cDNA expression vectors; (5) the molecular basis of enzymatic P-450 polymorphisms in man and rodents; (6) evolutionary, structural, and regulatory analysis of cellular peroxidases and the role of these enzymes in carcinogen metabolism and tumorigenesis.

The nature of the common human drug oxidation defect, characterized by the lack of ability to metabolize debrisoquine and other drugs, has been elucidated. By analysis of human liver specimens it was discovered that individuals who cannot metabolize debrisoquine do not express a specific form of P-450 designated P-450dbl. This lack of expression was found to be due to mutant P-450dbl genes. These defective genes can be detected by assays of human lymphocyte DNAs providing the basis for a potential clinical test for this drug oxidation defect.

To evalute the enzymology of human P-450s we are isolating full-length P450 cDNAs from human liver cDNA libraries. P-450s are being produced using a variety of cDNA expression systems, including yeast, COS-cell-simian virus-40 (SV40), vaccinia virus, retrovirus and bacculo virus systems. Several human and rat cDNAs have been expressed in these systems and the P-450s are being evaluated for their enzymatic specificities toward drugs and carcinogens. These cDNA-expressed enzymes are also being evaluated for their ability to activate carcinogens and to produce mutagenic metabolites. By these studies we hope to enzymatically catalog all human P-450s. This data in conjunction with studies on P-450 polymorphisms and variability in the human population may allow us to determine if P-450 expression is associated with susceptibility or resistance to chemically induced cancer.

To determine the mechanism by which P-450s are regulated during development and by dietary inducing factors, we have isolated several P450 genes. The promotor regions of the genes are being studied using a variety of assays, including i) cell transfection using promoter-driven heterologous genes; ii) direct DNA binding assays using cell nuclear extracts and upstream region of the genes are being conducted; and iii) in vitro transcription assays are also being carried out using nuclear transcription extracts. These studies should provide insight into the DNA _cis_-acting elements and the _trans_-acting protein factors involved in controlling the transcription of P-450 genes.

The cDNA and gene coding for human thyroid peroxidase has been cloned. These studies have revealed the presence of an alternate splice of the peroxidase gene primary transcript that results in the production of two distinct proteins. The enzymology of these proteins is being investigated using the Vaccinia virus cDNA

expression system. Comparison of the thyroid peroxidase protein sequence with that of human myeloperoxidase has revealed that these two enzymes are evolutionarily related. This finding is very surprising since these enzymes are produced in totally different organelles and have distinct physiological roles. The gene coding for the thyroid peroxidase has been cloned and sequenced and found to share some similarities with only the myeloperoxidase gene. Other cellular peroxidase cDNAs are also currently being isolated.

Protein Section - Studies (1) the relationship between chromatin structure and gene expression and (2) mechanisms by which chromosomal proteins affect the structure and function of chromatin.

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes is being investigated. Present efforts are concentrated on determining the cellular function of two non-histone chromosomal proteins, HMG-14 and HMG-17. These two proteins are the only known nucleoproteins whose main binding site in the nucleus is on the nucleosome. Our experiments suggest that they may be involved in modulating the structure of transcriptionally active chromatin. We have isolated and sequenced the chicken cDNA coding for chromosomal protein HMG-14 and the entire gene coding for chicken HMG-17. The chicken HMG-17 gene displays features characteristic of both cell-cycle regulated and housekeeping genes. We discovered a consensus structure of the transcripts coding for the HMG-14/-17 protein family. We found that the human HMG-14 and -17 multigene families are the largest known retropseudogene families. Genes coding for the functional human HMG-14 and HMG-17 have been isolated by use of oligonucleotide probes. Expression of human HMG-14 and HMG-17 in COS cells and in yeast reveals that there is a mechanism which regulates the cellular content of these proteins and that expression of the protein alters the protein profile of yeast cells. Study of the DNA binding domain of this family of proteins reveals a high degree of evolutionary conservation. These studies will advance the understanding of gene structure and function in normal and neoplastic cells and provide insights into the evolution of the nucleosome structure, a hallmark of all eukaryotic organisms.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04496-11 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Chromosomal Proteins and Chromatin Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Laboratory	Institute
PI:	Michael Bustin	Acting Section Chief	LMC	NCI
Others:	David Landsman	Visiting Associate	LMC	NCI
	Thyagarajan Srikantha	Visiting Associate	LMC	NCI
	James Pash	IRTA Fellow	LMC	NCI
	Massimo Crippa	Visiting Fellow	LMC	NCI
	Nirmolini Soares	Biologist	LMC	NCI
	O. Wesley McBride	Section Chief	LB DCBD	NCI

COOPERATING UNITS (if any)

Chester Beatty Laboratories, England (Dr. Graham Goodwin)
Laboratory of Biochemistry, Georgetown University (Dr. M. Smulson)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.6	3.6	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes is being investigated. Present efforts are concentrated on determining the cellular function of two non-histone chromosomal proteins, HMG-14 and HMG-17. These two proteins are the only known nucleoproteins whose main binding site in the nucleus is on the nucleosome. Our experiments suggest that they may be involved in modulating the structure of transcriptionally active chromatin. We have isolated and sequenced the chicken cDNA coding for chromosomal protein HMG-14 and the entire gene coding for chicken HMG-17. The chicken HMG-17 gene displays features characteristic of both cell-cycle regulated and housekeeping genes. We discovered a concensus structure of the transcripts coding for the HMG-14/-17 protein family. We found that the human HMG-14 and -17 multigene families are the largest known retropseudogene families. Genes coding for the functional human HMG-14 and HMG-17 have been isolated by use of oligonucleotide probes. Expression of human HMG-14 and HMG-17 in COS cells and in yeast reveals that there is a mechanism which regulates the cellular content of these proteins and that expression of the protein alters the protein profile of yeast cells. Study of the DNA binding domain of this family of proteins reveals a high degree of evolutionary conservation. These studies will advance the understanding of gene structure and function in normal and neoplastic cells and provide insights into the evolution of the nucleosome structure, a hallmark of all eukaryotic organisms.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Michael Bustin	Acting Section Chief	LMC	NCI
David Landsman	Visiting Associate	LMC	NCI
Thyagarajan Srikantha	Visiting Associate	LMC	NCI
James Pash	IRTA Fellow	LMC	NCI
Massimo Crippa	Visiting Fellow	LMC	NCI
Nirmolini Soares	Biologist	LMC	NCI
O.W. McBride	Section Chief	LB DCBD	NCI

Objectives:

To understand the mechanism of gene regulation and its relation to neoplasia by studying the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes in normal and transformed cells. Studies are designed to give insights into the chemical nature of chromosomal proteins, their immunological specificities, the manner in which they interact with DNA, and the regulation of the expression of genes coding for these proteins.

Methods Employed:

The regulated expression of the genetic information encoded in DNA is dependent on specific protein-nucleic acid interactions. We have elicited antibodies against specific proteins and used them to study the structure and function of specific chromosomal proteins and their interactions with DNA. Proteins are purified from isolated nuclei by differential precipitation, size exclusion chromatography, and ion exchange chromatography. Synthetic peptides are prepared by solid phase synthesis. Polyclonal and monoclonal antibodies are elicited in rabbits and mice, respectively. Chromatin is isolated from purified nuclei. The antigenic activity of the purified chromatin and isolated proteins is measured by enzyme linked solid phase assay (ELISA), immunoblotting and radioimmunoassays. Nucleosomes are prepared by nuclease digestion. Immunoaffinity columns are prepared by the cyanogen bromide procedure using purified immunoglobin (Ig). cDNA clones are isolated from expression libraries prepared from the mRNA isolated from transformed human cells. The clones are characterized, propagated in plasmids, and the DNA sequence determined. The cDNA clones are used to isolate the human genes coding for the proteins and to study gene expression in various tissues by analyzing the RNA. Genomic clones are isolated from human and chicken libraries using the cDNA fragment as a probe. Vectors used to transfect the cDNA into COS and yeast cells are constructed by inserting the cDNA fragment at selected restriction sites in the vector. COS cells are transformed by the diethyl amino ethyl (DEAE) cellulose method and yeast cells by the lithium acetate method. Transcription is determined by Slot blot and Northern analysis. Translation of the HMG protein is determined by extraction of whole cells with 5% perchloric acid (PCA) and polyacrylamide gel electrophoresis. The cellular location of the protein is determined by immunofluorescence.

Major Findings:

The entire cDNA coding for chicken HMG-14 has been isolated and sequenced. The results indicate that the transcript has features typical of all other cDNAs coding for HMG-14 and HMG-17. Only 25% of the transcript contains the open reading genome; the 3' untranslated region is relatively long (60% of transcript) and the 5' untranslated region is extremely GC-rich. Based on this sequence and those previously determined in our laboratory, we conclude that the cDNAs coding for the HMG-14/-17 chromosomal proteins have a characteristic, evolutionally conserved structure.

HMG-14 and HMG-17 are encoded by multigene families. To date, the multigene families coding for these proteins are the largest known human retropseudogene families. Since the chicken genome contains a single gene for HMG-14 and -17 the chicken HMG-17 gene has been isolated and sequenced. The gene, spanning 4165 base pairs contains 6 exons. The DNA binding domain is contained in 2 exons. The gene has an extremely high content of G+C (75%) in a 1150 b.p fragment starting with 500 bp 5' to the putative cap site. The HMG-17 gene has features characteristic of cell cycle regulated housekeeping genes. The regulatory sequences in the 5' region have been identified.

The functional human HMG-17 and HMG-14 genes have been isolated using sets of oligonucleotides which could distinguish between the sequence of the functional gene and those of the respective retropseudogenes. The sequence determination and chromosome localization of the genes are in progress.

Study of the amino acid sequence of all the known HMG-14 and HMG-17 proteins revealed a remarkable conservation in the DNA binding domain of these proteins. The evolutionary conservation suggests strict structural requirements for interaction with nucleosomes. Since the nucleosome is also a highly conserved structure, understanding of the mechanism of binding of HMG to nucleosomes may provide insights into evolutionary differences between prokaryotes and eukaryotes.

Expression of human HMG-14 and HMG-17 in COS cells indicated that the stability of the mRNAs is independently regulated and that there is an upper limit to the cellular content of HMG proteins.

The human proteins have been expressed in yeast. Expression of the protein does not affect the growth characteristic of the yeast; however, two-dimensional polyacrylamide gel analysis reveals that the mobility of certain proteins has been altered.

Publications:

Bustin M. Immunochemical analysis of the structure and function of chromosomal proteins. Cytometry 1987;8:251-9.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04517-12 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

DNA Repair in Human Cancer-Prone Genetic Diseases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: K.H. Kraemer Res. Scientist LMC NCI

Others:

Name	Title	Affiliation
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T. Runger	Guest Researcher	LMC NCI
M. Seidman	Guest Researcher	LMC NCI
R. Tarone	Biometrician	BB DCE NCI
D. Brash	Staff Fellow	LHC NCI
J. Robbins	Sr. Invest	DB NCI
H. Waters	Tech (Biol)	LMC NCI
F. Kanai	Guest Res.	LMC NCI
G. Peck	Sr. Invest.	DB NCI
K. Sanford	Sr. Invest.	LCMB NCI
Y. Pommier	Sr. Invest	LMP NCI

COOPERATING UNITS (if any)

Dept of Pathology, NJ School of Medicine (W.C. Lambert); NY Blood Center (J. German); Dept of Dermatol Hosp Univ of Pa (W. H. Clark); Wistar Institute, Phila, PA (M. Herlyn); Bureau of Devices and Radiological Health, FDA (J. Sagripanti)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Molecular, cellular, and clinical abnormalities in patients with xeroderma pigmentosum (XP), with the dysplastic nevus syndrome (DNS) of familial cutaneous melanoma, and with Bloom's syndrome (BS) are being studied. We have developed new assays utilizing plasmids as tools to measure DNA repair, DNA ligation and mutagenesis at the molecular level in cultured human cells. We utilized a shuttle vector plasmid, pZ189, to determine that there is a restricted spectrum of mutations induced in ultraviolet (UV)-treated DNA replicating in XP cells of complementation groups A and D. The generation of plasmids with multiple base substitution mutations was related to an error-prone polymerase activity acting on nicked DNA which may be relevant to generation of immunoglobulin diversity. DNS cells introduced more mutations into UV-treated pZ189 than normal cells but had fewer tandem mutations. The major UV photo-product, the T-T cyclobutane dimer, was found to be only weakly mutagenic in XP, DNS and normal lines. We determined that photoproduct frequency was not the major determinant of UV mutation frequency in plasmids replicated in human cells. BS cell lines were reported by others to have diminished ligase activity in vitro. Utilizing a linearized replicating plasmid we demonstrated reduced ability of BS cells to ligate plasmids in vivo. The BS cells also introduced more deletion mutations than normal cells into the recircularized plasmids. A Registry of XP patients has been established. Utilizing an assay of G2 phase chromosomal hypersensitivity, collaborative studies have, for the first time, detected XP heterozygotes. A 3-year clinical trial of cancer chemoprevention demonstrated that high dose (2 mg/kg/da) oral 13-*cis* retinoic acid (Accutane) is effective in preventing formation of new skin cancers in patients with XP.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K.H. Kraemer	Research Scientist	LMC	NCI
S. Seetharam	Visiting Fellow	LMC	NCI
H. Waters	Biologist	LMC	NCI
T. Runger	Guest Researcher	LMC	NCI
F. Kanai	Guest Researcher	LMC	NCI
M. Seidman	Guest Researcher	LMC	NCI
G. Peck	Senior Investigator	DB	NCI
R. Tarone	Biometrician	BB DCE	NCI
K. Sanford	Chief, In Vitro Carcinogenesis Sect.	LCMB	NCI
D. Brash	Staff Fellow	LHC	NCI
Y. Pommier	Visiting Associate	LMP	NCI
J. Robbins	Senior Investigator	DB	NCI

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 to 1) understand the molecular basis of their cellular hypersensitivity, 2) correlate cellular hypersensitivity with clinical abnormalities, 3) determine if there is genetic heterogeneity within such groups, 4) explore methods of cancer prevention in these patients, and 5) educate the medical community to the importance of early recognition and diagnosis of these disorders.

Methods Employed:

Plasmids for measurement of DNA repair or mutagenesis are treated in vitro with ultraviolet radiation, with copper plus hydrogen peroxide, with damage-modifying enzymes such as photolyase to remove cyclobutane dimers, enzymes or with restriction endonucleases to create linear plasmids. The extent and sites of damage are assessed by endonuclease-sensitive site assay, by polymerase chain termination assay with DNA sequencing, or with end labeling (Maxam-Gilbert) DNA sequencing. DNA-mediated gene transfer (transfection) is used to introduce the plasmids into cultured human cells. DNA repair is measured by transient expression of the encoded bacterial gene chloramphenicol acetyl transferase (CAT), or by autoradiographic measurement of unscheduled DNA synthesis (UDS). Plasmid mutation frequencies are measured by isolation of replicated plasmids from the human cells and transformation of indicator strains of bacteria. Mutant plasmids are isolated from bacterial colonies and purified. The DNA sequence of mutated plasmids is determined using a primer-directed dideoxy-sequencing technique with double-stranded plasmids.

Patients with XP or with DNS are examined with particular emphasis on cutaneous abnormalities. Cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory analysis. Physicians treating patients with XP are contacted and are encouraged to fill out a Xeroderma Pigmentosum Registry questionnaire about their patients. New clinical forms of XP are investigated in-depth. XP patients with multiple cutaneous neoplasms are being treated with oral 13-cis retinoic acid in a long-term study to attempt to reduce their rate of new skin tumor formation.

Major Findings:

We have developed host cell reactivation assays utilizing expression vector plasmids as tools to measure DNA repair and mutagenesis in cells from patients with cancer-prone genetic diseases. Transient expression of damaged plasmids depends on the competence of cellular repair enzymes. We found that there were 100-fold differences in expression between normal and XP groups A and D cells. In XP-A and XP-D cells, one pyrimidine dimer inactivated expression of the transfected gene. Selective enzymatic removal of pyrimidine dimers by pre-treatment with photoreactivating enzyme revealed that XP cells also cannot repair non-dimer photoproducts.

The shuttle vector plasmid, pZ189, was used to measure replication and mutagenesis after UV treatment and transfection in normal, XP-A and XP-D, and DNS cells. Plasmid survival was reduced in the XP cells reflecting their repair deficiency and cellular hypersensitivity to the cytotoxic effects of UV. Plasmid survival was normal in the DNS line reflecting the normal UV survival of the DNS cell line. The frequency of mutations introduced by the cells into the UV damaged pZ189 was greater than normal with the XP-A, XP-D and DNS lines, reflecting the cellular UV-hypermutability of these disorders. We demonstrated, for the first time, that there was a markedly reduced spectrum of mutations found with the XP-A, XP-D and DNS lines in comparison to the spectrum with normal cells. With the XP-A and XP-D lines there was a significant reduction in the frequency of plasmids recovered with multiple base substitutions and in transversion mutations. The XP-D line, arising from a patient who had several cutaneous melanomas, also had a reduced frequency of plasmids with tandem mutations. The DNS line, derived from a patient with multiple melanomas, was similar to the XP-D line in having a reduced frequency of plasmids with tandem mutations. Different mutagenic hotspots were present with each cell line. With all lines, more than 85% of the mutations were G:C to A:T transitions.

We found that the major UV photoproduct, the T-T cyclobutane dimer, was only minimally mutagenic in UV-treated pZ189 introduced into all cell lines. By eliminating 99% of the cyclobutane dimers (T-T, T-C, C-T, and C-C) by photoreactivating enzyme prior to transfection, we found increased plasmid survival, decreased mutation frequency, and an altered spectrum of types of mutations. We determined that both dimer and non-dimer photoproducts (especially those at T-Cs) were mutagenic in plasmids replicated in human cells. Cytosine-containing photoproducts produced 90-95% of the mutations. We measured photoproduct production at more than 50 sites of adjacent pyrimidines in the marker gene. There were large variations in the frequency of cyclobutane dimers and in formation of 6-4 pyrimidine-pyrimidone photoproducts at individual sites.

Surprisingly, there was no correlation between photoproduct frequency and mutation frequency at the same sites. Thus, photoproduct frequency was not the major determinant of UV mutation hotspots or coldspots in plasmid DNA replicated in human cells. Presumably, photoproduct-induced secondary structural alterations (that may be sequence-specific and cell polymerase-specific) are responsible for the mutational hotspots but these are not adequately measured by photoproduct frequency.

We found evidence for activity of an error-prone polymerase in human cells. About 20-30% of the mutant plasmids recovered from normal cells had multiple base substitution mutations. These were found to be clustered in a 150 base pair region, while an adjacent region of similar size was found to be free of mutations. These multiple mutations were found in less than 4% of the plasmids from the XP-A cells, but increased in frequency to nearly 66% if a single-strand nick was introduced prior to transfection. These results may indicate the action of an error-prone polymerase in human cells that acts over a limited region. This activity could play a role in generation of immunoglobulin gene diversity.

Cells from patients with Bloom's syndrome (BS) were reported by Lindahl's group to have in vitro DNA ligase I activity 30 - 50% of normal. This enzyme is believed to be important in DNA replication and to have the unique ability to ligate double-stranded DNA pieces with blunt ends (in addition to being able to ligate pieces with overlapping ends). We utilized plasmid vectors to determine if this defect was also present in vivo. The non-replicating plasmid, pRSVcat, and the replicating plasmid, pZ189, was cut with restriction endonucleases to produce linear plasmids with either blunt or overlapping ends. These were transfected into BS and normal fibroblasts and lymphoblastoid cell lines, and recovery of CAT activity and ability of pZ189 to transfer ampicillin resistance was measured. No difference was found between the ability of BS and normal cells to express CAT activity with the linearized, non-replicating plasmid. However, there was a significantly reduced ability of the BS cells to repair pZ189 which must pass through a circular stage in order to replicate. In addition there was an approximately 10-fold higher frequency of mutations recovered in pZ189 replicated in BS in comparison to replication in normal cells. These results are consistant with the thesis that the ligase defect is present in vivo in BS.

A survey of the literature, our own unpublished data and unpublished information from others using pZ189 for mutagenesis studies, was performed to identify the sensitivity of the supF gene for detection of mutations. We determined that nearly 90% of the 85 base pairs in the tRNA region of the supF gene, when mutated, will reduce suppressor activity and thus be detectable. The supF gene is thus an excellent marker for detection of mutations in shuttle vector plasmids.

We identified a unique point mutation in the supF gene of pZ189 (A:T to T:A at position 136) that results in the plasmid being 5- to 80-fold more sensitive to inhibition of transforming ability by UV-B radiation (295 nm) than the wild-type plasmid. A non-dimer photoproduct appears to be responsible since the hypersensitivity is still present after removal of cyclobutane dimers by

enzymatic photoreactivation. This plasmid is not hypersensitive to UV-C radiation (254 nm). The mechanism of this hypersensitivity is under investigation.

Oxidative damage to DNA was examined by treating pZ189 with low levels (10^{-3} to 10^{-6} M) of copper and hydrogen peroxide. Cu(II) plus H_2O_2 was found to create DNA single-and double-strand breaks and to inactivate plasmid transforming ability at concentrations of each agent alone that did not alter the plasmid. Sequence analysis revealed site-specific DNA damage at sites of two or more adjacent guanosine residues.

Nevus cells and melanocytes were cultured from a patient with xeroderma pigmentosum who had defective DNA repair. We found a similar reduction in DNA repair in the cultured pigment cells as was present in the fibroblasts of this patient. This indicates that the DNA repair defect may be implicated in the high rate of melanomas in XP patients.

In a collaborative study designed to detect carriers of the XP gene, blood samples were obtained from patients with XP and from their parents (obligate heterozygotes). Coded samples were cultured and then exposed to X-rays. The frequency of chromosome breaks and gaps introduced by X-irradiation during the G2 phase of the cell cycle was determined. XP heterozygotes were found to have a level of chromosome breaks and gaps intermediate between the XP patients and the normal controls. This assay may thus form the basis for a test of XP heterozygote detection.

The XP Registry has collected clinical information on about 100 patients. Literature on XP has been sent to more than 500 physicians who requested information. Clinical consultation concerning diagnosis or treatment of XP patients is provided by telephone to physicians who contact NIH at the rate of about 2-3 per month.

A collaborative 3-year clinical trial of skin cancer prevention utilizing oral 13-cis retinoic acid (Accutane) in patients with XP was completed. XP patients were selected who had a documented high rate of skin tumor formation. All pre-existing tumors were surgically removed and high dose (2 mg/kg/da) oral 13-cis retinoic acid (Accutane) was given for 2 years. The drug was then stopped for an additional 1 year observation period. In the 5 XP patients who completed the study there was a greater than 60% reduction in frequency of skin cancer formation during the time of drug treatment and an increase to pre-treatment levels when the drug was withdrawn. All patients experienced the side effect of mucocutaneous toxicity, in addition some patients developed elevated liver function tests, elevated triglycerides and calcification of the tendons and ligaments. Patients who have completed the high dose protocol are presently being treated with a lower dose to determine if the effectiveness will remain with less toxicity. This is the first controlled trial to demonstrate skin cancer chemoprophylaxis in humans.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01OP05086-10 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Phenotyping of Cytochrome P-450s in Animals and Human Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	S.S. Park	Expert	LMC	NCI
Others:	H.V. Gelboin	Chief	LMC	NCI
	D. Ray	Chemist	LMC	NCI
	L. M. Anderson	Special Expert	LCC	NCI

COOPERATING UNITS (if any)

Univ. of Oulu, Finland (O. Pelkonen); IARC, Lyon, France (E., Hietanen, H. Bartsch); Univ. Hosp., Sweden (A. Rane); VA Med. Center, Cleveland, Ohio (W. A. Khan, H. Mukhtar)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Human individuals differ in their capability to metabolize and detoxify drugs and xenobiotics. Cytochrome P-450 (P-450) is a key component of the mixed function oxidase system which metabolizes many drugs and xenobiotics. The type and quantity of specific forms of cytochrome P-450 determine the extent of activation and/or detoxification of particular substrates. Monoclonal antibodies (MAbs) to P-450 were prepared and used for identification and phenotyping of P-450 in tissues and organs of mice, rats and humans by radioimmunoassay (RIA), Western blotting, histochemical staining and inhibitory effects on catalytic activity. When C57BL/6 mice were treated with 3-methylcholanthrene (MC), MC inducible P-450s (MC-P-450s) were identified in liver, lung and kidney but not in those organs of DBA/2 mice. When neonatal rats were treated with the carcinogenic compound 3-nitrofluoranthene, a nitrated product of environmental fluoranthene, MC-P-450s were found to be the predominant forms. In untreated rats, the MC-P-450s were increased during the first month after birth. The MC-P-450s were also identified in human fetal and adult livers. The inducibility of MC-P-450 was modulated by diet and increased by feeding rats a high cholesterol diet. These results indicate that epitope-specific MAbs are very useful probes for identification of P-450s in various tissues and for the study of P-450 changes during development and under different environmental conditions.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S S. Park	Expert	LMC	NCI
H.V. Gelboin	Chief	LMC	NCI
D. Ray	Chemist	LMC	NCI
L.M. Anderson	Special Expert	CC DCE	NCI

Objectives:

The objective of this work is to use monoclonal antibodies (MAbs) to identify cytochrome P-450 in tissues and organs of animals and humans at different developmental as well as sexual stages, and under different diet and environmental conditions. The project is designed to understand the role of P-450s in individual differences in sensitivities to drugs and chemical carcinogens.

Methods Employed:

MAbs were prepared against different rat liver microsomal P-450 isozymes. Microsomal and histological preparations were made from liver, intestine, lung, kidney and skin of mouse, rat and human. Histological preparations from various tissues were incubated with specific MAbs and specific cytochrome P-450s were identified with avidin-biotin peroxidase systems. Microsomal preparations were utilized for the identification of P-450 by immunoblotting and immunoinhibition of catalytic activity.

Major Findings:

When aryl hydrocarbon hydroxylase (AHH)-inducible C57/BL/6 mice were treated with 3-methylcholanthrene, MC-inducible MAb 1-7-1-specific P-450s (MC-P-450s) were identified in liver, lung and kidney, but not in those organs of AHH non-inducible DBA/2 mice. When neonatal rats were treated with carcinogenic 3-nitrofluoranthene, a nitrated product of environmental fluoranthene, MC-P-450s were detected as MAb 1-7-1-specific forms. In untreated rats the MC-P-450s were increased during the first month after birth. The MC-P-450s were also identified in human fetal and adult livers. However, the inducibility of MC-P-450 was modulated by diet and enhanced by a high (2%) cholesterol diet. These results indicate that epitope-specific MAbs are very useful probes for phenotyping different forms of P-450 and understanding the individual differences in xenobiotics metabolism and sensitivity to chemical carcinogens during growth and sexual development and under different diets and environments.

Publications:

Anderson LM, Ward JM, Park SS, Jones AB, Junker JL, Gelboin HV, Rice JM: Immunohistochemical determination of inducibility phenotype with a monoclonal antibody to a methylcholanthrene-inducible isozyme of cytochrome P-450. Cancer Res 1987;47:6079-85.

Khan WA, Asokan P, Park SS, Gelboin HV, Bickers DR, Mukhtar H: Use of monoclonal antibodies to characterize P-450 dependent mixed function oxidase system to nitrofluoranthenes. Carcinogenesis 1987;8:1679-84.

Hietanen E, Ahotupa M, Bereziat JC, Park SS, Gelboin HV, Bartsch H: Monoclonal antibody characterization of hepatic and extrahepatic cytochrome P-450 activities in rats treated with phenobarbital or methylcholanthrene and fed various cholesterol diets. Biochem Pharmacol 1987;36:3973-80.

Pacifici GM, Park SS, Gelboin HV, Rane A. 7-ethoxycoumarin and 7-ethoxyresorufin O-deethylase in human fetal and adult liver: studies with monoclonal antibodies. Dev Pharmacol Ther (In Press).

Pacifici GM, Park SS, Gelboin HV, Rane A: Ontogenic development of 7-ethoxycoumarin and 7-ethoxyresorufin O-deethylase in the rat and effect of monoclonal antibodies. Pharmacol Toxicol 1988;62:101-3.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05125-08 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Preparation and Characterization of Monoclonal Antibodies to P450

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S.S. Park	Expert	LMC	NCI
Others:	H.V. Gelboin	Chief	LMC	NCI
	D. Ray	Chemist	LMC	NCI

COOPERATING UNITS (if any)

Dana-Farber Cancer Institute, Boston, MA (D. J. Waxman); Univ. of Connecticut, CT (J. S. Schenkman)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Numerous xenobiotics as well as endobiotics such as prostaglandin, fatty acids and steroids are metabolized by mixed function oxidase (MFO) systems. The system consists of three components: cytochrome P-450 (P-450), NADPH-P-450-reductase and phospholipids. Cytochrome b5 (b5) is an additional electron carrier. P-450 is a key component of the MFO system and substrate specificity is directly associated with individual P-450 isozymes. Monoclonal antibodies (MAbs) were prepared to P-450 isozymes, b5 and reductase in order to identify and characterize each component of MFO systems in tissues and organs and to understand their role in differential xenobiotic metabolism and sensitivity to chemical carcinogens. MAbs to P-450 2c/RLM5 were found to be regiospecific in steroid metabolism. Hybridization between myeloma cells and spleen cells derived from mice immunized with b5 yielded 29 independent hybrid clones which produced either IgG1(κ), IgG2b(κ), IgG3(κ) or IgM (κ) type MAbs. Out of 29 hybrids, 15 clones produced MAbs which were inhibitory up to 77% on b5-reductase activity. Eleven MAbs were specifically bound to a liver microsomal protein which corresponded to the chemically purified b5 on Western blots. These MAbs would be very useful for identification of each component of MFO systems in tissues and organs and analysis of their roles in MFO systems.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. S. Park	Expert	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI
D. Ray	Chemist	LMC	NCI

Objectives:

Cytochrome P-450 (P-450), NADPH-P-450 reductase and phospholipid are the major three components of mixed function oxidase (MFO) systems. Cytochrome b5 (b5) is an additional electron carrier. MFO systems metabolize many xenobiotics and endobiotics such as prostaglandins, fatty acids and steroids. In order to identify the components of MFO systems in tissues and organs, monoclonal antibodies (MAbs) to P-450, reductase and b5 were prepared for use as molecular probes and to determine the role of each component.

Methods Employed:

Balb/c female mice were immunized with purified P-450, b5 or reductase. The primed spleen cells were fused with myeloma cells and hybrid cells were grown in selective medium (HAT) and screened by radioimmunoassay (RIA) against each immunogen. The specificity of MAbs was determined by RIA, double immunodiffusion analysis, and Western blotting. The effect of MAbs on catalytic activity was also measured in the microsomal and reconstituted reaction systems of purified P-450, b5 and reductase systems.

Major Findings:

MAbs to P-450 2c/RLM5 were found to be regiospecific in steroid metabolism and they are distinct from other P-450s which metabolize steroid. Hybridization between myeloma cells and spleen cells derived from mice immunized with b5 yielded 29 independent hybrid clones which produced either IgG1(κ), IgG2b(κ), IgG3(κ), or IgGM(κ) type MAbs. Out of 29 hybrids, 15 clones produced MAbs which inhibited b5-reductase activity up to 77%, indicating that interaction between b5 and b5-reductase was interfered with by MAbs binding to b5. Eleven MAbs were specifically bound to a liver microsomal protein which corresponded to the chemically purified b5 on Western blots. Fifteen independent hybrid clones producing MAbs to P-450-NADPH-reductase were also obtained and are under investigation for their specificities. These MAbs would be very useful for identification of the components in tissues and organs and analysis of their roles in MFO systems.

Publications:

Dong Z, Hong J, Ma Q, Li D, Bullock J, Gonzalez FJ, Park SS, Gelboin HV, Yang CS. Mechanism of induction of cytochrome P-450ac (P-450j) in chemically-induced and spontaneously diabetic rats. Arch Biochem Biophys (In Press).

Gelboin HV, Park SS, Battula N. DNA recombinant and monoclonal antibody detected methods for determining cytochrome P-450 specificity. Biochem Pharmacol 1988;37:97-8.

Malaveille C, Brun G, Park SS, Gelboin HV, Bartsch, H. A monoclonal antibody against cytochrome P-450 enhances mutagenic activation of N-nitrosodimethylamine by mouse liver S9: Studies on the mode of action. Carcinogenesis 1987;8:1775-79.

Song BJ, Veech RL, Park SS, Gelboin HV, Gonzalez FJ. Structure and regulation of the ethanol-inducible cytochrome P-450j. In: Stimmel, B, ed. Advances in alcoholism and substance abuse. New York: Academic Press (In Press).

Waxman DJ, Lapenson DP, Park SS, Attisano C, Gelboin HV. Monoclonal antibodies to rat hepatic cytochrome P-450: P-450 form specificities and use as inhibitory probes for cytochrome P-450-dependent steroid hydroxylations. Mol Pharmacol 1987;32:615-24.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05208-08 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Phenotyping of Human Cytochrome P-450

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Lab	Institute
PI:	Fred K. Friedman	Research Chemist	LMC	NCI
Others:	Haruko Miller	Bio. Lab. Tech.	LMC	NCI
	Jewell Wilson	Biotech. Fellow	LMC	NCI
	Sang S. Park	Sr. Staff Fellow	LMC	NCI
	Harry V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

University of Oulu, Finland (O. Pelkonen)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.2	0.6

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The individual forms of cytochrome P-450 display unique substrate specificity and reactivity profiles toward a variety of drugs and carcinogens. Differences in cytochrome P-450 phenotype may relate to individual differences in sensitivity to certain drugs and susceptibility to chemical carcinogenesis. Monoclonal antibodies (MAbs) to various forms of rat liver cytochrome P-450 have been used as specific probes for the cytochrome P-450s in human liver. Western blot analysis with MAb to rat ethanol-induced P-450, a form with high nitrosamine metabolizing activity, detected a related P-450 in human liver microsomes. Individual variation in the level of this P-450 was observed. A radioimmunoassay (RIA) to this P-450 was developed with MAb 1-98-1. Using this assay we were able to detect and quantitate the MAb-specific cytochrome P-450 in human liver microsomes. This human P-450 was immunopurified and analyzed by peptide maps and amino acid sequencing.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Fred K. Friedman	Research Chemist	LMC	NCI
Haruko Miller	Bio. Lab. Tech.	LMC	NCI
Jewell Wilson	Bio. Tech. Fellow	LMC	NCI
Sang S. Park	Sr. Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

The profile of cytochrome P-450s in a tissue may influence metabolism that results in either activation or detoxification of potential carcinogens. Monoclonal antibodies (MAbs) are utilized to identify and characterize the P-450s in human tissues in order to relate P-450 phenotype to individual differences in sensitivity to drugs and carcinogens.

Methods Employed:

MAbs were prepared to a variety of rat liver microsomal cytochrome P-450s. Human liver microsomes were subjected to Western blot analysis, using these MAbs for immunodetection. A competitive radioimmunoassay (RIA) was developed using radiolabeled MAbs and rat liver microsomes as competing antigen. Sepharose-MAb immunoadsorbents were used to immunopurify human P-450 from liver microsomes.

Major Findings:

Human liver microsomes were analyzed by Western blots using MAbs to various rat P-450s. MAbs to the ethanol-induced P-450et and pregnenolone 16 -carbonitrile-inducible PCN-P-450 forms detected cross-reactive P-450s in these microsomes. Differences in band intensity were observed among the samples obtained from different individuals.

A competitive RIA using MAb 1-98-1 to rat P-450et was developed and detected variable levels of MAb-specific P-450 in the human liver microsome samples.

P-450et has been immunopurified using MAb 1-98-1 and analyzed by peptide mapping and amino acid sequence analysis. The human and rat P-450s displayed primary structural differences by both of these criteria.

P-450et has been partially purified from rat liver microsomes using a combination of hydrophobic and ion-exchange chromatography. A high performance liquid chromotographic (HPLC) method for more efficient isolation of this P-450 is being developed.

Two P-450-dependent activities, 7-ethoxyresorufin O-deethylase (ERDE) and aryl hydrocarbon hydroxylase (AHH) were measured in human fetal liver microsomes. MAb 1-7-1 to a 3-methylcholanthrene-induced P-450 and MAb 2-66-3 to phenobarbital-induced rat P-450 were used to measure the contributions of MAb-specific P-450 to the total activities. MAb 1-7-1 inhibited fetal hepatic ERDE activity to a variable extent (from 0 to 100%, 34 in average), but had only negligible effects on AHH activity. MAb 2-66-3 inhibited ERDE activity by 18% and AHH activity by 12%. In comparison with earlier studies on adult liver and placental microsomes, the present results with fetal liver suggest differences in P-450-associated ERDE and AHH activities between these tissues, which might be due to different tissue-specific isoenzyme patterns.

<u>Publications</u>:

Pasanen M, Pelkonen O, Kauppila A, Park SS, Friedman FK, Gelboin HV. Characterization of human fetal hepatic cytochrome P-450 associated 7-ethoxyresorufin O-deethylase and aryl hydrocarbon hydroxylase activities by monoclonal antibodies. Dev Pharmacol Ther 1987;10:125-32.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05318-06 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structure and Regulation of Cytochrome P-450

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Fred K. Friedman	Research Chemist	LMC	NCI
Others:	Richard C. Robinson	Biologist	LMC	NCI
	Haruko Miller	Bio. Lab. Tech.	LMC	NCI
	Sang S. Park	Expert	LMC	NCI
	Harry V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

Benedict College, Columbia, SC (K. Alston)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	0.8	1.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The cytochromes P-450 metabolize a wide array of compounds, including xenobiotics such as drugs and carcinogens, and endogenous compounds such as steroids and prostaglandins. The focus of this project is the characterization of structure-function relationships and regulation of the multiple forms of this enzyme. Monoclonal antibodies (MAb's) to rat P-450's are a tool in these studies. Using a MAb to a nitrosamine-metabolizing ethanol-inducible rat liver P-450, a P-450 has been immunopurified from both rat and human liver. These differed in primary structure as evidenced by different amino terminal sequences and peptide maps. The membrane organization of P-450's was probed by a crosslinking study which revealed specific association among certain forms of P-450. Such interactions may influence the disposition of P-450 substrates in secondary metabolism. The induction of rat liver microsomal and nuclear envelope P-450's by 2-acetyl-aminofluorene (AAF) was immunochemically probed with MAb's to P-450c and P-450d. These P-450's were induced in both microsomes and envelopes, although AAF decreased total P-450 in envelopes but not microsomes.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional personnel Engaged on this Project:

Fred K. Friedman	Research Chemist	LMC	NCI
Richard C. Robinson	Biologist	LMC	NCI
Haruko Miller	Bio. Lab. Tech.	LMC	NCI
Sang S. Park	Expert	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

To characterize the structure-function relationships and regulation of the multiple forms of cytochrome P-450 in animal and human tissues.

Methods Employed:

Monoclonal antibodies (MAbs) were prepared to several rat liver P-450s. MAbs linked to Sepharose were used for immunopurification of P-450s from tissue microsomes. Cross-linking of microsomal membrane proteins was carried out with the cross-linker sulfosuccinimidyl(4-azidophenyldithio)propionate. Cross-linked P-450s were identified after disulfide cleavage. Analytical methods employed include gel electrophoresis, Western blots, spectral analyses, and amino acid sequencing. A laser flash photolysis apparatus was assembled from various components and used to monitor the kinetics of carbon monoxide (CO) binding to P-450.

Major Findings:

Human P-450s have not been studied as extensively as the rat liver P-450s owing to the relative unavailability of human tissues and difficulty in purification by conventional methods. The ethanol-inducible form of P-450, P-450et, has high nitrosamine metabolizing activity and may play a role in metabolism of these carcinogens in humans. This P-450 was immunopurified from both rat and human liver using MAb 1-98-1. These P-450s differed in molecular weight, peptide maps, and N-terminal sequence.

The distribution of ethanol-induced P-450 in rabbit tissues was studied by catalytic activity measurements and immunoblots using antibody to rat P-450et. A related P-450 was observed in liver and kidney, but was undetectable in lung.

Organization of P-450s in the microsomal membrane was studied by chemical cross-linking of associated membrane proteins and subsequent immunopurification with MAbs to P-450. The results showed that P-450c was not monomeric, but was associated with other proteins, including the pregnenolone 16α-carbonitrile inducible form of P-450. Since P-450 substrates are often acted upon by multiple P-450s, the distribution of metabolites may be influenced by interactions among specific P-450s.

The influence of dietary 2-acetylaminofluorene (AAF) on the P-450 content of rat liver microsomes and nuclear envelope was immunochemically probed with monoclonal and polyclonal antibodies to P-450c and P-450d. P-450d, but not P-450c, was immunodetected in microsomes and nuclear envelopes from untreated rats. An AAF diet elevated the levels of both P-450c and P-450d in each of these fractions. Since dietary AAF markedly reduces total P-450 in nuclear envelopes but not microsomes, the results demonstrate selective induction and depression of induced P-450s, and provides additional evidence for independent regulation of the drug metabolizing system in nuclear envelope and microsomes.

The active site structure of microsomal P-450 was examined in binding studies using the substrate benzphetamine in conjunction with MAbs to the P-450 that metabolize this substrate. Flash photolysis experiments of CO recombination to the P-450 heme yielded parallel kinetic data on active site dynamics. The combined results show that a MAb that inhibits P-450 activity more effectively limits active site accessibility to a CO probe than noninhibitory MAbs.

Publications:

Ueng TH, Friedman FK, Miller H, Park SS, Gelboin HV, Alvares AP. Studies on ethanol-inducible cytochrome P-450 in rabbit liver, lungs and kidneys. Biochem Pharmacol 1987;36:2689-91.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CP05436-04 LMC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Expression of Cytochrome P-450s and Their Role in Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H.V. Gelboin	Chief	LMC	NCI
Others:	N. Battula	Expert	LMC	NCI
	G.K. Townsend	Biologist	LMC	NCI
	F.J. Gonzalez	Sr. Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)
Dept. of Pharmacology, Medical School, State Univ. Pennsylvania, Hershey, PA (E. Vesell); Dept. of Pharmacology, USUHS, Bethesda, MD (S. K. Yang); Roswell Park Memorial Institute, Buffalo, NY (H. Gurtoo)

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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

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

Cytochrome P-450s metabolize xenobiotics such as drugs and carcinogens as well as endobiotics such as steroids and prostaglandins. Multiple forms of enzymes are expressed constitutively or after administration of inducers. A single cytochrome P-450 may metabolize multiple substrates and a single substrate may be acted upon by either a single or several cytochrome P-450s. Cytochrome P-450 may engage normal detoxification or catalyze deleterious carcinogen or mutagen activation. This project has used previously cloned cDNA of two P-450s to construct the two cDNAs into two recombinant viruses, infectious vaccinia and retrovirus. These vectors express the incorporated cDNA into single P-450s when infected into host cells. In both the vaccinia and retrovirus systems, proteins were expressed with the appropriate molecular weights and exhibit enzyme activity typical of each P-450. Studies are in progress to determine the specificity of each expressed protein in the 1) stereochemistry of polycyclic aromatic hydrocarbon metabolism; (2) aflatoxin metabolism; and (3) the metabolism of aminopyrine, antipyrine and theophylline. The results from the above studies indicate a variability of specificity depending on the substrate, and a high degree of specificity for one form of P-450 with some substrates and cross-reactivity for both P-450s with other substrates. In a different project various expression systems are being developed with a large variety of cDNAs and different vectors. Success in this effort will enable us to define the contribution of each of these enzymes to mutagenesis and cell transformation mediated by chemical carcinogens.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

H.V. Gelboin	Chief	LMC	NCI
N. Battula	Expert	LMC	NCI
G. K. Townsend	Biologist	LMC	NCI
F. Gonzalez	Sr. Staff Fellow	LMC	NCI

Objectives:

The goal of this project is to (a) express from recombinant vaccinia virus and recombinant retrovirus vectors two individual cytochrome P-450s, P1-P450 and P3-P450, into enzymatically active proteins; (b) to analyze the expressed proteins for enzymatic activities; and (c) to test for mutations and cell transformation after exposure to carcinogens of cells expressing the P-450 enzymes. To determine specificity and stereospecificity of the expressed P_1-450 and P_3-450 for benz(a)anthracene, aflatoxin, aminopyrine, antipyrine, theophylline and for a variety of other substrates.

Methods Employed:

Construction of recombinant viruses requiring recombinant DNA methodology, DNA separation procedures, DNA transfections, cell culture techniques, virological procedures and genetic selection of cells and viruses. Detection and isolation of expression products requires subcellular fractionation procedures, protein separations by electrophoresis and chromatography, immunological procedures employing monoclonal and polyclonal antibodies. Functional evaluation of the expressed proteins require a variety of enzymatic assays such as aryl hydrocarbon hydroxylase, ethoxyresorufin O-deethylase, 7-ethoxycoumarin O-deethylase; and acetanilide hydroxylase, benz(a)anthracene, aflatoxin, theophylline, aminopyrine and antipyrine metabolite assays.

Major Findings:

Infectious recombinant vaccinia viruses and retroviruses containing selectable genetic markers and full-length cDNAs for mouse cytochromes, P1-450 and P3-450, were constructed and characterized. Cells infected with the virus efficiently expressed their respective proteins. The newly synthesized protein products are translocated into the microsomes and their characterization by immunochemical analysis indicates that the size of the polypeptides expressed were indistinguishable from the authentic cytochrome P1-450 and P3-450. Functional analysis of each of the proteins by spectral and enzymatic analysis indicates that the expressed proteins have incorporated heme and holoenzyme and displayed catalytic activities characteristic of the respective cytochrome P-450 enzymes.

The P1-P450 expressed from the vaccinia vector shows a high degree of specificity in the formation of a single enantiomer of a benz(a)anthracene metabolite. Similar findings were observed with the P_3-450 for an aflatoxin metabolite.

Antipyrine and aminopyrine were metabolized actively by both the P_1-450 and P_3-450, whereas theophylline was metabolized only by P_3-450.

Publications:

Gelboin HV, Gonzalez FJ, Park SS, Sagara J, Battula N. Cytochrome P-450 function analyses with monoclonal antibodies and cDNA expression vectors. In: Torino, FB, ed. Proceedings international meeting on chemical carcinogenesis. New York: Plenum Press. (In Press).

Gelboin HV, Park SS, Battula N. DNA recombinant and monoclonal antibody detected methods for determining cytochrome P-450 specificity. Biochem Pharmacol 1988;37:97-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05520-02 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structure and Regulation of N-Nitrosodimethylamide Demethylase Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Frank Gonzalez	Sr. Staff Fellow	LMC	NCI
Others:	Harry V. Gelboin	Chief	LMC	NCI
	Morio Umeno	Visiting Fellow	LMC	NCI
	O. Wesley McBride	Section Chief	LB	NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Microbiology, NIAID (C. Kozak); Laboratory of Metabolism and Molecular Biology, NIADA (B.J. Song); Department of Biochemistry, University Medical and Dental School of New Jersey, Newark, N Jersey (C.S. Yang)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The principal enzyme involved in the metabolism and activation of small nitrosamine compounds is the ethanol-inducible P450IIE1. This enzyme is induced by ethanol, fasting and diabetes through a post-transcriptional mechanism. The IIE1 gene becomes transcriptionally activated soon after birth.

To study the regulation of the IIE1 gene, rat and human genomic clones were isolated and completely sequenced. Both genes contained nine exons similar to other genes in the P450II gene family. Significant nucleotide similarity between both genes was detected within 150 bp of the genes' cap-site. The promoter regions were able to drive transcription of the luciferase gene when transfected into rat hepatoma cells. The IIE1 was localized to mouse chromosome 7 and human chromosome 10 by use of the somatic cell hybrids mapping strategy. Analysis of the cytosine methylation status of the IIE1 gene revealed that sites upstream of the rat IIE1 transcription start site are demethylated coincident with transcriptional activation within 24 hours after birth. Methylation changes in the IIE1 gene were also detected in humans during development. A Taq I restriction fragment length polymorphism was detected in the human IIE1 gene by use of the IIE1 cDNA probe.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Frank Gonzalez	Sr. Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Morio Umeno	Visiting Fellow	LMC	NCI
O. Wesley McBride	Section Chief	LB	NCI

Objectives:

1. Determine the structure of the rat and human IIE1 genes.

2. Determine the chromosome localization of the rat and human IIE1 genes.

3. Analyze human subjects for restriction fragment length polymorphisms.

4. Analyze changes in cytosine methylation patterns in the IIE1 genes during development.

5. Western blot quantitation of IIE1 in human liver.

Methods Employed:

Rat and human genomic libraries were constructed using a EMBL3 vector. The libraries were screened using the rat and human IIE1 cDNA probes. DNA sequence was determined by shotgun cloning into M13 and by the Sanger deodeoxy sequencing method. Sequence data were assembled and analyzed by use of the Beckman Microgenie program. The chromosomal localization in mouse and human was determined by the somatic cell hybrids mapping strategy. Restriction fragment length polymorphisms were determined on lymphocyte DNAs by Southern blot analysis using the IIE1 cDNA. Methylation analysis was determined using the cytosine methylation-sensitive enzymes, HpaII and HhaI. Western blot analysis was carried out on human liver specimens using the anti-rat IIE1 antibody.

Major Findings:

The complete sequences of the rat and human genes were determined. The rat and human IIE1 gene spanned 10,373 and 11,413 base pairs, respectively, and contained 9 exons. The IIE1 gene is located on mouse chromosome 7 and human chromosome 10. Comparison of the upstream sequence of the rat and human genes revealed high nucleotide similarities within 150 base pairs of the polymerase start site.

The rat IIE1 gene becomes transcriptionally activated within one day after birth. Western blot analysis of human liver specimens also revealed that the IIE1 protein is not expressed until after birth. Analysis of the methylation pattern of the rat IIE1 gene was carried out using the methylation-sensitive enzymes, HpaII and HhaI. Coincident with transcriptional activation of the IIE1 gene was

the demethylation of cytosine residues in the 5' upstream region. Cytosines in the middle and 3' region of the IIE1 gene remained hypermethylated regardless of the transcription state of the gene. It is still unclear whether this demethylation phenomenon is the cause of or result of the developmental transcriptional activation of the IIE1 gene.

Publications:

Umeno M, Song BJ, Kozak C, Gelboin HV, Gonzalez FJ. The rat P450IIE1 gene: complete intron and exon sequence, chromosome mapping, and correlation of developmental expression with specific 5' cytosine demethylation. J Biol Chem 1988;263:4956-62.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05521-02 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Polymorphic Drug Oxidation: The Human and Rat Debrisoquine 4-Hydroxylase Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Frank Gonzalez	Sr. Staff Fellow	LMC	NCI
Others:	Harry V. Gelboin	Chief, LMC	LMC	NCI
	Shioko Kimura	Visiting Associate	LMC	NCI
	Morio Umeno	Visiting Fellow	LMC	NCI
	Eiji Matsunaga	Visiting Fellow	LMC	NCI
	O. Wesley McBride	Section Chief	LB	NCI
	Roderick Moore	Biological Aid	LMC	NCI

COOPERATING UNITS (if any)

Argonne National Laboratory, Argonne, IL (James P. Hardwick); Biocenter, University of Basel, Switzerland (Urs A. Meyer, Radek Skoda)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Individual subjects vary considerably in their abilities to metabolize drugs. In addition, genetic influences are thought to govern individual susceptibility to chemically-induced cancers. The mixed function monooxygenase system is composed of multiple forms of P450s that are the principle enzymes associated with drug and carcinogen metabolism. These enzymes can serve to detoxify and hasten the elimination of foreign agents or they can activate inert chemicals to harmful electrophilic metabolites that damage DNA and initiate the carcinogenic process. We have begun to study polymorphic drug oxidation in humans by establishing the mechanism of the debrisoquine 4-hydroxylase polymorphism. This polymorphism effects from 5% to 10% of the North American and European Caucasian population. These individuals, referred to as poor metabolizers, or PMs, cannot metabolize debrisoquine and many other related drugs, while the rest of the population, called extensive metabolizers, or EMs, rapidly hydroxylate this drug resulting in the inactivation of its therapeutic form. To examine the mechanism of this defect we have isolated the rat P450 that metabolizes debrisoquine, designated IID1, and prepared a specific antibody that reacts with both the rat and human IID1 protein. We also isolated the human IID1 genes. By analysis of lymphocyte DNA we are able to phenotype PMs using Southern blots and the IID1 cDNA. The human IID1 locus contains three related genes and is located on the long arm of chromosome 22.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional personnel Engaged on this Project:

Frank Gonzalez	Sr. Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Shioko Kimura	Visiting Associate	LMC	NCI
Morio Umeno	Visiting Fellow	LMC	NCI
Eiji Matsunaga	Visiting Fellow	LMC	NCI
O. Wesley McBride	Section Chief	LB	NCI
Roderick Moore	Biological Aid	LMC	NCI

Objectives:

1. Isolate and characterize human IID1 cDNA.

2. Determine the mechanism of the debrisoquine 4-hydroxylase polymorphism.

3. Determine the chromosomal location of the IID1 locus.

4. Isolate and sequence the genes in the IID1 locus.

5. Develop a method to phenotype poor metabolizer of debrisoquine.

Methods Employed:

1. Construction of cDNA libraries in the λgt11 vector was carried out by reverse transcription of mRNA and strand replacement.

2. Shotgun cloning into m13 phage and sequencing by the Sanger dideoxynucleotide method. Sequence data were compiled and analyzed by use of the Beckman Microgenie program.

3. Northern blotting was carried out by use of formaldehyde-agarose gels and the hybridization technique of Church and Gilbert.

4. Genomic cloning was performed using the λEMBL3 vector and partial Sau3A digested human DNA.

5. Southern blotting was carried out by alkaline transfer of DNA to nylon filters and hybridization using 50% formamide.

6. Phenotyping of individuals for debrisoquine metabolism was done by urine analysis after a sub-therapeutic dose of drugs.

Major Findings:

Western immunoblot analysis of 29 human liver samples phenotyped in vitro for the presence of the debrisoquine defect revealed that the IID1 protein was absent in specimens that did not have debrisoquine 4-hydroxylase or bufuralol 1'-hydroxylase activity.

The human and rat debrisoquine 4-hydroxylase cDNAs, designed hIID1 and rIID1 respectively were sequenced and their deduced amino acid sequences were 73% similar. The human cDNA was found to code for IID1 that catalyzed debrisoquine 4-hydroxylase and bufaralol 1'-hydroxylase activities when inserted into an expression vector and transfected into COS-cells.

Direct cloning and sequencing of RNA transcripts produced in livers in which the IID1 protein is absent revealed the presence of mutant IID1 genes. Two mutant transcripts were identified that were the result of mutations affecting pre-mRNA splicing. One transcript contained the IID1 5th intron and another transcript contained the 6th intron. These variant transcripts are incapable of producing functional IID1 protein.

By use of somatic cell hybrids of human and rodent cells in which one or more human chromosomes are retained and by Southern hybridization of the DNA with the IID1 cDNA probe, the IID1 locus was mapped to the long arm of chromosome 22. Further studies using hybrids containing fragments of chromosome 22 revealed that the IID1 gene was linked to the sis oncogene.

The IID1 genomic locus was directly cloned and sequenced. A genomic library was constructed from lymphocyte DNA of a homozygous extensive metabolizer. The IID1 gene and two closely related genes were isolated and sequenced. All three genes possessed nine exons and were located on a single contiguous 50 kbp fragment on chromosome 22. The two related genes, IID5 and IID6, shared 95% and 89% deduced amino acid similarity, respectively, with the IID1 gene. IID5 was found to have an insertion of a single base in its second exon resulting in an amino acid reading frame shift. The IID6 gene had two independent base deletions causing protein coding frame shifts. These two genes, therefore, appeared to be recently inactivated P450 genes. Neither gene was found to be expressed in over 120 liver specimens examined.

To identify restriction fragment length polymorphisms (RFLPs) linked to the IID1 gene, we examined a large population of unrelated PMs and EMs with five families having at least one PM proband. These individuals were phenotyped for debrisoquine metabolism and their DNAs were examined by restriction endonuclease digestion. RFLPs were found linked to two separate mutant IID1 allele. About 75% of all PMs could be phenotyped by RFLP analysis.

Publications:

Gonzalez FJ, Skoda RC, Kimura S, Umeno M, Zanger UM, Nebert DW, Gelboin HV, Hardwick, JP, Meyer UA. Characterization of the common genetic defect in humans deficient in debrisoquine metabolism. Nature 1988;331:442-6.

Gonzalez FJ, Vilbois F, Hardwick JP, McBride OW, Nebert DW, Gelboin HV, Meyer UA. Human debrisoquine 4-hydroxylase (P450IID1): cDNA and deduced amino acid sequence and assignment of the CYP2D locus to chromosome 22. Genomics (In Press).

Skoda RC, Gonzalez FJ, Demierre A, Meyer UA. Identification of mutant alleles of the P450DB1 gene associated with deficient metabolism of debrisoquine. Proc Natl Acad Sci USA (In Press).

Patents:

Gonzalez FJ, Gelboin HV, Hardwick JP and Meyer UA. U.S. Patent (pending): A Genetic Probe for the Human Debrisoquine 4-hydroxylase Defect.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05522-02 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structure and Characterization of Human Thyroid Peroxidase

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Shioko Kimura Visiting Associate LMC NCI

Others: None

COOPERATING UNITS (if any)

Miyazaki Medical College Hospital, Miyazaki, Japan (Sachiya Ohtaki, Tomio Kotani)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Peroxidases are widely distributed throughout plants, animals, and microorganisms and carry out a variety of biosynthetic and degradative functions related to the consumption of hydrogen peroxide. Free radicals are produced during this reaction and can bind irreversibly to DNA, which is thought to be a cause of tumorigenesis. The similarity of the peroxidative reaction to one of a series of steps in the reaction of cytochrome P450 with substrates has been well documented. This may suggest the evolutionary relationship between cytochrome P450s and peroxidases. Studies on the structure-function relation of peroxidases, therefore, will help in the understanding of cytochrome P450s. In the case of animals, thyroid peroxidase and myeloperoxidase are the most well-studied peroxidases. Thyroid peroxidase is involved in thyroid hormone synthesis and recently has been indicated to be one of the major antigens of thyroid autoimmune diseases such as Graves' disease and Hashimoto's thyroiditis. On the other hand, myeloperoxidase is involved in bacteriocidal function of leukocytes and has completely different physicochemical properties from those of thyroid peroxidase. We have successfully cloned human thyroid peroxidase. The comparison of its deduced amino acid sequence with that of the recently reported human myeloperoxidase revealed a surprising fact that both enzymes are diverged from a common ancestral gene and belong to the same gene family. We further isolated and partially sequenced genomic clones of human thyroid peroxidase and found that several exon-intron junctions are well conserved between two enzymes.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional personnel Engaged on this Project:

Shioko Kimura	Visiting Associate	LMC NCI

Objectives:

1) Isolate and sequence genomic clones for human thyroid peroxidase.

2) Determine the mechanism of regulation of peroxidase levels in thyroid tissue.

3) Express human thyroid peroxidase in tissue culture cells and characterize the expressed peroxidase.

4) Examine individual variations in the expression of thyroid peroxidase in thyroid tissues from thyroid disease patients and normal individuals.

Methods Employed:

A genomic library in EMBL3 and a cosmid library were constructed from human lymphocyte DNA by a partial Sau3A digestion. These libraries were screened with human thyroid peroxidase cDNA and clones isolated. EcoRI digested fragments from the isolated clones which hybridized to the cDNA were further isolated and used to make M13 shotgun libraries for sequencing. Sequencing was done by the method of dideoxy-nucleotide chain termination. Messenger RNA for thyroid peroxidase was analyzed by Northern blot analysis, and genomic DNA characterization and mapping of genomic clones were done by Southern blot analysis. Human thyroid peroxidase was expressed by means of the vaccinia virus expression system in tissue culture cells. Expressed protein was analyzed by Western blot and enzyme activity was determined spectrophotometrically.

Major Findings:

Two human thyroid peroxidase cDNAs previously isolated and sequenced are almost identical except that the second cDNA has lost 171 nucleotides in the middle of the sequence. This short stretch of sequence has intron splicing characteristic base pairs, GT and AG, at the beginning and the end of the sequence. These two mRNAs are expressed in all the tissues examined including 19 Graves' disease, 1 normal and 6 adenoma thyroid tissues, although the level of each mRNA varies from tissue to tissue. These results and the genomic cloning results suggest that two thyroid peroxidase mRNAs might be generated through alternative splicing of the same pre-mRNA transcript. When the nucleotide and the deduced amino acid sequences of human thyroid peroxidase was compared with those of the reported human myeloperoxidase, the similarity of 46% and 44%, respectively, was observed. These results clearly indicate that thyroid peroxidase and myeloperoxidase are members of the same gene family and diverged from a common ancestral gene. This is rather surprising since two enzymes have been believed to be completely different in terms of physicochemical and physiological properties and their

localization in specific target tissues. Further amino acid comparison with other known plants, yeast, and fungi peroxidases allowed us to predict the possible location of heme-binding histidine residue in both thyroid peroxidase and myeloperoxidase. Several conserved features were also observed in this proximal histidine residue-containing region among all peroxidases compared, suggesting the presence of a peroxidase gene superfamily.

Using human thyroid peroxidase cDNA, several genomic clones were isolated from a λEMBL3 and a cosmid library. Gene mapping and sequencing of M13 shotgun clones which hybridized to the thyroid peroxidase cDNA, prepared from EcoRI digested fragments of the isolated genomic clones, revealed that the thyroid peroxidase gene probably spans 100-200 kbp of human chromosome 2 and consists of 17 exons. This strongly contrasts with the myeloperoxidase gene which is only 12 kbp long and contains 12 exons. In spite of these differences, several exon-intron junctions are well conserved, which again suggests an evolutionary relation between two enzymes.

Two human thyroid peroxidase cDNAs were expressed by means of the vaccinia virus expression system in a TK^- cell line. Two different molecular weight proteins corresponding to two different size cDNAs began to appear 6 hr after infection and reached maximum levels of expression at 24-48 hr. Once we are able to obtain a large enough amount of two different size proteins, it will be interesting to see whether or not these two proteins have the same type of activity (iodination and/or coupling of tyrosine residues on thyroglobulin) and level of the activities.

Publications:

Kimura S, Ikeda-Saito M. Human myeloperoxidase and thyroid peroxidase, two enzymes with separate and distinct physiological functions are evolutionarily related members of the same gene family. PROTEINS: structure, function, and genetics 1988;3:113-20.

Kimura S, Kotani T, McBride OW, Umeki K, Hirai K, Nakayama T, Ohtaki S. Human thyroid peroxidase: complete cDNA and protein sequence, chromosome mapping, and identification of two alternatively spliced mRNAs. Proc Natl Acad Sci USA 1987;84:5555-59.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05561-01 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Transcriptional Regulation of Cytochrome P-450 Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Frank Gonzalez Sr. Staff Fellow LMC NCI

Others: Harry V. Gelboin Chief LMC NCI
Minoru Nomoto Visiting Associate LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

The majority of P-450 genes are regulated at the transcriptional level. During distinct stages of development, P-450 genes became transcriptionally activated. We are studying several of these P-450 genes that are activated during development. The genes have been cloned and completely sequenced and their promoter regions have been identified. The genes being examined are IIA1, IIA2, IIC7, and IIE1. IIA1 and IIE1 became transcriptionally activated immediately after birth in both males and females. IIA1, however, becomes specifically suppressed in males at puberty. IIA2 becomes activated in males when they reach puberty and is never expressed in females. IIC7 is activated in both males and females at puberty. To study the <u>cis</u>- and <u>trans</u>-acting elements responsible for these gene activations, we are using a cell-free in vitro transcription system derived from rat liver. Promotor regions of each of the above genes were faithfully transcribed in the cell-free system. Efforts are under way to characterize DNA sequence elements and protein factors responsible for activating transcription of these genes.

PHS 6040 (Rev 1/84)

GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Frank Gonzalez	Sr. Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Minoru Nomoto	Visiting Associate	LMC	NCI

Objectives:

1. Develop an in vitro cell-free transcription system that faithfully transcribes P450 gene promoters.
2. Determine the DNA sequence elements that are necessary for gene activation during development.
3. Characterize the protein factors that are needed to activate transcription during development.
4. Determine the sequences that bind to developmental specific protein factors that activate transcription.

Methods Employed:

Cell-free transcription extracts derived from rat liver were prepared by a modification of the method described by Gorski, Carneiro, and Schibler. Transcription was monitored by primer extension analysis. Human TK and rat albumin promoters were included in the transcription extracts as internal positive controls. Upstream DNA was modified by deletion analysis using restriction enzymes and exonucleases. Factor-binding assays were performed using the gel retardation assays of kinased labeled fragments derived from upstream DNA sequence.

Major Findings:

Transcription extracts were prepared from rat liver that accurately transcribed several P-450 gene promoters. The assays were found to be highly reproducible; however, the results did not reflect in vivo transcription activities. For instance the IIC7 gene was found to be transcribed in vitro efficiently from extracts made from livers of 2-week-old and 9-week-old rats. In vivo, however, the IIC7 gene is transcriptionally inactive in 2-week-old rats. The reason for this discrepancy is currently under investigation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05562-01 LMC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Assessment of Human P-450 Catalytic Activities by cDNA-Directed Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Frank Gonzalez	Sr. Staff Fellow	LMC	NCI
Others:	Harry V. Gelboin	Chief	LMC	NCI
	Toshifumi Aoyama	Visiting Fellow	LMC	NCI
	Amha Asseffa	IRTA	LMC	NCI
	Shioko Kimura	Visiting Associate	LMC	NCI

COOPERATING UNITS (if any)

Argonne National Laboratories, Argonne IL (James P. Hardwick); Biocenter, Basel, Switzerland (Urs A. Meyer); St. Mary's Hospital Medical School, London (Jeffrey R. Idle)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.6	3.6	0.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

P-450s are a group of hemoproteins that are the terminal components of the mixed function monooxygenase system. These enzymes are products of a gene superfamily and up to 50 distinct forms may exist in mammals. The bulk of P-450s are expressed in liver as enzymes responsible for the metabolism of foreign chemicals. In some cases endogenous steroid catabolism is associated with distinct species of P-450s; however, the physiological role of these reactions is questionable. Of principal importance is the participation of P-450s in chemical carcinogen activation. The general method for study of P-450 enzymology is through standard purification and assay of the enzymes. Two major drawbacks to this approach are the difficulty in obtaining highly purified preparations and the fact that these membrane-bound enzymes must be reconstituted with artificial lipid bilayers and the enzyme NADPH-P-450-oxidoreductase. Further human P-450s are even more difficult to purify based on the paucity of an available tissue source. We have been studying the enzymology of rat and human P-450s by using cDNA-directed expression.

Several P450s have been expressed using a variety of expression systems. Rat IIA1, IID1 and IVA1 and human IIC8, IIC9, IID1 and IIA1 P450s have been expressed using the COS-cell system. Mouse IA1 and IA2, and rat IIA1 and IVA1 were expressed using the T7-vaccinia virus system. Rat IIA1 and IVA1 were expressed using a yeast expression system. These enzymes and others are also being expressed using baculo virus and retrovirus.

Expressed P-450s have been evaluated for their catalytic activities toward a variety of drugs and carcinogens. DNA binding and mutagenesis assays are being used to evaluate the carcinogen-activating properties of human P-450s.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Frank Gonzalez	Sr. Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Toshifumi Aoyama	Visiting Fellow	LMC	NCI
Amha Asseffa	IRTA	LMC	NCI
Shioko Kimura	Visiting Associate	LMC	NCI

Objectives:

1. Express rat and human P-450s using the lytic vaccinia virus, baculo virus, and COS-cell cDNA expression systems.

2. Express rat and human P-450s using the stable retrovirus cDNA expression system.

3. Determine the enzymatic specificities of expressed P-450s toward drugs and carcinogens.

4. Evaluate the ability of expressed P450 to activate carcinogens to electrophilic DNA-binding mutagenic metabolites.

Methods Employed:

Rat and human cDNAs were isolated from lambda-gtll libraries using both antibody and DNA probes. The cDNAs were sequenced to establish if they contained the full protein coding region. The cDNAs were then ligated into the various expression vector plasmids. In lytic systems, they were first ligated to the T7 vaccinia virus and baculo virus recombination vectors. Viruses harboring the P-450 coding sequence were then constructed by spontaneous recombination and used to express P-450s. The p91023(B), a simian virus-40 (SV40)-adenovirus derived vector, was also used to express several cDNAs. Retrovirus work was carried out using the pXT-1 vector containing the human herpesvirus TK promoter to drive expression of the cDNA. P-450 expression was monitored by spectral analysis, enzyme assays, and carcinogen mutagenesis.

Major Findings:

Several systems have been evaluated for cDNA-directed expression of rat and human P-450s. The yeast expression was used to express rat IIA1 and IVA1, testosterone 7 omega-hydroxylase and lauric acid omega-hydroxylase activities. The COS-cell system was used to express human debrisoquine 4-hydroxylase and nifedipine oxidase activities from the IID1 and IIIA1 cDNAs, respectively. Human IIC8 and IIC9 P-450s were also expressed in COS-cells and assayed for mephenytoin 4-hydroxylase and tolbutamide hydroxylase activities.

The mouse IA1 and IA2 cDNAs were expressed in the T7-vaccinia virus system. This vector yielded five- to tenfold higher levels of the expressed enzymes than the standard vaccinia virus system. Several cell lines were assayed for their ability to accumulate vaccinia virus-expressed proteins. High levels of benzo(a)pyrene hydroxylase and acetanilide hydroxylase activities were obtained from IA1 and IA2 expressed in T7-vaccinia virus, respectively. The rat IIA1 was also expressed in this system and produced testosterone 7 alpha-hydroxylase activity.

Publications:

Gonzalez FJ, Schmid BJ, Umeno M, McBride OW, Hardwick JP, Meyer UA, Gelboin HV, Idle JR. Human P-450 PCN1: sequence, chromosome localization and direct evidence through cDNA expression that P450 PCN1 is nifedipine oxidase. DNA 1988;7:79-86.

ANNUAL REPORT OF

THE RADIATION EFFECTS BRANCH
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The Radiation Effects Branch (REB), established in response to Public Law 95-622, plans, directs and administers a program consisting of grants and contracts investigating the means by which exposure to ionizing and non-ionizing radiations, particularly at low doses or dose rates, leads to molecular and cellular events and processes resulting in mutagenesis, cell transformation, and carcinogenesis, and the associated dose-effect relationships; directs and administers selected epidemiological studies investigating the effects of radiation exposure in humans; provides a broad spectrum of information, advice, and consultation to scientists and to institutional science management officials relative to the National Institutes of Health (NIH) and the National Cancer Institute (NCI) funding and scientific review policies and procedures, preparation of grant applications, and choice of funding instruments; maintains contact with other Federal agencies and institutions and with the broader relevant scientific community to identify new and needed research in, and related to, the fields of radiation mechanisms and effects; provides NCI management with recommendations concerning funding needs, priorities, and strategies for the support of relevant research areas consistent with the current state of development of individual research elements and the promise of new initiatives; provides information, advice, and guidance to NCI management and staff on radiation-related issues; implements the mandates of Public Law 97-414, Section 7(a), and Public Law 98-542, Sections 7(a)(2) and 7(b); and represents the Department of Health and Human Services on the Science Panel of the Committee on Interagency Radiation Research and Policy Coordination, which is located within the Office of Science and Technology Policy, Office of the President.

The extramural activities of the Branch are accomplished through contractual agreements with universities and other Federal agencies, and through traditional individual research grants, program project grants, and conference grants with universities and research organizations. At present the Branch administers over 100 research activities with an annual budget in excess of 14 million dollars (Tables I and II). The program consists of two broad categories of research: mechanisms of radiation damage and repair, and radiation carcinogenesis. In addition, the NIH and the NCI have assigned to the Branch responsibility for the implementation of sections of two Public Laws addressing radiation-related issues emanating from Congressional policy concerns.

Section 7(a) of Public Law 97-414, the Orphan Drug Act, requires the Secretary to conduct scientific research and prepare analyses necessary to develop valid and credible (1) assessments of the risks of thyroid cancer that are associated with thyroid doses of Iodine-131, (2) methods to estimate the thyroid doses of Iodine-131 that are received by individuals from nuclear weapons fallout, and (3) assessments of the exposure to Iodine-131 that the American people received from the Nevada atmospheric nuclear bomb tests. A working committee consisting of relevant expertise within and outside of the government, including foreign nationals, has been formed and is addressing these issues. The committee is

organized into three task groups addressing the risk of thyroid cancer per unit dose of Iodine-131 to the thyroid, the dose of Iodine-131 to the thyroid per unit of exposure to Iodine-131, and the development and verification of models to estimate the exposure of the American people to Iodine-131 resulting from radioactive fallout associated with atmospheric nuclear weapons tests at the Nevada Test Site. A number of meetings of the several task groups have been held during this year. In particular, considerable effort has been expended with respect to the required exposure and dose reassessments; these are being carried out via interagency agreements and with the assistance of staff expertise acquired for this purpose. An interim report to the Congress is near completion and a number of research needs are being addressed. However, a final report will not be available for some time.

Public Law 98-542 requires the Director of the National Institutes of Health (NIH) to conduct a review of the reliability and accuracy of scientific and technical devices and techniques which may be useful in determining previous radiation exposure (e.g., among military personnel who participated in atmospheric nuclear weapons tests conducted by the United States or in the American occupation of Hiroshima or Nagasaki, Japan), including the availability of such devices and techniques, the categories of exposed individuals for whom the use of such devices and techniques may be appropriate, and the reliability and accuracy of dose estimates which may be derived from such devices and techniques. Technical reviews and analyses were conducted by working groups to assess the several relevant devices and techniques (e.g., whole body counting, bioassay studies, chromosomal alterations). A final report was submitted to the Director, NIH, and transmitted through the Department to the Congress during this reporting year.

The Mechanisms of Radiation Damage and Repair program includes, but is not limited to, studies on molecular and cellular changes resulting from exposure to radiation, DNA damage and repair following radiation exposure, the hypermutability, mutagenesis, and malignant transformation of cells exposed to ionizing and non-ionizing radiation, mutagenicity-carcinogenicity relationships following exposure to radiation, interspecies comparisons, and the interaction between cocarcinogens.

The Radiation Carcinogenesis program addresses the effects of exposure to radiation, including, for example, the role of oncogenes, studies of the sensitivity of the embryo or fetus to ionizing radiation, the effect of dose rate and linear energy transfer on radiation-induced effects, dose-effect relationships, interspecies comparisons, cocarcinogenesis, the incidence of selected diseases as they may relate to exposure from radioactive fallout, and synthesis of radiobiological data in the assessment of risk and the establishment of appropriate radiation protection practices.

TABLE I
RADIATION EFFECTS BRANCH
(Extramural Activities - FY 1988 - Estimated)

	No. of Contracts/Grants	$ (Millions)
Research Contracts	4	0.54
Research Grants	107	14.07
Traditional Project Grants (103 grants; $12.44 million)		
Conference Grants (3 grants; $0.02 million)		
Program Project Grants (1 grant; $1.61 million)		
TOTAL	111	14.61

TABLE II
RADIATION EFFECTS BRANCH
(Contracts and Grants Active During FY 1988)

	FY 88 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	$ (Millions)	No. of Grants	$ (Millions)
Radiation Mechanisms and Carcinogenesis	3	0.54	102	13.83
Office of the Chief	1	0.00	5	0.24
TOTAL	4	0.54	107	14.07

Research activities are concerned with a wide variety of radiation effects including mechanisms of damage and repair of DNA by ionizing and non-ionizing radiation, and radiation carcinogenesis. The majority of the 107 grants (84) support investigations relating to mechanisms of radiation damage and repair of cellular DNA, 58 of which investigate the effects of exposure to ionizing radiation and 26 of which study the consequences of exposure to ultraviolet (UV) radiation, microwaves and ultrasound. Seventeen grants and three contracts fund studies in radiation carcinogenesis, and research addressing radiation risks and the compilation and assessment of information is supported by six grants and one contract.

Mechanisms of Radiation DNA Damage and Repair: The REB provides substantial support for basic research related to mechanisms of mutagenesis and malignant transformation by radiation. This information is essential for providing both insight into the mechanisms of carcinogenesis and the rationale for the carcinogenic effects seen following exposure to radiation. Such studies contribute to the evaluation of risks to individuals and to populations and thereby assist in providing a data base upon which radiation standards might be developed.

A. Ionizing Radiation: Though most work in DNA repair at this time involves the nucleotide excision repair mechanism, evidence continues to accumulate indicating that DNA repair is far more complex than anticipated from our present understanding of this pathway. It is now apparent that classical nucleotide excision is integrated with other equally important mechanisms, such as postreplication repair, to maintain the integrity of DNA. Earlier investigations studying the preferential formation of DNA-protein cross-links and single strand DNA breaks in areas of the genome containing transcriptionally active genes found that after x-irradiation two nonhistone proteins which are minor components of the nuclear matrix become covalently bound to the DNA. During the repair process, the protein linked to DNA in regions of transcriptionally active areas is removed, following which other areas of the genome are bound, and both actively transcribing genes and newly synthesized DNA are preferentially broken and cross-linked with protein in the nuclear matrix. Recent investigations have found that x-ray damage induces slightly more DNA-protein cross-links than double strand breaks. Unlike m-AMSA [4′-(9-acridinylamino)methane sulfon-m-anisidide] treatment, radiation induces cross-links with proteins other than topoisomerase II. These DNA-protein cross-links occur in both active actin genes and inactive globin genes in mouse L929 cells. Early depletion of actin sequences and enrichment of globin sequences in protein cross-links suggest the faster repair of the active actin genes. Novobiocin (an inhibitor of topoisomerase II) dampens the radiation effect on actin sequences which indicates a role for topoisomerase II-mediated supercoiling in radiation sensitivity. Kinetic analysis of DNA-protein cross-links in newly replicated DNA shows that these cross-links are not responsible for the inhibition of DNA replication that accompanies radiation damage. These results underscore the complexity of cellular responses and show that the DNA target genome is differentially sensitive to x-ray exposure throughout both its physical length and its position in the cell cycle.

Understanding the mechanisms of resistance and sensitization to ionizing radiation is important for designing radiotherapy protocols and for estimating cellular damage. Based on earlier work, an investigator proposed that nitrous oxide and low concentrations of oxygen sensitize the DNA to radiation through the same mechanism, while sensitization by nitrous oxide and high concentrations of

oxygen is mediated through a different mechanism. This concept is now supported by recent experimental results testing cell sensitivity to x-rays in mixtures of oxygen and nitrous oxide; damage from nitrous oxide sensitization does not add to the damage from low concentrations of oxygen, but it does add to the damage from high oxygen concentrations. These results were confirmed using a series of mutant cell lines, some of which are not sensitized by nitrous oxide. Further, measurement of the glutathione levels (GSH + 1/2 GSSG) in the insensitive cell strains indicates that the failure to sensitize is not related to the glutathione content. These unanticipated results suggest that new hypotheses are needed to understand the complexities of radiation sensitization. Another investigator has observed that beta-arabinofuranosyladenine in nonlethal concentrations is a potent sensitizer of nonproliferating cultured human cells to the lethal effects of x-irradiation. This phenomenon was observed in normal human diploid cells and in certain tumor cell lines, and may have significant implications for the radiotherapy of cancer.

The thymine anion is now accepted by a number of prominent research groups as accounting for 50 percent of initial damage in DNA exposed to the direct effects of ionizing radiation, i.e., the sole repository of electron addition. The remaining 50 percent, resulting from electron loss, is assigned to the guanine cation. An investigator has tested this concept with an electron spin resonance (ESR) Q-band system that allows the measurement of electron attachment to nucleotide oligomers in microgram quantities. Results to date clearly indicate that electrons are trapped preferentially on both thymine and cytosine. Further, it is evident that previous analyses reported on the simulation of free radicals trapped in DNA have resulted in ESR parameters for the thymine anion that are incorrect. These results raise questions regarding the interpretation that the remaining portion of the spectrum is due to only the guanine cation.

Currently, the most widely used methods for measuring mutagenesis in cultured mammalian cells are adaptations of the bacterial techniques developed by Ames. Previous studies by one laboratory indicated that these approaches may be seriously in error because they ignore the fact that the chromosome selected to contain the nonvital marker gene also contains many other genes which are essential for reproduction of the test cell. As a consequence, these tests generally are insensitive to damage consisting of deletions (>30 kb), chromosomal nondysjunctions and translocations. Further, these systems concentrate on point mutations within the marker gene so that only a very small target is utilized for mutagenesis; this requires relatively high or acute doses of mutagens rather than low chronic exposures, which are of primary interest. The method devised to circumvent these problems makes use of somatic cell hybrids containing a single human chromosome on which the marker genes are carried and allows the use of doses of mutagenic agents sufficiently low that cell killing is infrequent. This then permits direct measurement of mutations without the need to resort to controversial extrapolation procedures customarily used to estimate lower dose effects. Recent results from experiments using this approach indicate that the mutagenic frequency at low doses of x-rays or neutrons (as low as 0.01 Gy) is at least 100 times greater than that obtained by conventional methods (5-8 Gy). The dose response curve has no threshold for mutation and exhibits a downward concavity so that the mutational efficiency (mutations per rad) is maximal at low doses. Equations have been developed to explain this curve in terms of cell killing and chromosome translocation. The data strongly suggest that both potentially lethal damage (PLD) and sublethal damage (SLD) are different manifestations of the same processes operating on the same basic chromosomal

lesions. These results may have significant implications in evaluating the effects of exposure to low doses of radiation.

Another laboratory, using the same mammalian somatic cell hybrid system and the C3H 10T1/2 model, has completed studies on the relative biological effectiveness (RBE) of mono-energetic neutrons having a range of energies from 220 keV to 14 MeV. This corresponds to the energy range to which individuals were exposed at Hiroshima. The most biologically effective neutron energy for transformation was found to be 350 keV, with effectiveness falling off slowly for energies above and below this value. This peak in RBE coincides with previous findings for cell killing and for inhibition of the growth of vicia seedlings which strongly suggests the broad generality of these results. Additional studies have been carried out for oncogenic transformation and mutations in these same two model systems and dose response curves were generated for charged particles having a range of linear energy transfer (LET) values up to 120 keV per micron. The relative biological effectiveness of high LET particles was found to be higher for mutation as an endpoint than for cell lethality. Another finding was that high LET helium ions interact in a super-additive way with asbestos fibers to produce oncogenic transformation. Further, the radiation-induced transformation incidence is enhanced by the presence of thyroid hormone, and inhibited by many agents, including selenium.

The concept that deletions or DNA rearrangements are important in mutagenesis and, therefore, in neoplastic transformation is strongly supported by recent experiments on transposon mobility in Drosophila and its interaction with x-ray-induced damage. Chromosome damage resulting from the combined effect of x-rays (5.5 Gy) and P element mobility was found to be highly synergistic for transmission distortion of the major autosomes, for X/Y chromosome loss and for the loss of two X chromosome markers on the Y chromosome. No synergism was found in induction of X-Y chromosome translocations. These findings support the hypothesis that chromosome breaks induced by the two mutator systems can interact, thus adversely affecting chromosome lesions. The highly x-ray-sensitive, temperature- and age-dependent increase in sterility of hybrids originating from a novel laboratory-derived Harwich P strain correlates with a greater sensitivity to chromosome loss. A cross of Muller-5-Birmingham females, known to bear defective P elements, with the high sterility-inducer Harwich males resulted in a preferential suppression of gonadal dysgenesis in hybrid daughters. These findings provide insight into the molecular-genetic basis of hybrid dysgenesis and strongly support the idea that recombination events are important processes in mutagenesis and transformation.

When Chinese hamster ovary (CHO) cells are exposed briefly to x-rays, in addition to the suppression of division (lethality), other manifestations of damage are also registered. Among these is damage which results in a reduced rate of population increase which is very likely due to "lethal sectoring" or "lethal mutations" among the progeny of a surviving cell. Recently an investigator has found that at a dose rate of 1.85 cGy/hr cells grow as rapidly as unirradiated cells. Even though large total doses were accumulated (1800 cGy), almost all evidence of lethal sectoring had disappeared. Hence, these results indicate that the damage responsible for the appearance of sterile progeny lines among the progeny of a surviving cell is repaired during exposure at a reduced dose rate.

Of the laboratories studying mechanisms of cell damage and repair by radiation, the overwhelming number focus on DNA as the sensitive material in cells.

However, there are other reasonable possibilities. For example, cell membranes have been implicated in the initiation and control of cell cycling, and these membranes are readily damaged by radiation-produced oxidants. Also, the radiation oxidants easily reduce the sensitive sulfhydryl groups of enzymes, thus inhibiting crucial cellular reactions. One investigator studying these oxidation processes has found that in irradiated solutions of glutathione, cysteine and penicillamine, thiyl radicals react with oxygen to form sulfinyl radicals in competition with reactions to form other radicals, e.g., perthiyl radicals and disulfide radical anions. In addition, it was found that carbon-centered peroxyl radicals react directly with thiols to form sulfinyl radicals. This reaction is found to occur rapidly with a variety of biologically important thiols.

B. Ultraviolet Radiation: Ultraviolet radiation is a ubiquitous carcinogenic agent in the environment. However, the REB is not only interested in its direct effect, but also in its plausible role as a cocarcinogen and the relationship of its repair pathways to the repair pathways for ionizing radiation. Sorting out and documenting the various biochemical pathways and properties has historically provided new insights, new methods and biological reagents for further advances, both practical and conceptual.

In the past two and one-half years a series of studies showed that ultraviolet light-induced pyrimidine dimers in active genes are more efficiently repaired than are those in nontranscribing regions of the genome in mammalian cells. Thus, repair is much more efficient in the active c-able proto-oncogene than in the inactive c-mos proto-oncogene in the same Swiss mouse 3T3 cells. These observations have important implications for the relationship between overall DNA repair levels and biological end points such as cell survival, mutagenesis or transformation. They also raise questions about the applicability of rodent test systems as models for human carcinogenesis because of the quantitative differences in the intragenomic heterogeneity in DNA repair between rodent and human cell systems. These observations were extended to the analysis of intragenomic heterogeneity in the formation and repair of chemical adducts to DNA. In particular it was shown that not only are photoadducts of psoralen formed more readily in active genes, but also that they were repaired much more efficiently in actively transcribing genes than in the overall human genome. These studies generally support the hypothesis that repair of "bulky" lesions in DNA is essential for transcription of genes but not for DNA replication. Now an investigator has observed that the preferential DNA repair that occurs in active genes in mammalian cells is actually due to selective repair of the transcribed DNA strands, i.e., the plus DNA strands, not the minus strands, are repaired. These results provide convincing evidence for the hypothesis that preferential repair is designed to permit transcription of essential active genes, but that it is not needed for genomic replication. The significance of measuring repair in defined genes is that it demonstrates that essential expressed genes are still being efficiently repaired, even in differentiated cells or tissues which exhibit low overall levels of repair. This is important to our understanding of basic mechanisms of mutagenesis and carcinogenesis.

Much has been written regarding the relationship of DNA repair capacity and the life span of individuals and species. Generally, investigators intuitively feel that there is an important relationship between longevity and repair capacity. Most relevant experiments lead to observations which are of a correlative nature, i.e., that there is a tendency for repair capacity to be proportional to life span in species such as mice, rats, cows, monkeys, apes, elephants and humans;

however, there are numerous exceptions. Recently, an investigator developed radioimmunoassays (RIAs) which specifically monitor ultraviolet light-induced DNA lesions such as cyclobutane dimers, (6-4) photoproducts and Dewar pyrimidones. The use of these RIAs permits a more direct approach to investigating this relationship. Studies using the nematode, Caenorhabditis elegans, which has a demonstrated developmental pattern in DNA repair capacity, have shown that there is a loss of the ability of this organism to remove (6-4) photoproducts as it progresses through its life cycle. By the use of recombinant inbred nematode strains with different mean life spans, it was shown that there was no correlation between DNA repair capacity and the aging process in this animal. These results indicate that if there is a relationship between repair capacity and longevity, it is not a simple one.

Several years ago two groups concluded that the lesion in xeroderma pigmentosum, complementation group A, was inaccessible to the enzyme repair complex rather than that the cells were deficient in the incision enzyme. This conclusion was based on the observation that cell-free extracts were as effective as extracts from normal cells at incision of DNA devoid of the various chromatin proteins. Later, another worker reported that the cell-free experiments were in error and that the results were an artifact involving divalent metal ions. Recently, however, an investigator has transfected the cultured cells of a xeroderma pigmentosum patient (group A) with DNA from the mammal Potorous, which is highly efficient in photoreactivation, and screened for cells which demonstrated photoreactivating enzyme activity. Transfectants have been obtained which show greatly increased resistance to ultraviolet radiation, and also which show increased survival when exposed to photoreactivating light after ultraviolet exposure. These results provide important evidence that enzyme deficiency is likely to be of greater significance than is the lack of enzyme accessability to the DNA lesion.

In the past few years a number of prokaryotic and eukaryotic DNA repair genes have been cloned and the protein products have been characterized. In keeping with the generally more complex structure and organization of the eukaryotic genome, it appears that the repair enzymes also are more complex. For example, an investigator has recently compared the E. coli excision repair genes with an excision gene, RAD1, found in Saccharomyces cerevisiae. In contrast to the bacterial enzyme, the yeast excision enzyme also functions in genetic recombination which, in turn, can be involved in postreplication repair.

Radiation Carcinogenesis: This program focuses on efforts to establish dose-effect relationships, including factors of dose rate and type of radiation; to determine whether there is a level of exposure to these agents which might be considered "safe"; to explore possibilities for intervening or ameliorating detrimental levels of exposure; and to expand the data base from which risk estimates are derived.

The risk of developing thyroid neoplasms following therapeutic radiation exposures is being studied in over 5,000 patients with a history of external irradiation to the head and neck for benign conditions during childhood. Analysis of factors influencing the risk associated with developing radiation-related thyroid neoplasms is continuing. Specifically, numerous sibling groups were identified within the cohort. A study involving these sibling groups was completed during the past year which indicated a definite, albeit small, familial component in the risk of developing radiation-induced thyroid neoplasms. Thus,

if one member of a sibship develops a thyroid neoplasm, other members also may be at higher risk. With the cooperation of the Radiation Epidemiology Branch of NCI, efforts have been initiated to obtain precise organ-specific dosimetry data for each member of the cohort. These dosimetry data will form the basis for new analyses of the dose-response curves and risk factors, and will allow more meaningful comparisons with data from other exposed groups. In addition, changes in the carbohydrate structure of thyroglobulin reportedly have been related to thyroid neoplasms, and the use of such structural changes is being investigated with respect to their usefulness as a tumor marker.

The incidence of leukemia and thyroid disease in Utah is being assessed in relation to fallout from the Nevada Test Site between 1951 and 1962. As many as possible of the nearly 5,000 exposed and unexposed children identified and examined for thyroid disease in the 1960s have been located, and thyroid reexaminations of these persons, which constitute a "thyroid cohort" study, have been completed. Dose reassessments of subjects in this cohort study and in a leukemia case-control study are nearly complete.

The role of oncofetal antigen (OFA) in radiation carcinogenesis is being investigated. Particular focus is on the immunobiological reaction of the host to the expression of these potential autoantigens on lymphomas and leukemias induced by sublethal x-irradiation. Primary and in vitro cultured lymphomas of the RF/M mouse have universally expressed a 44 kilodalton OFA at the surface of radiation-induced lymphoblastic lymphoma cells. The various radiation-induced tumors used as models in the study of radiation biology, including the UV-induced sarcomas, also express these antigenic markers. New information shows that all head and neck squamous cell carcinomas of humans as well as most other classes of human carcinoma express this conserved transformation marker. This antigenic marker is normally present in pregnant humans and mice in the amniotic fluid. The investigators have purified the 44 kD OFA glycoprotein and have demonstrated the capacity of OFA to induce solid tumor resistance in mice. They have shown that the OFA cross-protects against challenge between different radiation-induced lymphomas and that the immunity is cell mediated. They also have observed that the lymphoblastic lymphomas can reversibly alter their expression of the major histocompatible complex of antigens (H-2) at the cell surface in vitro. These H-2 antigens are essential for recognition of the OFA marker (both OFA and H-2 must be recognized simultaneously) by cytotoxic lymphocytes so that they may be effective in tumor cell destruction. If these lymphomas can differentially control the expression of H-2 antigens and thereby escape immune attack against the OFA, an additional mechanism may have been identified for leukemia and lymphoma development in the face of potent host immune responses.

Mammary cancer is a major malignant disease among American women and a major cause of morbidity and mortality among those exposed to ionizing radiation. It has been hypothesized that the mammary cells from which malignant tumors arise are the same clonogenic cell subpopulation necessary for tissue repair and repopulation following radiation injury. Researchers have developed quantitative rat mammary cell transplantation and endocrine manipulation procedures and short-term culture techniques to measure the concentration of mammary clonogens, i.e., the cells which, when transplanted to hormonally modified recipients, give rise to multicellular clonal glandular units. These techniques have been used to define the radiation dose-survival response of mammary clonogens after exposure to radiation, and to demonstrate that radiogenic initiation of cancer is a common cellular event and is dependent on the number of clonogenic cells present at the

time of radiation exposure. It is known from other cell systems that normal cells inhibit the expression of the transformed phenotype. Using similar techniques, other investigators have found that following exposure of mice to 1 Gy of gamma radiation or 75 micrograms of 7,12-dimethylbenz[a]anthracene (DMBA), altered cell populations can be detected by their expression as ductal dysplasias within outgrowths derived from transplantation of dissociated mammary epithelial cells from these mice. These damaged cells are detectable soon after administration of relatively low doses of carcinogen, yet they closely resemble those cells which appear to be precursors of mammary carcinoma in the mouse. The results also suggest that the acquisition of the altered growth potential, which resulted in ductal dysplasias, and the ability of these cells to gain some autonomy from growth-regulatory mechanisms are separate events that occur at different times after carcinogen treatment.

Means of ameliorating potential consequences resulting from exposure to radiation in particular situations are being investigated. Several studies are concerned with the possibility of reducing cancer risk from internally deposited radionuclides by chelation therapy. It has been shown that subcutaneous administration of either N-(2,3-dimercaptopropyl)phthalamidic acid (DMPA) or meso-dimercaptosuccinic acid (DMSA), water-soluble analogs of British Anti-Lewisite (BAL), increases the survival of rats after they have received a lethal dose of polonium-210. In addition, DMPA has been found to be a potent decorporating agent for polonium-210 in body tissues. This radionuclide, a radioactive daughter of radon, is an alpha emitter and thus potentially hazardous if inhaled or ingested. It has been an occupational hazard for uranium miners and is of increasing concern to the general population because of radon, and consequent polonium-210, concentrations in the interior air of buildings and homes. Groups of beagle dogs injected with plutonium-239 or Americium-241 are on lifetime observation to determine the effectiveness of radionuclide removal by Ca-DTPA (calcium diethylenetriaminepentaacetate) or zinc-DTPA. The use of these chelating agents appears to decrease the risk of cancer and delay the time to tumor appearance. New chelating agents for the removal of actinide and lanthanide elements are being developed which can be absorbed from the intestinal tract after oral administration and which have improved target organ specificity, primarily liver and skeletal tissue.

A potato-derived protease inhibitor, chymotrypsin inhibitor 1, has been found to be as effective (at concentrations of 10 micrograms per ml or higher) as the Bowman-Birk soybean-derived protease inhibitor in preventing radiation-induced malignant cell transformation. The fact that this compound does not inhibit trypsin, as does the Bowman-Birk inhibitor, is a distinct advantage for the potato chymotrypsin inhibitor if protease inhibitors are ever used as cancer chemopreventive agents in human populations. Possible toxic side effects of dietary protease inhibitors are thought to be caused by trypsin inhibition, and such side effects would not be associated with chymotrypsin inhibition. As it is chymotrypsin inhibition which is necessary for anticarcinogenic activity, potential side effects from trypsin inhibition could be precluded with use of the potato chymotrypsin inhibitor.

Comparative studies with rodents and dogs are expected to provide information that would help to establish a unifying concept of radiation damage that will provide a basis for interspecies extrapolation and scaling to man. Study variables have included dose rate, total dose, type of exposure (single, fractionated, protracted, or duration-of-life), sex, and age at exposure.

One investigation is measuring the relative importance of dose rate and total dose to the late effects of protracted whole-body gamma irradiation on beagles at four total doses (4.5-30 Gy) at four dose rates (3.8-26.3 cGy/day; 22 hr/day). The preliminary results indicate that (a) there is no evidence for a dose rate effect, (b) death from tumors is proportional to total dose, (c) time of fatal tumor onset is shortened by increased total dose, and (d) increased total dose increases the number of nonfatal tumors. The final results, when compared to results of similar ongoing studies on rodents, are expected to contribute to the data base upon which a unifying concept may be established.

Evidence is accumulating that cocarcinogenesis is an important aspect of the carcinogenesis process. Studies have shown that combined exposures to gamma radiation and DMBA lead to an incidence of tumors which, under certain exposure conditions, is more than additive. For example, exposure of mice to 0.5 Gy of gamma radiation followed by 75 micrograms of DMBA leads to significantly more mammary tumors than expected based on additivity. In order to examine the basis for these observations, cellular studies were conducted with an in vitro epithelial focus technique. The results indicated that this exposure regimen did not produce more mammary cells with altered in vitro growth potential but did tend to increase the frequency of cells which were subculturable. The effect of a lower chemical dose was examined in an experiment with mice treated with only 2.5 micrograms of DMBA together with 0.5 Gy of gamma radiation given either one week earlier or later. At these doses, radiation followed by DMBA resulted in very few mammary tumors, while DMBA followed by radiation resulted in a synergistic tumorigenic effect. The relationship of these findings to initiation-promotion concepts is being further investigated.

It is apparent that ultraviolet light, as well as chemicals, can be a cancer-promoting agent, but that, depending on the conditions, UV also can inhibit tumor induction; both effects can be induced systemically. Studies have been conducted to determine the influence of dorsal UVB-irradiation upon subsequent tumorigenesis induced chemically on the ventral surface of mice. In one experiment, dorsal UVB-irradiation followed by ventral treatment with benzo[a]pyrene (BP) resulted both in enhanced skin tumor induction which was dependent on the dose of BP and in a tumor-free survival time which was significantly shortened in the irradiated mice as compared to the unirradiated mice. In another experiment, dorsal UVB-irradiation of mice resulted in inhibition of subsequent tumor induction by ventral initiation with DMBA and promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). Thus, UVB-irradiation induced systemic effects which increased subsequent chemical carcinogenesis by a single stage protocol, but inhibited carcinogenesis induced by an initiation-promotion protocol. These findings broaden the potential role of ultraviolet radiation in human cancers.

There is concern that the irradiation of a developing embryo and fetus at low levels of exposure may have long-term detrimental effects not apparent in the early months after birth. Although not yet complete, studies are investigating the sensitivity of the embryo and fetus to ionizing radiation as a function of stage of development. The current results from a study with dogs exposed in utero 14 years earlier indicate that 1) there is an increased risk for neoplasia in young dogs up to four years of age after both late fetal and neonatal irradiation, 2) there is an increased risk for fatal cancer in later life after both late fetal and neonatal irradiation, 3) there is an increased risk for fatal

thyroid cancer after juvenile irradiation, and 4) there is an earlier onset of all these types of neoplasms after irradiation during development.

In studies of the effect of prenatal irradiation on the development of the immune system, canine fetuses were exposed at 35, 40 or 45 days of gestation. Irradiation at all gestation times resulted in the reduction of thymic lobular volume and absolute thymic weight. Damage to the thymic medulla, as measured by compartment size, appeared to be more severe than to the cortex by 10 days after irradiation. The degree of radiation-induced damage was greater after exposures earlier in gestation. There also was a distinct reduction of the size and number of Hassal's corpuscles in thymic medulla after irradiation, suggesting that fetal thymic epithelium is a radiosensitive cell population. Further, in vitro studies have shown that while the epithelium is radiosensitive, the thymic stroma is much more so. This suggests that in vivo epithelial loss after irradiation may be a function of an interference with the normal epithelial induction by mesenchyme during fetal development. The development of lymphoid components of lymph nodes and spleen also were delayed by the irradiation, and hematopoiesis was reduced in liver and bone marrow.

Various investigators have suggested that oncogenes have a central role in malignant transformation in a variety of model animal systems as well as in human cancer. There are now over 40 genes described as oncogenes but none of them are clearly established as an essential component of the process of malignant transformation in human systems. Earlier investigations suggested that the elusive and changing roles of various oncogenes may arise from the specificity of both the cell system and the carcinogenic agent. For example, one investigator found that mouse mammary tumors induced with dimethylbenz[a]anthracence manifest an activated Ha-*ras* oncogene, whereas tumors induced by murine mammary tumor virus have the *int* genes activated. However, this investigator had been unable to detect any activated oncogenes in radiation-induced mammary tumors. Now, his laboratory has detected two candidate oncogenes in the radiation-induced mouse mammary tumors using two different techniques. One candidate oncogene appears to be an unusual form of the Ha-*ras*-1 p21 antigen which appears in 40 percent of the tumors. Although the same gene is activated in the DMBA-induced tumors, the chemical activates Ha-*ras*-1 by a different molecular mechanism than does irradiation. The second candidate transfects NIH 3T3 cells to form tumors, but DNA analysis proves that the transforming elements are not related to Ha-*ras*-1 so it must be another oncogene. Another investigator working with rat skin tumors induced by ionizing radiation had found earlier that the K-*ras* oncogene was activated in 6 of 12 tumors. In addition, Southern blot analysis indicated that c-*myc* was activated in others as well as coactivated with K-*ras*. Now, however, he has found that the tumor stage may be a very important factor. When smaller tumors which have not developed as extensively are characterized, the investigator found that c-*myc* was not activated, rearranged or amplified in any of ten tumors assayed. These results raise important questions about the relationship of the activation of oncogenes and the events that lead to malignant transformation versus the progression of tumors resulting from the genetic instability of transformed cells.

The REB also provides grant support to several national and international advisory bodies which analyze and disseminate information concerning, and provide guidance on matters pertaining to, occupational and public radiation protection issues: the National Council on Radiation Protection and Measurements (NCRP), the International Commission on Radiation Units and Measurements (ICRU), and the

International Commission on Radiological Protection (ICRP). During the year, the ICRP adopted and published two reports on 1) "Use of Computers in External Beam Radiotherapy Procedures with High-Energy Photons and Electrons" and 2) "Clinical Neutron Dosimetry -- Part I: Determination of Absorbed Dose in a Patient Treated by External Beams of Fast Neutrons." The NCRP published four reports during FY 1988 on the following subjects: 1) "Public Radiation Exposure from Nuclear Power Generation in the United States," 2) "Ionizing Radiation Exposure of the Population of the United States," 3) "Exposure of the Population in the United States and Canada from Natural Background Radiation," and 4) "Radiation Exposure of the U.S. Population from Consumer Products and Miscellaneous Sources."

RADIATION EFFECTS

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. ALDERFER, James L. New York State Department of Health 3 R01 CA 39027-03S1	Effects of Light on Nucleic Acids
2. ALDERFER, James L. New York State Department of Health 2 R01 CA 39027-04	Effects of Light on Nucleic Acids
3. ANANTHASWAMY, Honnavara N. University of Texas System Cancer Center 5 R23 CA 40454-03	Relationship Between UV-Associated Antigens and Transformation
4. ASHMAN, Charles R. University of Chicago 5 R01 CA 45336-02	Specificity of Ionizing Radiation Mutagenesis
5. BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 5 R23 CA 32729-06	Oncogenesis from Low-Dose-Rate Irradiation
6. BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 5 R01 CA 42820-02	Mechanism of Microwave Carcinogenesis in Vitro
7. BARROWS, Louis R. University of Utah 7 R01 CA 41561-03	Repair of X-Ray-Induced DNA Damage: Genetic Basis
8. BASES, Robert E. Yeshiva University 2 R01 CA 36492-04A2	X-Ray Damage and Repair of Primate Cell Alpha-DNA Sequences
9. BEDFORD, Joel S. Colorado State University 5 R01 CA 18023-14	Dose and Time Factors in Cellular Radiosensitivity
10. BENJAMIN, Stephen A. Colorado State University 5 R01 CA 36456-04	Prenatal Thymic Radiation Injury and Immune Development
11. BERNHARD, William A. University of Rochester 5 R01 CA 32546-13	Solid State Radiation Chemistry of Nucleic Acid Bases
12. BOWDEN, George T. University of Arizona 5 R01 CA 42239-03	Radiation Induced Skin Tumors and Oncogene Activation

13.	BOX, Harold C. Roswell Park Memorial Institute 5 R37 CA 25027-21	Transfer Mechanisms in Irradiated Biological Systems
14.	BOX, Harold C. Roswell Park Memorial Institute 1 R01 CA 46838-01	Analysis of Radiation Damage in DNA
15.	BRENT, Thomas P. St. Jude Children's Research Hospital 5 R01 CA 14799-14	Enzymes and Reactions for Repair of DNA in Human Cells
16.	CALOS, Michele P. Stanford University 5 R01 CA 45365-02	LacI Shuttle Vectors for Study of Ionizing Radiation
17.	CARDIFF, Robert D. University of California, Davis 5 R01 CA 36493-05	Radiation Activation of Oncogenes
18.	CLAYCAMP, Gregg H. University of Iowa 5 R01 CA 43324-03	Radiation Biochemistry of DNA Base Damage
19.	CLIFTON, Kelly H. University of Wisconsin, Madison 2 R37 CA 13881-16	Radiation In Vitro - Mammary Neoplasia
20.	COGGIN, Joseph H., Jr. University of South Alabama 5 R01 CA 39698-04	Role of Oncofetal Antigens in Radiation Carcinogenesis
21.	COOPER, Priscilla K. University of California, Berkeley 5 R01 CA 32986-06	Inducible Responses to Carcinogenic DNA Damage
22.	CORNFORTH, Michael N. Univ. of California/Los Alamos Nat. Lab. 5 R29 CA 45141-02	Cytogenetic Effects Pertaining to Low Doses of Radiation
23.	DEMPLE, Bruce Harvard University 5 R01 CA 37831-04	Oxidative DNA Damage: Repair and Cellular Responses
24.	DOETSCH, Paul W. Emory University 5 R01 CA 42607-03	Repair of Oxidative and Radiation-Induced DNA Damage in Human Cells
25.	DRITSCHILO, Anatoly Georgetown University 5 R01 CA 45408-02	Molecular Studies of Radiation Resistant Tumor Cells

26.	ELKIND, Mortimer M. Colorado State University 5 R01 CA 33701-05	Cell Radiation Response at Low Dose Rates
27.	ELKIND, Mortimer M. Colorado State University 5 R37 CA 41483-03	Radiation Transformation and Its Modulation by Chemicals
28.	ELKIND, Mortimer M. Colorado State University 1 R35 CA 47497-01	Radiobiology of Lethality, Mutation, & Transformation
29.	ESSIGMANN, John M. Massachusetts Inst. of Technology 5 R01 CA 33821-06	Genetic Effects of Ionizing Radiation
30.	EVANS, Helen H. Case Western Reserve University 5 R37 CA 15901-14	Mutants and Altered Radioresponse to Cells and Tumors
31.	EWING, David Hahnemann Univ. School of Medicine 5 R01 CA 28932-06	Lethal Damage from O2 and OH in Irradiated Cells
32.	EWING, David Hahnemann Univ. School of Medicine 5 R01 CA 30921-03	Mechanisms of Damage in Irradiated Cells
33.	FRY, R. J. Michael University of Tennessee 1 R13 CA 48803-01	Radiation Environments and Effects in Space
34.	FRY, R. J. Michael University of Tennessee 1 R13 CA 49108-01	Low Dose Radiation - Basis of Risk Assessment
35.	GARTE, Seymour J. New York Univ. Medical Center 3 R01 CA 43199-01S1	Oncogene Activation in Radiation Carcinogenesis
36.	GARTE, Seymour J. New York Univ. Medical Center 5 R01 CA 43199-02	Oncogene Activation in Radiation Carcinogenesis
37.	GENSLER, Helen L. University of Arizona 5 R29 CA 44504-02	UV Modulation of Chemical Carcinogenesis
38.	GILCHREST, Barbara A. Tufts University 5 R01 CA 45687-02	Retinoids and UV-Induced Melanogenesis

39. GLICKMAN, Barry
York University, Canada
5 R01 CA 45498-02
Radiation-Induced Mutation in Mammalian Cells

40. GRIFFITHS, T. Daniel
Northern Illinois University
5 R01 CA 32579-07
DNA Replication after Insult with UV

41. GRIGGS, Henry G.
John Brown University
2 R01 CA 18809-12
Ultraviolet and Ionizing Radiation Damage

42. GUERNSEY, Duane L.
University of Iowa
5 R01 CA 36483-05
X-Irradiation-Induced Oncogene in Mouse Embryo Cells

43. HALL, Eric J.
Columbia University
2 P01 CA 12536-17
The Effects of Small Doses of Radiation

44. HALL, Eric J.
Columbia University
5 R01 CA 37967-05
Oncogenic Transformation and High LET Radiations

45. HANAWALT, Philip C.
Stanford University
5 R01 CA 35744-04
Molecular Basis of DNA Repair Deficiency in Xeroderma Pigmentosum

46. HARRISON, George H.
University of Maryland
5 R01 CA 40223-04
Ultrasound and Malignant Transformation In Vitro

47. HENNER, William D.
Dana-Farber Cancer Institute
5 R01 CA 35767-05
Ionizing Radiation-Induced DNA Damage and Repair

48. HILL, Colin
Univ. of Southern California
2 R01 CA 42808-04
Neutron Energy: Dose Protraction Effect on Transformation

49. HUBBELL, Howard R.
Hahnemann University
5 R01 CA 37020-03
Oncogenes in Chronic Myelogenous Leukemia

50. HUBERMAN, Eliezer
University of Chicago
5 R01 CA 33974-06
Mutation-Transformation: Neutron Damage and Repair

51. HUMPHREY, Ronald M.
Univ. of Texas System Cancer Center
5 R01 CA 04484-30
DNA Repair and Recovery in the Mammalian Cell Cycle

	Investigator / Institution / Grant	Title
52.	HUMPHREY, Ronald M. Univ. of Texas System Cancer Center 5 R01 CA 24540-09	The Importance of DNA Damage and Repair for Cell Survival
53.	ILIAKIS, George Thomas Jefferson University 2 R01 CA 42026-04A1	Has Cellular Repair a Common Molecular Base?
54.	ILIAKIS, George Thomas Jefferson University 1 R01 CA 45557-01	Radiosensitization by BrdUrd/IdUrd: Cellular Molecular Effects
55.	KASID, Usha N. Georgetown University 1 R29 CA 46641-01	RAF Oncogene Analysis and Radiation Resistant Tumor Cell
56.	KENNEDY, Ann R. Harvard University 5 R01 CA 34680-06	Hormones, Radiation, and Malignant Transformation
57.	KOVAL, Thomas M. George Washington University 5 R01 CA 34158-07	Insect Cells: A Basis for Radioresistance
58.	LANGE, Christopher S. Downstate Medical Center 5 R01 CA 39045-03	Radiosensitivity Prognosis Based on DNA Repair Assay
59.	LAUGHLIN, John Memorial Sloan-Kettering Cancer Center 1 R13 CA 43355-01	Conference on Radiation Carcinogenesis and Dosimetry
60.	LITTLE, John B. Harvard University 5 R01 CA 11751-18	Effects of Radiation on Stationary Cells
61.	LITTLE, John B. Harvard University 5 R01 CA 34037-06	Radiation Mutagenesis in Human Cells
62.	LITTLE, John B. Harvard University 1 R35 CA 47542-01	Effects of Radiation on Mammalian Cells
63.	MARGULIES, Lola New York Medical College 5 R01 CA 35580-05	Ionizing Radiation and Transposon Mobility
64.	MERUELO, Daniel New York University 2 R01 CA 35482-04	Reverse Genetics of a Leukemia Susceptibility Locus

65. MILLER, Scott C.
University of Utah
1 R01 CA 47659-01

Occupational Risk Reduction by Radiotoxin Chelation

66. NAIRN, Rodney S.
Univ. of Texas System Cancer Center
5 R01 CA 36361-05

Repair and Recombination in Radiation Sensitive Cells

67. NELSON, William H.
Georgia State University
5 R01 CA 36810-03

Radiation Chemistry of Purines in the Solid State

68. NORDLUND, Thomas M.
Univ. of Rochester Medical Center
5 R01 CA 41368-03

DNA Damage Studied by Ultrafast Spectroscopy

69. OLEINICK, Nancy L.
Case Western Reserve University
5 R01 CA 15378-15

Radiation-Induced Modifications in Protein Synthesis

70. PEAK, Meyrick J.
University of Chicago
5 R01 CA 34492-05

Solar UV Damage in Human Cells

71. PEAK, Meyrick J.
University of Chicago
5 R01 CA 37848-05

Biological Effects of Solar-UV-Generated Oxygen Species

72. PIEPKORN, Michael W.
University of Utah
3 R29 CA 41591-02S1

Glycosaminoglycans of Skin Tumors

73. PIEPKORN, Michael W.
University of Utah
5 R29 CA 41591-03

Glycosaminoglycans of Skin Tumors

74. PRAKASH, Satya
University of Rochester
2 R01 CA35035-06

Excision Repair of UV Irradiated DNA in Yeast

75. PRAKASH, Satya
University of Rochester
5 R01 CA 41261-03

Repair of UV-Irradiated DNA: Excision Genes of Yeast

76. RAABE, Otto G.
University of California, Davis
1 R01 CA46296-01

Cancer and Injury Risk Assessment for Radionuclides

77. RALEIGH, James A.
Cross Cancer Institute
1 R01 CA 46548-01

Molecular Radiobiology of Nucleic Acids

78.	RAMANATHAN, Brinda Washington State University 5 R23 CA 43079-03	Nuclear Protein Modifications in UV Damaged Human Cells
79.	REDPATH, John L. University of California, Irvine 5 R01 CA 39312-03	Radiobiological Studies of Human Hybrid Cell Lines
80.	REYNOLDS, Richard J. University of California 5 R01 CA 42390-03	Radiation Damage of Eukaryotic DNA and Its Repair
81.	RINALDY, Augustinus Vanderbilt University 5 R01 CA 43769-02	Molecular Cloning of Human DNA Repair Gene(s)
82.	ROSSI, Harald H. Columbia University 5 R01 CA 15307-14	Cell Irradiations with Molecular Ions
83.	SCHNEIDER, Arthur B. Michael Reese Hospital & Med. Ctr. 5 R01 CA 21518-12	Radiation-Induced Thyroid Cancer
84.	SEVILLA, Michael D. Oakland University 5 R01 CA 45424-02	Radiation Induced Lipid & Sulfhydryl Autoxidation
85.	SINCLAIR, Warren K. National Council on Radiation Protection 5 R01 CA 18001-22	Radiation Protection and Measurements
86.	SMITH, Hylton Intl. Com. on Rad. Protection 2 R01 CA 30163-07	Recommendations on Radiological Protection
87.	SMITH, Kendric C. Stanford University 5 R01 CA 06437-27	Molecular Basis of Radiation Lethality
88.	SMITH, Kendric C. Stanford University 2 R01 CA 33738-06	Ionizing Radiation Mutagenesis in Escherichia coli
89.	STAMATO, Thomas D. Wistar Institute 5 R01 CA 45277-02	Isolation of Radiation Sensitive Mammalian Cell Mutants
90.	STAMATO, Thomas D. Wistar Institute 1 R01 CA 48636-01	Poly(ADP-Ribose) and Repair of Radiation Induced Damage

No.	Investigator / Institution / Grant	Project Title
91.	SUMMERS, William C. Yale University 5 R01 CA 45300-02	Molecular Basis of DNA Damage by Ionizing Radiation
92.	SUTHERLAND, Betsy M. Associated Univ./Brookhaven Natl. Lab. 5 R01 CA 23096-11	DNA Repair: Human E. coli Photoreactivating Enzymes
93.	SUTHERLAND, Betsy M. Associated Univ./Brookhaven Natl. Lab. 5 R01 CA 26492-08	UV Transformation, DNA Repair in Human Cells and Skin
94.	TAYLOR, GLENN N. University of Utah 5 R01 CA 28314-09	Reducing Cancer Risk by Radionuclide Chelation
95.	TAYLOR, William D. Pennsylvania State Univ. 1 R01 CA 44658-01A1	Mutagenic & Recombinogenic Effects of Gamma Rays
96.	TAYLOR, Yvonne C. Washington Univ. School of Medicine 1 R29 CA 47855-01	Chromatin Conformation and PLD Repair
97.	UHLENHOPP, Elliott L. Grinnell College 1 R15 CA 47656-01	DNA Damage and Repair
98.	ULLRICH, Robert L. Martin Marietta Energy Systems 2 R01 CA 43322-03	Carcinogenic Interactions of Radiation and Chemicals
99.	VERMA, Surendra New England Medical Center, Inc. 5 R01 CA 36195-05	Membrane Composition and Radiation Damage
100.	WALDREN, Charles A. Eleanor Roosevelt Inst. for Cancer Res. 5 R01 CA 36447-03	Cell Genetic Damage at Low Doses and Dose Rates
101.	WALLACE, Susan S. New York Medical College 5 R37 CA 33657-07	Repair of DNA Damage Induced by Ionizing Radiation
102.	WARD, John F. Univ. of California, San Diego 5 R37 CA 26279-09	Mechanisms in Shouldered Survival Curves
103.	WARD, John F. Univ. of California, San Diego 1 R01 CA 46295-01A1	Studies of Biologically Significant Damage in DNA

104.	WILLIAMS, Jerry R. Johns Hopkins University 5 R01 CA 39543-05	X-Ray Induction of Cellular Hypersensitivity
105.	YASUI, Linda S. University of Utah 5 R29 CA 45011-02	Cytotoxicity of ^{125}I Decay Produced Lesions in Chromatin
106.	ZAIN, Sayeeda B. University of Rochester 5 R01 CA 36432-05	Oncogenes, Oncogene Products in Radiation-Induced Tumors
107.	ZAIN, Sayeeda B. University of Rochester 1 R01 CA 46625-01	C-abl Oncogene in Radiation Induced Thyroid Carcinoma

CONTRACTS ACTIVE DURING FY 88

	Investigator/Institution/Contract Number	Title
108.	BENJAMIN, Stephen A. Food and Drug Administration Y01-CP-50506	Neoplasia in Beagles after Irradiation During Development
109.	FRITZ, Thomas E. Department of Energy Y01-CP-50503	Late Effects of Protracted Irradiation in Dogs
110.	HOFFMAN, F. Owen Department of Energy Y01-CP-60504	Pasture Grass Interception and Retention of Iodine-131
111.	STEVENS, Walter University of Utah N01-CO-23917	Assessment of Leukemia and Thyroid Disease in Relation to Fallout in Utah

ANNUAL REPORT OF

THE CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The Chemical and Physical Carcinogenesis Branch (CPCB) plans, coordinates, and administers a national extramural program of basic and applied research consisting of grants and contracts, collectively concerned with the occurrence and the inhibition of cancer, caused or promoted by chemical or physical agents acting separately or together, or in combination with biological agents; plans, organizes, and conducts meetings of scientists and otherwise maintains contacts with scientists-at-large, to identify and evaluate new and emergent research in, and related to, the fields of chemical and physical carcinogenesis; provides a broad spectrum of information, advice, and consultation to scientists and to institutional science management officials, relative to the National Institutes of Health (NIH) and National Cancer Institute (NCI) funding and scientific review policies and procedures, preparation of grant applications, and choice of funding instrument, based on individual need; plans, develops, maintains, and allocates research resources necessary for the support of carcinogenesis research of high programmatic interest; and provides NCI management with recommendations concerning funding needs, priorities, and strategies relative to the support of chemical and physical carcinogenesis research, consistent with the current state of development of individual research elements and the promise of potential, new initiatives.

Research and related activities supported under this program bear upon a broad range of subject matter areas, with principal emphasis on environmental carcinogenesis, mechanisms of action of chemical and physical carcinogens; the role of DNA damage and repair in carcinogenesis; properties of cells transformed by chemical and physical agents; inter- and intraspecies comparisons in the response to carcinogen exposure; the role of tumor promoters, hormones, and other cofactors in cancer causation; experimental approaches to the inhibition of carcinogenesis; the role of diet and nutrition in carcinogenesis; the role of tobacco products and smoking in carcinogenesis; and in vitro carcinogenesis studies on human and other mammalian cells, tissues, and subcellular fractions. The program also supports the synthesis, acquisition, and distribution of a considerable spectrum of chemical standards, critically needed in the field of carcinogenesis research.

The Branch utilizes a variety of funding instruments to accomplish its objectives. These include: traditional individual research project grants (R01), program project grants (P01), first independent research support and transition (FIRST) awards (R29), conference grants (R13), cooperative agreements (U01), contracts (N01), small business innovative research (SBIR) grants (R43/44), SBIR contracts (N43/44), academic research enhancement awards (AREA) (R15), outstanding investigator grant (OIG) awards (R35), and the method to extend research in time (MERIT) awards (R37). Currently, the Branch administers 490 research grants with an annual budget of approximately 73.3 million dollars.

Grants and contracts administered by the staff of this Branch support six complementary categories of chemical and physical carcinogenesis research and associated resources: Biological and Chemical Prevention, Carcinogenesis Mechanisms, Diet and Nutrition, Molecular Carcinogenesis, Smoking and Health, and Research Resources.

The Biological and Chemical Prevention component is concerned with the experimental inhibition of carcinogenesis caused by chemical, physical, and biological agents. Efforts are devoted to the identification, development, and testing (both in vitro and in vivo) of agents intended to inhibit carcinogenesis. Areas of prime interest include mechanisms of action of candidate preventive agents, binding proteins and receptors, structure-function relationships, and the experimental use of combinations of preventive agents.

The Carcinogenesis Mechanisms category relates to the absorption and body distribution of carcinogens; metabolism, activation, and inactivation of carcinogens; identification of proximate and ultimate carcinogenic forms; molecular structure-carcinogenicity relationships; carcinogen-mutagen relationships; isolation, identification, and synthesis of suspect carcinogens and their metabolites; factors which alter carcinogen activity; the characterization of carcinogen metabolizing enzymes; and the role of hormones in carcinogenesis.

The Diet and Nutrition category supports basic studies on the carcinogenic and anticarcinogenic effects of diet and specific nutrients in animal systems and human cells in vitro.

The Molecular Carcinogenesis component focuses on changes in biological macromolecules and in cell functions as a result of carcinogen exposure; DNA damage and repair following exposure to carcinogens; identification of biochemical and molecular markers and properties of cells transformed by carcinogens; the development of analytical procedures for the identification and quantitation of carcinogens present in biological specimens; interspecies comparisons in carcinogenesis; the role of tumor promoters and the mechanism of tumor promotion in carcinogenesis; and studies on the genetics and mechanism of cell transformation and of the genetics and regulation of enzymes characteristically associated with the carcinogenesis process.

The Smoking and Health category supports studies on the toxicology and pharmacology of smoking and tobacco-related exposures. Both grant and contract mechanisms are used to support these activities.

The Research Resources component, consisting solely of contracts, is principally concerned with the synthesis and distribution of selected chemical carcinogens and certain of their metabolites through a repository. The inventory of over 700 compounds includes natural products, nitrosamines, dioxins, aromatic amines, and asbestos. Particular emphasis has been given to polynuclear aromatic hydrocarbon carcinogens, their metabolic intermediates, and analogous heterosubstituted compounds.

A Program Announcement on the "Biological Role of Exocyclic Nucleic Acid Derivatives in Carcinogenesis" was issued from the Molecular Carcinogenesis component of the Branch in FY 1987 and was open for three rounds of grants. This announcement sought applications on basic mechanistic studies focused on

determining the formation, repair, and relevance to mutagenesis and carcinogenesis of exocyclic nucleic acid derivatives. The compounds of interest which are known or are likely to form exocyclic nucleic acid derivatives include: vinyl halides (vinyl chloride, vinyl bromide), alkyl carbamates (ethyl and vinyl carbamate), halonitrosoureas (1'3-bis[2-chloroethyl]-1-nitrosourea [BCNU], 1'3-cyclohexyl [2-chloroethyl]-1-nitrosourea [CCNU]), monofunctional unsaturated aldehydes (acrolein, crotonaldehyde), bifunctional aldehydes (glyoxal, malonaldehyde, glycidaldehyde), beta-propiolactone, acrylonitrile, N-nitrosopyrrolidine and related cyclic nitrosamines, and some halogenated ethers and aldehydes (chloro- and bromoacetaldehyde). A total of four grants have now been awarded as a result of this announcement totaling over $600,000 in total costs.

Another Program Announcement simultaneously encouraged basic mechanistic studies on the role of omega-3 polyunsaturated fatty acids in cancer prevention. Among the areas of particular interest were: (1) anticarcinogenesis studies in various organ systems, particularly those organ systems in which the type and level of fat have been shown to play a role; (2) determination of whether efficacy is obtained during the initiation period by modifying the susceptibility of the host to early events, or whether these fatty acids modulate the carcinogenic response in the post-initiation period, or both, and including determination of efficacy over the lifetime of the animal; (3) pharmacokinetic studies on the absorption, distribution, metabolism and excretion of these fatty acids, including such studies performed under the experimental conditions demonstrating cancer prevention; (4) studies on toxicology of the agents, including lifetime administration studies under defined dietary conditions in several species of animals; (5) comparative metabolic studies in human versus animal systems; (6) in-depth studies of mechanisms of action, especially as related to conditions known or demonstrating anticarcinogenic efficacy. Four grants totaling over $500,000 have now been awarded and are assigned to the Biological and Chemical Prevention component of the Branch.

The Biological and Chemical Prevention component sponsored a workshop in December of 1987 on "Protease Inhibitors as Cancer Chemopreventive Agents." The workshop was co-chaired by Drs. Deitrich Hoffman and Lee Wattenberg and was attended by thirty investigators in Dedham, Massachusetts. The workshop highlighted the current state of our knowledge on protease inhibitors and their efficacy as chemopreventive agents as well as research needs in the field. The recommendations from this workshop will lead to one or more concept recommendations for consideration by the Division of Cancer Etiology (DCE) Board of Scientific Counselors (BSC) in FY89.

During the last year, the Branch awarded one new outstanding investigator grant of 7 years duration to Dr. Larry Marnett (1 R35 CA47479-01), Wayne State University.

In addition, The Branch was successful in nominating four research grant applicants for the Method to Extend Research in Time (MERIT) award. These investigators were Dr. Paul Hollenberg (2 R37 CA16954-13), Wayne State University; Dr. Malejka-Giganti (2 R37 CA28000-09), University of Minnesota; Dr. Gary Stoner (2 R37 CA28950-07), Medical College of Ohio at Toledo; Dr. George Teebor (2 R37 CA16669-13), New York University.

As a result of a reorganization of the NCI Organ Systems Program, it was decided this year that the active grant portfolios would be reassigned to the operating

Divisions of the Institute. The transfer will be completed late this fiscal year. The CPCB will receive 11 grants dealing with breast cancer totaling $2.24 million, five grants dealing with bladder cancer totaling $0.95 million, five grants dealing with cancer of the large bowel totaling $0.69 million, and three grants on prostate cancer for a total of $0.27 million. This total inventory of $4.15 million will be reported on annually by the CPCB in a Division report and will be collated within the regular annual report of the Branch and reported here as well in the future.

Table I summarizes the number of grants and contracts in the Branch and the total cost for each type of funding instrument. In Table II the distribution of the grants and contracts by program component is summarized.

TABLE I
CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
(Extramural Activities - FY 1988 - Estimated)

	No. of Contracts/Grants	$ (Millions)
Research Contracts	3	0.30
Research Grants	490	71.50
Traditional Research Grants (R01) (384 grants; $47.84 Million)		
Conference Grants (R13) (13 grants; $0.04 Million)		
FIRST Awards (R29) (12 grants; $1.60 Million)		
New Investigator Research Grants (R23) (7 grants; $0.05 Million)		
Program Project Grants (P01) (12 grants; $9.94 Million)		
Cooperative Agreements (U01) (7 grants; $3.93 Million)		
Small Business Grants (R43/R44) (4 grants; $0.30 Million)		
Outstanding Investigator Grants (R35) (5 grants; $3.16 Million)		
RFAs (R01) (30 grants; $1.55 Million)		
MERIT Awards (R37) (11 grants; $3.09 Million)		
AREA Grants (R15) (5 grants; $0.00 Million)		
Research Resource Contracts	6	1.47
	499	73.27

TABLE II
CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
(Contracts and Grants Active During FY 1988)

FY 1988

	CONTRACTS		GRANTS	
	No of Contracts	$ (Millions)	No. of Grants	$ (Millions)
Biological & Chemical Prevention	2	0	73	11.90
Carcinogenesis Mechanisms	0	0	88	13.29
Diet and Nutrition	0	0	42	3.98
Molecular Carcinogenesis	0	0	276	40.03
Smoking and Health	1	0.30	11	2.30
Research Resources	6	1.47	0	0.00
TOTAL	9	1.77	490	71.50

SUMMARY REPORT

BIOLOGICAL AND CHEMICAL PREVENTION

The Biological and Chemical Prevention component of the Branch is responsible for research on agents that inhibit, arrest, reverse or delay the development of cancer in humans. Agents can derive from naturally occurring products such as foods consumed by man, from chemical synthesis, or from various biological sources. At the present time, there are 73 grants in this program area with FY 88 funding of approximately $11.90 million, two contracts with zero FY 88 funding, and seven cooperative agreements with FY 88 funding of approximately $3.93 million.

The research grants consist predominately of Traditional Research Projects (56 R01s), as well as Research Program Projects (3 P01s), New Investigator Research Awards (2 R23s), a First Independent Research Support and Transition Award (1 R29), Academic Research Enhancement Awards (3 R15s), a Conference Grant (1 R13), and a MERIT Award (1 R37). The research grants can be categorized into retinoid (22 grants); antioxidant (8 grants); natural inhibitor (9 grants); micronutrient including selenium, vitamin C and vitamin E (10 grants); carotenoid (3 grants); protease inhibitor (2 grants); omega-3 polyunsaturated fatty acids (2 grants); and miscellaneous (18 grants) areas. The miscellaneous area includes grants on protein kinase C inhibitors, vitamin D analogs, suicide substrates of cytochrome P-450, isothiocyanates, inhibitors of the arachidonic acid cascade, dehydroepiandrosterone, interferon, and nucleophilic compounds active against direct-acting carcinogens. They support diverse types of studies including the experimental inhibition of carcinogenesis, the inhibition or suppression of malignant transformation in culture, mechanisms of action and metabolism of preventive agents, synthesis of chemopreventive compounds, structure-function relationships, pharmacologic disposition, and toxicologic investigations. The most frequently used experimental approach is to study inhibition of carcinogenesis induced by chemical, physical, or biological agents against several stages of the tumorigenic process, and against the development of cancer at many organ sites. The modifying effects of anticarcinogens are investigated relative to a large number of biochemical and biological endpoints, which, in addition to tumorigenesis and transformation themselves, include the activity of the mixed-function oxidase system, free radical generation and quenching, cell proliferation, differentiation, activation/detoxification of carcinogens, DNA damage and repair, binding proteins or receptors for preventive agents, preneoplastic states, and selective attack and prevention of oncogene-specific neoplastic disease.

The Biological and Chemical Prevention component sponsored a workshop this year at the Endicott House in Dedham, Massachusetts, entitled "Protease Inhibitors as Cancer Chemopreventive Agents." Dr. Dietrich Hoffmann, a member of the DCE Board of Scientific Counselors, and Dr. Lee W. Wattenberg, a recent member of the Board, served as cochairmen of the workshop which was organized by Drs. Ann R. Kennedy and Walter Troll. Thirty investigators were in attendance to discuss the status of the field and to identify areas of additional experimentation necessary to discover new protease inhibitors relevant to cancer prevention, determine their range of preventive activity, understand their basic mechanisms of action, and expedite their availability to the research community. A summary of the workshop is being prepared for publication by the organizers, and it is anticipated that a concept statement will be presented to the Board of Scientific Counselors for their consideration in the near future.

The "National Collaborative Chemoprevention Projects (NCCPs)" initiated by request for application (RFAs) in FY 86 and FY 87, provide a mechanism to enhance and expand multidisciplinary/interdisciplinary investigations in chemoprevention through a funding mechanism that permits combination of diverse research expertise from one or more institutions and the facilitating resources of the NCI. The stated objectives of the projects are to generate new approaches and strategies in the inhibition or suppression of the carcinogenic process and to bridge development from individual grant/contract-supported work up to the stage of preclinical/clinical testing of new agents for the protection or prevention of neoplasia. It was indicated last year that three new NCCPs were initiated in FY 87. Since it was found possible to fund one additional project, the number actually funded in FY 87 was four, to give a total of seven. This new project emphasizes the development and study of organoselenium compounds with particular emphasis on inhibition of colon carcinogenesis.

The program initiative developed by a Program Announcement in FY 86 and FY 87 on "The Role of Omega-3 Polyunsaturated Fatty Acids in Cancer Prevention" has not been particularly successful, judged from the standpoint of high ranking priority scores enabling projects to be funded. At the present time, 4 grants have been funded in response to this Program Announcement out of 26 applications reviewed. Of the 26 applications reviewed, 6 were revised applications so that a total of 20 investigators responded to the Announcement. Two major difficulties, among others, in the applications have been failures in proper dietary design relative to caloric density and the lack of careful consideration in protecting these highly unsaturated fats, and diets containing them, from autooxidation. Aside from these technical and scientific considerations, however, this area might be better assessed in relation to any future program emphasis after knowledge is obtained from the recently-initiated projects and after a workshop(s) is held directed at cancer prevention and these unique dietary constituents.

The two contracts supported by this component deal with studies on the toxicology and pharmacology of preventive agents and the inhibition and/or ameliorization of their toxicity. Studies on mechanisms of agent toxicity are also pursued. Research accomplishments on a number of these grant and contract supported endeavors are detailed later in this report.

Grants Activity Summary:

Retinoids: One project exists in the Biological and Chemical Prevention component of the Branch specifically devoted to the design and synthesis of new retinoids, assay of their activities in several in vitro and in vivo systems, exploration of their mechanisms of action, and determination of their toxicologic and pharmacologic properties. The first period of this recently renewed program project has resulted in several notable achievements. Four unique chemical classes of retinoids have been designed, synthesized, and studied during this grant period. One class has been the ether derivatives of retinol, one of which, retinyl propynyl ether, appears to be more advantageous in prevention of mammary carcinogenesis than previously tested retinoids. Not only did this retinyl ether show good chemopreventive activity against carcinogen-induced rat mammary cancer, but it also showed no evidence of toxicity, did not accumulate itself in the liver nor produce elevated levels of liver retinyl palmitate. A second and new class of retinoid that has been synthesized is that of terminal bifunctional analogs. These retinoids have two functional groups at the terminal position of the retinoid side chain portion of the molecule. Examples of such bifunctional

analogs are the ester-acid types, the amide-ester types, and the amide-acid types. The structures of these analogs were determined by proton nuclear magnetic resonance (NMR) analyses and studies of Nuclear Overhauser Enhancements. Stability determinations indicate that they would remain essentially unchanged in bioassay media, or during administration or dosing in other biological assays. Those analogs having a free carboxyl group were shown to bind to cellular retinoic acid binding protein, demonstrating that the presence of a bulky substituent at the alpha position does not interfere with binding. Some of these analogs possessed some degree of activity in four out of five assays in which they were tested. Although little activity was found in inhibiting the development of mouse skin papillomas, all of the bifunctional analogues induced differentiation of mouse F9 embryonal carcinoma cells. A third class of retinoid analogue that has been synthesized is that of the retinoylamino acids. Several of the retinoylamino acids proved to be effective in inducing differentiation of mouse F9 embryonal carcinoma cells and several inhibit protein kinase C as effectively as retinoids known to inhibit experimental carcinogenesis. Perhaps the most interesting result found with this class of analogs, in particular with all-trans-retinoylleucine, is its potency in assays for immunostimulation. This analog enhances the immune response at concentrations as low as 10^{-14} to 10^{-15}M. Although all-trans-retinoic acid itself is active at these low levels, biochemical studies on microsomal amidases and on the cis- and trans-retinoylleucine derivatives show that they are not cleaved to retinoic acid (RA). Furthermore, the all-trans-compound did not show overt toxicity in an efficacy evaluation, and was very much less toxic than retinoic acid in a detailed toxicology study. The fourth class of retinoid analogues synthesized in the program project is that of the 3-substituted-4-oxo-retinoids. It has been known for some time that 4-hydroxy-RA and 4-oxo-RA (and their 13-cis-isomers) are natural RA metabolites that represent products of elimination or detoxification. Such retinoids may have chemopreventive activity, and may have advantage in being less toxic. To this end a number of new 4-oxo-RA analogs (the 3-substituted-4-oxo-RA derivatives) were synthesized and subjected to a number of bioassays. Several of these compounds, which bind to cellular retinoic acid binding protein, proved to be notably effective in inhibiting phorbol ester-induced ornithine decarboxylase activity in mouse skin, in inhibiting carcinogen-induced papilloma formation in mouse skin, and in inducing differentiation of mouse embryonal carcinoma cells. Retinoids have been particularly effective in the inhibition or suppression of experimental mammary, bladder, pancreatic, and two-stage epidermal carcinogenesis. In the context of a development program for chemopreventive agents, a short-term reliable animal bioassay for screening purposes has obvious merits. In this regard, under this program project, such a bioassay has been developed for evaluating the efficacy of new retinoids (or other chemopreventive agents) in preventing mammary cancer. The procedure employs a modification of the well-known N-methyl-N-nitrosourea (MNU) model for induction of mammary cancer in Sprague-Dawley rats. The rapid assay procedure employs a small number of animals and provides data on the efficacy of a new agent in 90 days from the time of administration of the single dose of MNU. The new assay allows a greater number of agents to be assayed at considerable savings in time and cost, not only for the assay itself, but also the time and costs required for synthesizing sufficient quantities of chemopreventive agents required for the animal bioassay. Other accomplishments during this period have been the discovery of a novel, membrane-associated protein that binds retinoic acid, demonstration in vitro and in vivo that retinoids are involved in signal processes of cells of various types, and development of two novel strains of mice that have extreme sensitivity and resistance to tumorigenesis. The sensitive strain of mouse in the classical two-stage epidermal carcinogenesis model produces

100 percent incidence of tumors after just 5 weeks of promotion, with tumor multiplicities in excess of 20 papillomas per mouse. In addition, serum-free cultures of tracheal epithelial cells have been established which possess different morphological and biochemical properties which respond differentially to the cytotoxicity of carcinogens. These epithelial cell lines will be employed in basic studies and in bioassays on anticarcinogenic retinoids. Many high performance liquid chromatographic (HPLC) systems have been developed for metabolic and pharmacologic studies on retinoids. During this period, a new HPLC system has been developed which is a 30-fold more sensitive assay for retinoids. This sensitivity will allow detection of much lower levels of endogenous retinoids and importantly contribute to pharmacokinetic studies.

Finally, biochemical studies on retinoid cis-trans isomerization and on retinamide cleavage have contributed to the solution of some puzzling questions in retinoid toxicology and pharmacology. In this regard, it was demonstrated that low molecular weight compounds containing sulfhydryl or thione groups chemically catalyze retinoid cis-trans isomerization, and that rat liver microsomes contain an amidase that cleaves some retinamides, all of which were previously thought to be resistant to enzymic hydrolysis. The role of such cleavage to retinoic acid in retinamide toxicity requires further elucidation.

High interest continues in the chemopreventive and chemotherapeutic potentials of retinoids in anticarcinogenesis and anticancer action. Since the dual nature of this class of compounds as either preventive or promoting agents in cancer development continues to be shown, detailed studies on their mechanisms of action, pharmacology, and toxicology remain of high priority. Many hundreds of retinoids have been synthesized, but relatively few, even among those showing promise as chemopreventive agents, have had appropriate study of their pharmacologic and toxicologic properties. An example of such a retinoid, which has now received more detailed investigation, is retinyl methyl ether (RME). This synthetic retinoid has been known for at least 37 years. It is known to be a more effective inhibitor of rat mammary carcinogenesis induced by either 7,12-dimethylbenz(a)-anthracene (DMBA) or MNU than the naturally-occurring retinoid, retinyl acetate (ROAc). It is as active as ROAc in the differentiation assay in the hamster tracheal organ culture (HTOC) system for reversal of keratinization. It inhibits asbestos-induced hyperplasia and squamous metaplasia in the HTOC system. It is more active than the natural vitamin A metabolite all-trans-retinoic acid in reversing N-methyl-N-nitro'-N-nitrosoguanidine (MNNG)-induced hyperplasia in organ cultures of mouse prostate, and as active as RA in reversing benzo(a)pyrene-induced squamous metaplasia in these organ cultures. Retinyl methyl ether also suppresses the transformation of rat kidney fibroblasts caused by sarcoma growth factor, and inhibits the proliferation of a human carcinoma and a murine melanoma in cell culture. Furthermore, it is less active (less toxic) than the natural retinoids retinol, ROAc, and RA in degrading cartilage in various culture systems in vitro.

Since these reports have indicated favorable pharmacological and toxicological properties of RME relative to the natural reference retinoids, and since it is the prototypic reference retinyl ether itself, more detailed studies have been done on its disposition, toxicity, and target-organ effects.

Disposition studies have been performed in rats administered oral RME at 10 mg/kg and 40 mg/kg. At the high dose, RME was eliminated from plasma with a terminal half-life of 19.5 hours, but the terminal phase could not be determined at the low

dose. For both doses, the concentrations of RME in the liver, spleen, adrenals, and mammary glands were greater than in plasma. In the adrenal glands of rats administered the low dose, concentrations were as much as 10- to 100-fold higher. Concentrations in the mammary gland, where RME has chemopreventive activity, were also relatively high with an elimination phase half-life of 63-81 hours. The concentration of retinyl esters in the liver did not increase after RME administration and no retinyl esters were detected in the mammary gland. In 28 day toxicology studies, it was found that the toxic effects of RME were similar to those of ROAc. At equivalent doses, weight-gain depressions, bone fractures, elevations in serum triglycerides, anemia, elevations in cholesterol, and reductions in serum albumin were similar. Histopathologic findings, predominantly in high-dose groups, were in bone (osteodystrophy), skeletal muscle (hemorrhage), liver (hepatocellular vacuolization and hematopoietic cell proliferation), spleen (lymphoid hyperplasia and hematopoietic proliferation), forestomach (mucosal hyperplasia and hyperkeratosis), thymus (cortical epithelial depletion), and adrenals (cortical hyperplasia in males). Overall, since RME has greater anti-carcinogenic activity than ROAc and equal toxicity, its therapeutic index would be more favorable for chemoprevention studies (75).

Mechanisms of Anticarcinogenesis: One of the central mechanisms of chemical protection against carcinogenesis, mutagenesis, and other forms of electrophile toxicity is the elevation of enzymes involved in xenobiotic metabolism. This aspect of anticarcinogenesis is being investigated in great detail in a new program project at the Johns Hopkins University (62). Xenobiotic metabolizing enzymes can be classified into two groups, designated as Phase I (e.g., cytochromes P-450) and Phase II (conjugation) enzymes. Many studies show that chemoprotection against carcinogens occurs when Phase II enzymes, such as glutathione S-transferases, UDP-glucuronosyltransferases, and NAD(P)H:(quinone acceptor) oxidoreductase only are elevated in animal tissues. Phase II enzyme induction appears to be a necessary and sufficient condition for obtaining chemoprotection. Furthermore, such protection can be achieved by the administration of an astonishing variety of seemingly unrelated chemical agents including polycyclic aromatic hydrocarbons, azo dyes, flavonoids, phenolic antioxidants (e.g., butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), tert-butylhydroquinone), isothiocyanates, diterpenes, indoles, unsaturated lactones, 1,2-dithiol-3-thiones and thiocarbamates. These chemical protectors are all enzyme inducers, but fall into two categories according to the patterns of enzyme induction that they evoke. These two categories have been termed (62) bifunctional inducers and monofunctional inducers: bifunctional inducers elevate both Phase I and Phase II enzymes, while monofunctional inducers enhance the activities of Phase II enzymes selectively. Since Phase I (e.g., cytochrome P_1-450) induction is also an important mechanism for the activation of many carcinogens to ultimate carcinogens, whereas Phase II enzyme induction is often chemoprotective, an understanding of the mechanisms underlying these two types of induction is of critical importance for devising strategies for achieving chemoprotection in man.

The molecular mechanisms whereby bifunctional inducers (large planar aromatics) elevate Phase I enzymes are well established. These inducers bind to the Ah receptor which enhances the transcription of selected cytochrome P-450 genes and results in increased metabolism of the aromatic inducers. Because induction of Phase II enzymes by large planar aromatics generally occurs only in mouse strains and cultured hepatocytes with a functional Ah locus, the regulation of Phase II enzymes has been assumed to occur through the same mechanism as that regulating

Phase I enzymes. Yet efforts to establish this connection by genetic means have not been persuasive.

In earlier work this group showed that monofunctional inducers (of Phase II enzymes) have little structural similarity, and their mechanism does not appear to involve participation of a conventional receptor. Furthermore, monofunctional inducers elevate Phase II enzymes in mutant murine hepatoma cells defective in Ah receptor function and in mutant mice in which the Ah receptor is nonresponsive to polycyclic hydrocarbons. It was inferred that the action of monofunctional inducers does not depend on their morphological features but the presence or ability to generate metabolism by a common chemical signal that has now been identified as an electrophilic or Michael acceptor function. It had been suggested previously that by binding to the Ah receptor, bifunctional inducers specify enhanced synthesis of cytochrome P_1-450 which in turn converts these bifunctional inducers to metabolic products that have the same chemical reactivities as monofunctional inducers.

In order to clarify the relation between the induction mechanism whereby monofunctional and bifunctional inducers elevate Phase I and Phase II enzymes, a systematic analysis was undertaken of this relation by the use of 15 structurally dissimilar monofunctional and bifunctional inducers in two experimental systems: wild-type and mutant murine hepatoma cell cultures and genetically defined mice. Measurements of aryl hydrocarbon hydroxylase activity was used as an index of Phase I enzyme induction, and quinone reductase activity was used as a marker for Phase II enzyme induction. These experiments demonstrated the following:

a. There are two clearly distinguishable types of Phase I and Phase II enzyme inducers: monofunctional (Phase II only) and bifunctional (Phase I and Phase II). Bifunctional inducers may be further subdivided into those that are metabolized (e.g., polycyclic aromatic hydrocarbons, azo dyes) and those that are not significantly metabolized, e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin, (TCDD).

b. There are large differences in the ratios of the elevations (treated/control-specific activities) of quinone reductase and aryl hydrocarbon hydroxylase produced by different types of bifunctional inducers.

c. The concentrations of TCDD required to produce half-maximum induction of aryl hydrocarbon hydroxylase and quinone reductase are similar (50-100 pM). TCDD is also the most effective inducer in elevating aryl hydrocarbon hydroxylase (60-fold) but not the most effective inducer of quinone reductase.

d. Saturating concentrations of monofunctional and bifunctional inducers that produce maximal inductions singly, produce more than additive elevations of quinone reductase when present simultaneously.

e. In a mutant hepatoma cell line lacking Ah receptor function (because of defective transport of receptor-ligand to the nucleus), the quinone reductase activity is low (about 1/3 control), but monofunctional inducers evoke normal inductions of quinone reductase, whereas all bifunctional inducers (TCDD, polycyclics) are inactive. In a second hepatoma cell line mutant that has a normal Ah receptor but contains an inactive cytochrome P_1-450 gene product, the basal quinone reductase levels are high and they are inducible normally by monofunctional inducers. In this mutant, bifunctional inducers are weakly

active in elevating quinone reductase levels, but have no effect on the very low aryl hydrocarbon hydroxylase activity.

From these observations, a comprehensive model has been proposed for the relation between the mechanisms of induction by monofunctional and bifunctional inducers. Central to this model is the concept that bifunctional inducers enhance their own metabolism by elevating aryl hydrocarbon hydroxylase activity, and thereby generate molecules that have the electrophilic properties of monofunctional inducers. This would explain why there are such large differences in the potencies of induction of quinone reductase by bifunctional inducers since the rates of their metabolism to products that resemble monofunctional inducers is likely to be very different, and why a functional Ah receptor is required for the induction of quinone reductase by bifunctional but not by monofunctional inducers.

This group recognizes that the proposed metabolic cascade cannot explain some of the other experimental findings described above, i.e., the more than additive inductions of quinone reductase by combinations of maximally inducing concentrations of mixtures of monofunctional and bifunctional inducers. However, these investigators have suggested in a recent publication that the binding of bifunctional inducers to the Ah receptor might directly stimulate the transcription of the gene for quinone reductase by a separate mechanism not involving the generation of the aforementioned electrophilic chemical signal. This mechanism would account for the more than additive effects of maximally inducing concentrations of combinations of monofunctional and bifunctional inducers. Direct stimulation of gene transcription would also explain the action of TCDD, a nonmetabolizable bifunctional inducer that presumably cannot generate an electrophilic signal. The only experimental finding irreconcilable by this model is the high level of basal quinone reductase in the mutant that contains a functional Ah receptor but an inactive cytochrome P_1-450 gene product, and in which quinone reductase is normally inducible by monofunctional inducers and weakly inducible by bifunctional inducers. The latter findings are accommodated in the model at the present time by postulating an endogenous (or perhaps medium-derived) inducer that is normally inactivated by aryl hydrocarbon hydroxylase, but accumulates in mutants in which this enzyme is absent. This hypothetical endogenous inducer binds to the Ah receptor in the mutant murine hepatoma cell line to activate quinone reductase synthesis.

It is only 30 years since selenium was first demonstrated as an essential trace element for the normal growth and function of avian and mammalian species. Moreover, it is not much more than 10 years (except for one prescient paper almost 40 years ago) that its remarkable efficacy (at high doses) has been demonstrated in chemoprevention of carcinogenesis. In this regard, sodium selenite has been shown to inhibit experimental carcinogenesis, in both mice and rats, of tumors arising from the mouse mammary tumor virus and from treatment with chemical carcinogens, and of epithelial tumors arising in the mammary gland, colon, skin, liver, and stomach. The extent of inhibition in these studies has ranged from 48% to 88%. Selenium is known to inhibit/suppress carcinogenesis at both the initiation and post-initiation stages. For the mammary gland, selenium inhibits at both of these stages, perhaps more effectively when administered post-initiation, and is required on a continuous basis for inhibition to be maintained. The basic mechanisms of action of selenium either in animal and human nutrition at trace levels, or in the prevention of carcinogenesis at high levels, are not completely known. One aspect of selenium action that is well known is its mediation as part of the enzyme glutathione peroxidase in the cellular antioxidant

defense mechanisms. Glutathione peroxidase contains selenium as selenocysteine and functions to protect membrane lipids and nucleic acids from oxidative damage by catalytically reducing hydrogen peroxide, organic hydroperoxides, and nucleic acid peroxides, while simultaneously oxidizing the protective molecule glutathione. However, this mechanistic antioxidant function of selenium in glutathione peroxide, of known importance at physiological levels of selenium, has never been demonstrated as an important mechanism of its chemopreventive action at high levels. Other mechanisms for the anticarcinogenic activity of selenium that have been proposed include modification of carcinogen metabolism toward detoxification pathways, decreased binding of carcinogen/carcinogen metabolites to DNA, decreased specific adduct formation, antimutagenic activity of selenium, and increased DNA repair processes. However, many investigators feel that additional or alternate mechanisms of selenium action are necessary to explain its observed chemopreventive activities.

Such considerations have stimulated the search for selenoproteins other than glutathione peroxide that may play a role in the anticarcinogenic activity of selenium. Evidence for the existence of selenoproteins in mammalian systems has been reported by a number of investigators. These include a low molecular weight (M_r 10,000) selenoprotein having characteristics similar to the cytochromes, which has been isolated from lamb muscle and whose absence has been implicated in the cause of nutritional muscular dystrophy in selenium-deficient sheep, a selenocysteine-containing protein in rat and monkey plasma that plays a role in selenium transport and storage, and six selenocysteine-containing proteins, distinguishable from glutathione peroxidase, in rat tissue extracts whose specific enzymatic or biochemical functions remain unknown. Recently, the powerful technique of two dimensional polyacrylamide gel electrophoresis has been applied to determine the number and molecular weights of selenoproteins found in mouse mammary cells in order to determine their significance as a mediator of the biological role of selenium as a chemopreventive agent. Results show that selenium is incorporated into specific polypeptides of 11 different molecular weights which are resolvable as 25 distinct spots on a two-dimensional gel. The biochemical form of selenium in the major selenium-containing proteins appears to be predominately selenocysteine. Selenium-containing polypeptide fragments from several of the selenoproteins of differing molecular weights were generated by N-chlorosuccinimide cleavage which is known to cleave tryptophan residues selectively with high efficiency, and has been used to produce partial peptide maps from nanogram quantities of proteins. Differences in the molecular weights of the resulting selenium-containing polypeptide fragments indicate that several of the selenoproteins are probably unique and unrelated. These major proteins had relative molecular weights of 58,000, 26,000, and 22,000. Of interest was the fact that cleaved selenopolypeptides were not detected for selenoproteins having relative molecular weights of 18,000 and 14,000. Overall, this pioneering work provides evidence for the existence of selenium-binding proteins in addition to glutathione peroxide within mammary gland epithelial cells in vitro and murine tissues in vivo. Specifically, 11 subunit sizes were detected ranging in molecular weight from 78,000-12,000. The major selenoproteins had relative molecular weights of 58,000, 26,000, 22,000, 18,000, and 14,000, and were found consistently in cells cultured in vitro and in tissue homogenates from ^{75}Se-treated animals. These proteins were shown to contain selenium stably associated with protein in the form of selenocysteine. Such demonstration of the existence and characteristics of mammary gland epithelial cell selenoproteins is expected to contribute to biochemical understandings of the chemopreventive action of selenium against mammary carcinogenesis (44).

BIOLOGICAL AND CHEMICAL PREVENTION

GRANTS ACTIVE DURING FY88

	Investigator/Institution/Grant Number	Title
1.	ALWORTH, William L. Tulane University of Louisiana 1 R01 CA38192-01A3	Suicide Substrates of Cytochrome P-450 as Anticarcinogen
2.	AWASTHI, Yogesh C. University of Texas Med Br Galveston 5 R01 CA27967-08	Mechanism of Anti-Carcinogenic Effect of Antioxidants
3.	BAILEY, George S. Oregon State University 5 R01 CA34732-05	Mechanisms of Inhibition of Chemical Carcinogenesis
4.	BANERJEE, Mihir R. University of Nebraska Lincoln 2 R01 CA25304-07A1	Chemical Carcinogenesis Mammary Gland Organ Culture
5.	BELL, Robert M. Duke University 1 U01 CA46738-01	Protein Kinase C Inhibitors as Chemopreventive Agents
6.	BELMAN, Sidney New York University 5 R01 CA38156-03	Tumor Control by Onion, Garlic, and a Protease Inhibitor
7.	BERTRAM, John S. University of Hawaii at Manoa 2 R01 CA39947-03	Inhibition of In Vitro Transformation by Retinoids
8.	BRESNICK, Edward University of Nebraska Medical Center 5 R01 CA38150-03	Cruciferae and Carcinogenesis
9.	BROWN, Neal C. University of Massachusetts Medical Sch 5 R01 CA40893-03	Novel Inhibitor-Probes of the Ras Oncogene Protein P21
10.	BYUS, Craig V. University of California Riverside 1 R01 CA45707-01	Nutritional Modification of Cancer Cell Growth
11.	CHOPRA, Dharam P. Southern Research Institute 5 R01 CA35593-02	Mechanism of Retinoid Action Against Prostate Lesions
12.	CHUNG, Fung-Lung American Health Foundation 5 R01 CA41544-02	Glucosinolates and Environmental Nitrosamine Activation

13. COPE, Frederick O.
Southern Research Institute
5 R01 CA40894-03
Retinoid Receptor Control in Cytodifferentiation

14. CULLUM, Malford E.
Michigan State University
1 R29 CA45860-01
Function of 13-Cis-Retinoic Acid in HL-60 Cells

15. CURLEY, Robert W., Jr.
Ohio State University
5 R01 CA40967-03
Affinity Probes For the Retinoic Acid-Binding Proteins

16. DAWSON, Marcia I.
SRI International
5 R01 CA30512-06
Novel Retinoids For Chemoprevention of Cancer

17. DAWSON, Marcia I.
SRI International
5 R01 CA32428-06
Retinoid Tumor Inhibitory Activity-Toxicity Probe

18. DESCHNER, Eleanor E.
Sloan-Kettering Institute For Cancer Res
1 R01 CA46845-01
Omega-3 Fatty Acids: Inhibitors of Colon Cancer

19. DOERING, William V.
Harvard University
5 R01 CA41325-02
Semi-Rigid Conjugated Polyenes as Model Anticarcinogens

20. DURHAM, John P.
West Virginia University
5 R01 CA37060-03
Calcium/Lipid Protein Kinase in Myeloid Differentiation

21. FISCHER, Susan M.
University of Texas System Cancer Center
1 R01 CA46886-01
The Role of Omega-3 PUFA in Cancer Prevention

22. GLAUERT, Howard P.
University of Kentucky
1 R01 CA43719-01A1
Diet and Carcinogenesis by Peroxisome Proliferators

23. GOULD, Michael N.
University of Wisconsin Madison
5 R01 CA38128-04
Anticarcinogenic Agents in Orange Peel Oil

24. GUDAS, Lorraine J.
Dana-Farber Cancer Institute
1 R01 CA43796-01A1
Cellular Retinoic Acid Binding Protein

25. HECHT, Stephen S.
American Health Foundation
1 U01 CA46535-01
Isothiocyanates and Nitrosamine Carcinogenesis

26.	HILL, Donald L. Southern Research Institute 5 P01 CA34968-03	Development of Chemopreventive Retinoids
27.	HULTIN, Theresa A. IIT Research Institute 5 R23 CA40521-03	Pharmacogenetics of Retinoid Inhibition of Liver Cancer
28.	IP, Clement C. Y. Roswell Park Memorial Institute 2 R01 CA27706-07A1	Selenium Chemoprevention of Mammary Carcinogenesis
29.	IP, Clement C. Y. Roswell Park Memorial Institute 1 P01 CA45164-01	Mechanism of Selenium Chemoprevention of Carcinogenesis
30.	KENNEDY, Ann R. Harvard University 1 U01 CA46496-01	Cancer Prevention by Protease Inhibitors
31.	KENSLER, Thomas W. Johns Hopkins University 5 R01 CA39416-03	Modulation of Aflatoxin Toxicity by Dietary Antioxidants
32.	KOEFFLER, H. Phillip University of California Los Angeles 5 R01 CA33936-05	Action of Retinoids on Myeloid Leukemia Cells
33.	KOEFFLER, H. Phillip University of California Los Angeles 5 U01 CA43277-02	Vitamin D and Preleukemia/ Leukemia--Cancer Chemoprevention
34.	KRINSKY, Norman I. Tufts University 1 R13 CA45274-01	Eighth International Symposium on Carotenoids
35.	KRUMDIECK, Carlos L. University of Alabama at Birmingham 5 R01 CA40834-03	Folate Deficiency--Preventable Risk of Cancer
36.	LAM, Luke K. University of Minnesota of Mnpls-St Paul 5 R01 CA38932-03	Carcinogenesis of Butylated Hydroxyanisole
37.	LANDOLPH, Joseph R. University of Southern California 5 R01 CA41277-02	Inhibition of Chemical Trans-Formation by Aspirin
38.	LIEHR, Joachim G. University of Texas Med Br Galveston 5 R01 CA44069-02	Prevention of Estradiol-Induced Tumors by Vitamin C

39. LIPKIN, Martin
Sloan-Kettering Institute For Cancer Res
5 R01 CA40876-03
Natural Inhibitor of Colonic Cell Damage

40. LOTLIKAR, Prabhakar D.
Temple University
5 R01 CA40885-03
Mechanism of Anticarcinogenesis by Antioxidants

41. MACDONALD, Paul C.
University of Texas Hlth Sci Ctr Dallas
5 U01 CA43311-02
Dehydroisoandrosterone Therapeutics--Biomolecular Basis

42. MC CORMICK, Anna M.
University of Texas Hlth Sci Ctr Dallas
5 R01 CA31676-06
Metabolism of Chemopreventive Retinoids

43. MC CORMICK, David L.
IIT Research Institute
5 R01 CA40874-03
Arachidonic Acid Metabolism and Cancer Chemoprevention

44. MEDINA, Daniel
Baylor College of Medicine
5 R01 CA11944-15
Biology of Mammary Preneoplasias

45. MEHTA, Rajendra G.
IIT Research Institute
2 R01 CA34664-04A2
Hormone and Retinoid Interaction in Mammary Tissue

46. NAPOLI, Joseph L.
State University of New York at Buffalo
5 R01 CA42092-03
Determinants of Vitamin A Homeostasis

47. NILES, Richard M.
Boston University
2 R01 CA32543-06A1
Regulation of Growth and Differentiation by Retinoids

48. ONG, David E.
Vanderbilt University
5 R01 CA20850-10
Cancer and Vitamin A

49. PACKER, Lester
University of California Berkeley
1 R01 CA47597-01
Membrane Reactions of Vitamin E

50. PRASAD, Kedar N.
University of Colorado Hlth Sciences Ctr
5 R01 CA40619-03
Mechanism of Action of Vitamin E on Cancer

51. PROUGH, Russell A.
University of Louisville
5 R01 CA43839-03
Inhibitor Effects on Monooxygenase Function

52.	RAO, Damanna Ramkishan Alabama Agri and Mech University 1 R15 CA45160-01	Anticarcinogenic Activity of Lactic Cultures
53.	REDDY, Bandaru S. American Health Foundation 1 U01 CA46589-01	Chemoprevention of Colon Cancer by Organoselenium
54.	REDDY, Chinthamani C. Pennsylvania State University Park 5 R01 CA37979-02	Vit E, Selenium, and Fatty Acid-Dependent B(a)P Oxidation
55.	REDDY, Janardan K. Northwestern University 5 R01 CA32504-05	Modification of Peroxisome Proliferator Carcinogenesis
56.	REINERS, John J., Jr. University of Texas System Cancer Center 5 R01 CA34469-05	Inhibition of Chemical Carcinogenesis by Interferon
57.	REINERS, John J., Jr. University of Texas System Cancer Center 5 R01 CA40823-03	Inhibition of Chemical Carcinogenesis by Copper Chelates
58.	SANI, Brahma P. Southern Research Institute 5 R01 CA40756-02	Studies on Retinoic Acid-Binding Protein
59.	SCHWARTZ, Arthur G. Temple University 5 R01 CA38574-03	Studies on Cancer Preventive Effects of DHEA and Analogs
60.	SINHA, Dilip K. Roswell Park Memorial Institute 2 R01 CA36139-04	Protection Against Mammary Carcinogenesis by Pregnancy
61.	TALALAY, Paul Johns Hopkins University 1 P01 CA44530-01	Novel Strategies For Chemo-protection Against Cancer
62.	THOMPSON, Henry J. University of New Hampshire 5 R01 CA38265-03	Cancer Prevention and Vanadium
63.	THOMPSON, John A. University of Colorado at Boulder 5 R01 CA41248-02	Bioactivation of Dietary Phenols by Hemoproteins
64.	VERMA, Ajit K. University of Wisconsin Madison 5 R01 CA42585-02	Inhibition of ODC Induction by Retinoid

65. WALL, Monroe E.
Research Triangle Institute
5 R01 CA38245-03

New Natural and Synthetic Inhibitors of Carcinogenesis

66. WANG, Alexander Y.
University of Texas System Cancer Center
5 R01 CA35363-03

Vitamin E and Cancer

67. WANG, Sho-Ya
State University of New York at Albany
5 R23 CA44955-02

Differentiation of Teratocarcinoma Cells: Regulation

68. WATTENBERG, Lee W.
University of Minnesota of Mnpls-St Paul
5 U01 CA43285-02

Chemoprevention of Carcinogenesis by Nucleophiles

69. WEBB, Thomas E.
Ohio State University
5 R01 CA38125-03

B-Glucuronidase Inhibition and Chemical Carcinogenesis

70. WILL III, Oscar H.
Augustana College
1 R15 CA42048-01A1

Carotenes and Cytoprotection *Ustilago violacea*

71. WOLF, George D.
Massachusetts Institute of Technology
5 R01 CA13792-09

Vitamin A and Glycoproteins of Skin Tumors

72. WU, Reen
University of California Davis
5 R01 CA42097-03

Retinoids and the Growth of Respiratory Tract Epithelium

73. YAVELOW, Jonathan
Rider College
1 R15 CA43565-01

Anticarcinogenic Mechanisms of Bowman-Birk Inhibitors

74. HILL, Donald L.
Southern Research Institute
N01-CP-41005

Studies on Toxicology and Pharmacology of Biological and Chemoprevention Agents

75. MCCORMICK, David L.
IIT Research Institute
N01-CP-41063

Studies on Toxicology and Pharmacology of Biological and Chemoprevention Agents

SUMMARY REPORT

CARCINOGENESIS MECHANISMS

The Carcinogenesis Mechanisms component of the Branch includes studies of the metabolism, toxicity, physiological disposition, and mechanisms of action of carcinogens and their metabolites. Also included are syntheses of both known and suspect carcinogens or the development of derivatives for molecular structure-activity relationships. Other studies deal with the hormone-related biochemistry of cancer and cancerous hosts, the identification of reactive metabolites, and the isolation and characterization of carcinogen-metabolizing enzymes. In FY 88 there was one Research Program Project (P01), one Outstanding Investigator Grant (R35), four MERIT Awards (R37), one Small Business Innovative Research Award (R44), five Conference Grants (R13), and 76 Traditional Research Grants (R01) with a total funding level of $13.18 million.

Grants Activity Summary

Fish RFA: A request for application (RFA) for "Studies on the Etiology of Neoplasia in Poikilothermic, Aquatic Animals: Finfish and Shellfish" was issued on January 31, 1986. The purpose of the RFA was to develop a broad spectrum of studies that would facilitate the understanding of the etiology of neoplasia in finfish and shellfish. Fifty-four applications were received in response to the RFA and a total of nine awards were made. The RFA was jointly sponsored by the National Cancer Institute (CPCB, DCE), the National Institute of Environmental Health Sciences (NIEHS), and the Department of the Army (Medical Research and Development Command). The Army funds for this initiative are being administered by the NCI through an Interagency Agreement. The NCI and Army funds were used to award seven grants. Two other applications were given NIEHS primary assignment and were awarded by NIEHS early in FY 87.

Carcinogenesis is a complex, multistage process with both environmental and genetic components. Fish species are subject to developing a variety of neoplasms in both inbred and natural populations when exposed to polluted environments or experimentally treated with carcinogens. Of note is the fact that spontaneous neoplasms seem to be essentially nonexistent when fish are obtained from pristine waters. Study of fish models has accelerated in response to the finding of some similarities of fish tumors to cancers of mammalian species, and the realization that aquatic organisms represent potential environmental sentinels. It is also appreciated that many fish tumor systems offer reasonable and cost-effective models for experimental carcinogenesis. One of the "Fish RFA" grants involves the genetic mapping of biochemical markers of Xiphophorus fishes (Poeciliidae) (65). Analyses of allozyme phenotypes in interspecific backcross hybrids of platyfishes and swordtails of the genus Xiphophorus has resulted in the assignment of more than 50 protein-coding loci to multipoint linkage groups. The currently defined linkage groups (LGs) could reside on as many as 17 of the 24 chromosome pairs. An additional 17 polymorphic loci remain unassigned to the gene map. Comparison with mammalian gene maps identifies several potentially conserved gene arrangements: muscle pyruvate kinase and mannosephosphate isomerase (Xiphophorus LG II), glucosephosphate isomerase-1 and peptidase D [LG IV] and glyceraldehyde-3-phosphate dehydrogenase-3 and triosephosphate isomerase-1 [LG U4]. The striking similarity of linkage relationships in Xiphophorus and salmonid gene maps reveals extensive probable homology of gene arrangements in teleosts. When homologous map segments are compared between confamilial taxa such as Xiphophorus and

Poeciliopsis, similar estimates of recombination distance between homologous loci are observed. Such comparisons suggest that assembly of gene maps in a variety of fish taxa will allow prediction of gene arrangements in the ancestor of all vertebrates.

An epizootic of hepatic tumors had been described in the flatfish winter flounder (Pseudopleronectes americanus) in Boston Harbor. This disease was associated with locally high levels of carcinogenic polycyclic aromatic hydrocarbons (PAHs), suggesting a hypothesis that these compounds are involved in the origin of these tumors. The overall objective of one of the RFA awards was to examine fundamental aspects of the biochemistry and molecular biology associated with environmental tumorigenesis in these fish (77). Progress in the environmental tumorigenesis in winter flounder has proceeded on several lines. The work has emphasized analysis of oncogene activation and expression. DNA from liver tumors of winter flounder transfected into fibroblasts and subsequently into athymic (nude) mice has shown that DNA from more than 50% of the fish bearing histologic abnormalities produced tumors in nude mice. This is a higher percentage than achieved in similar analyses with tumor DNA of any type. The samples to date have all shown that the flounder k-ras proto-oncogene is present in the DNA from nude mice tumors. Direct sequence analysis of enzymatically amplified DNA from the primary flounder has shown that in more than 90% of the individuals examined, there was a k-ras proto-oncogene with a G-T mutation in the 12th codon; the wild type gene in fish lacking any gross abnormalities contained the same sequence in the first exon of the k-ras gene as present in the rat, with a GGT at the 12th codon.

The syndrome present in winter flounder livers includes a high prevalence of biliary proliferation and a cellular structure believed to be apoptosis. Analysis of ras gene product expression in livers from Boston Harbor fish has revealed a much greater level of p21 (ras protein) in hepatocytes and biliary cells from animals which possess apoptotic lesions. This result was obtained with monoclonal antibodies (RAP-5) to a segment of the first exon of p21. Pre-incubation of the antigen with the antibody eliminated the staining of p21 in immunohistochemical sections. The results clearly suggest that animals showing histologic abnormalities have also an altered expression of ras genes.

The RFA also supports research on the mechanistic and environmental aspects of a hemic neoplastic disorder of the marine mussel, Mytilus edulis (12). The goals of this project include the determination of the molecular basis of and relationship of the neoplasm to that of higher animals. Hemic neoplasia of bay mussels occurs in certain Puget Sound, Washington, populations at a prevalence of at least 45%. Experimental studies showed a progressive disease in 20/40 mussels with 60% of these dying from the disease. Remission occurred in 20% of the experimental mussels and resulted from infiltration of granulocytes, secretion of an extracellular matrix which immobilized neoplastic cells and the formation of granuloma-like structures. Neoplastic cells showed reduced phagocytosis in vitro, and diseased animals had reduced ability to clear injected bacteria compared to normal mussels. Further experiments demonstrated the ability to transplant hemic neoplasia to disease-free mussels with intact neoplastic cells, to transmit with cell-free homogenate, and transmit by the cohabitation of disease-free mussels with infected mussels. Cell cycling studies indicated that cell division of blood cell populations containing normal increments of DNA is first amplified as the disease process begins. Subsequently, discrete populations of cells with aneuploid DNA complements are formed from two anomalous cell cycles. DNA complements are as high as 14.7X haploid in the most advanced neoplastic population.

The DNA complement of discrete elements of abnormal cell cycles continously increases, while the cells cycle in relative incremental but aneuploid DNA increases. Reverse transcriptase assays, ultrastructural examination of density and velocity gradient-separated cell homogenate, and whole tissues have so far failed to demonstrate the etiologic agent of the disease. The studies indicate that neoplastic cells continuously increase their DNA content but that discrete populations corresponding to elements of a cell cycle are maintained. In addition, the results support a continuous and synchronous cell transition model of the disease, indicate its infectious nature, and the reduced immunocompetence of affected mussels.

Interest has heightened in the use of teleosts for bioassay testing, detection of carcinogens in the environment, and as potential comparative oncology models for human cancer. However, basic questions remain on the pathogenesis and biologic properties of fish tumors. The liver is sensitive to carcinogens in fish and higher vertebrates. To further develop fish as useful animal models, researchers in one laboratory have analyzed aspects of hepatic carcinogenesis in rainbow trout (Salmo gairdneri) and in Japanese medaka (Oryzias latipes) (33). In vivo exposure of young Oryzias latipes to the carcinogen, diethylnitrosamine (DENA), results in tumor formation after a brief latent period. During the serial analysis of cytologic changes accompanying neoplastic progression, cellular alterations and the appearance of enzyme-altered foci were reported. In addition, the hepatocellular neoplasms were shown to contain basophilic hepatocytes. These were arranged as thickened tubules, solid round cellular masses and elongated, triangular-shaped cells forming irregular extensions (poorly differentiated tumor). Common cytologic features of tumor cells and cells of early altered foci (enzyme and tinctorial) suggested a rapid neoplastic transformation of hepatocytes. The purpose of this study was to examine the ultrastructure of alterations occurring with the development of hepatic neoplasms in Oryzias latipes exposed to a tumorigenic concentration of DENA.

Due to their low water solubility, PAHs (especially those with greater than three aromatic rings) are predominantly associated with particulate matter and accumulate in sediment reservoirs worldwide. Aquatic organisms can accumulate PAHs through their diet. Recent laboratory experiments comparing uptake from food versus skin exposure to sediment indicated that dietary uptake may be more efficient. One would expect this route to be even more important in the transfer of sediment-associated PAH to pelagic fish which may be in contact with the sediments only in search of food. The goal of the proposed work by another "Fish RFA" awardee is to determine the possible trophic transfer of PAHs, their metabolites and their bound residues from marine worms (Nereis) to flounder. Studies will also determine if PAH metabolites and bound residues are available for accumulation and re-metabolism in predators, and ultimately whether they pose a health risk to the consumer (59). Winter flounder and green crab were collected locally from the Gulf of Maine and maintained in running seawater. In captivity, animals were fed a prepared diet containing cod liver oil, gelatin, and Tetramin fish food daily. Food was withheld from experimental organisms for 48 hours prior to administration of a single dose of [7-^{14}C-]benzo(a)pyrene (B(a)P). Organisms were sacrificed at periods of up to 12 days, and all major organs and tissues were collected for the determination of total radioactivity and the metabolic class determination, where appropriate. Preliminary determination of metabolite formation was made in flounder liver and crab hepatopancreas. Analysis of fish liver and crab hepatopancreas indicates that both organisms metabolize B(a)P very rapidly. In the flounder at all time points, 53 to 70% of recovered radioactivity

was found as conjugated metabolites, 11 to 31% as bound residues, with only 4 to 15% found as organic soluble material (parent and primary metabolites). B(a)P appeared to be less extensively metabolized in the green crab hepatopancreas, with 30 to 46% found as conjugated metabolites, 18 to 33% as bound residues, and 26 to 42% found in the organic extract. In summary, two species of locally abundant marine organisms were shown to metabolize dietary B(a)P in vivo extensively to conjugated and bound metabolites showing patterns of B(a)P accumulation similar to that reported for other closely related marine organisms. Although a relatively small proportion of the dose given was assimilated, significant levels of B(a)P-derived compounds remained in selected tissues of winter flounder and green crabs for at least 4 to 12 days, respectively. The importance of gastrointestinal tissues as a repository for B(a)P-derived radioactivity in both these species is also noteworthy.

Marine decapod crustaceans have a very low reported natural incidence of neoplasia, even though it has been shown that crabs and lobsters contain relatively high concentrations of procarcinogens, such as PAHs, when harvested from polluted waters. The reasons for this apparent resistance to neoplasia will be studied by the investigator of another grant, using the spiny lobster as model (36). Studies with several crustacean species have shown that monooxygenation of foreign chemicals (xenobiotics) occurs primarily in hepatopancreas microsomes, while endogenous substrates are also extensively oxygenated in other organs and organelles. As a group, crustacea metabolize xenobiotics more slowly than vertebrates but more rapidly than molluscs, although there are exceptions to this among some species or with other xenobiotic substrates. Chromatographic separation of solubilized hepatopancreas microsomes from spiny lobster and blue crab has provided evidence that multiple isozymes of cytochrome P-450 exist in these species. Functional differences between the isozymes are less well documented and at present the regulation of cytochromes P-450 in crustacea is not understood. Studies of hepatopancreas microsomal monooxygenase activity in crabs and lobsters have been hindered by the presence of reduced nicotinamide adenosine dinucleotide phosphate (NADPH) cytochrome P-450 reductase inhibitors in hepatopancreas. There is, as yet, no convincing evidence that cytochrome P-450 can be induced in crustacea by agents which induce cytochromes P-450 in vertebrates.

While chemical carcinogenesis is probably more thoroughly characterized in rainbow trout than in any other aquatic model, there are enormous gaps in our understanding of the basic processes of neoplasia in this model in comparison to mammalian species. One of the RFA awardees proposes to use the rainbow trout as a model system to study the fundamental aspects of tumor progression in kidney and liver (32). Histochemical markers for the detection of nephroblastoma in experimental animal models have not been previously documented. The resistance of nephroblastoma to the accumulation of iron was histochemically demonstrated in the Shasta strain rainbow trout (Salmo gairdneri). Embryos were exposed to 100 ppm aqueous N-methyl-N'-nitro-N-nitrosoguanidine for 30 minutes. The fish were raised in indoor tanks and fed a semipurified diet. At the sixth month, kidney tumors became grossly visible at necropsy. The fish were intraperitoneally "iron loaded" with a single dose of 0.20 mg iron/100 gram body weight as iron dextran. Kidney tumors were processed for iron and hematoxylin and eosin staining. Nephroblastomas exhibited iron resistance which distinctly defined the borders of the tumors. Normal renal tubule cells exhibited slight to moderate iron staining, while those from the nephroblastomas were iron resistant. Deposition of melanin in the nephroblastomas were notably reduced. Nephroblastoma iron resistance could be analogous to that expressed by neoplastic and preneoplastic hepatic lesions in

rodents and trout. Iron resistance may be a significant and practical histochemical marker in experimental animal models of nephroblastoma (Wilms' tumor).

Polycyclic Aromatic Hydrocarbons: PAHs are ubiquitous environmental pollutants which may be responsible for some cancer induction in man. The biological properties of PAHs, such as mutagenicity, carcinogenicity, and covalent binding to cellular macromolecules, require metabolic activation to an electrophilic species. Metabolic activation of PAH may occur by two main pathways: monooxygenation or two-electron oxidation and one-electron oxidation. Monooxygenation affords oxygenated metabolites by direct attack of an "oxene-like" activated oxygen generated by cytochrome P-450 monooxygenase. One electron oxidation produces radical cations. In an aromatic system a radical cation is formed by removal of one pi-electron, whereas one n-electron oxidation of a phenol or amine, followed by rapid loss of a proton, yields a radical.

Work from many laboratories on hepatic microsomal cytochrome P-450 from rabbits and rats has established that many different forms of cytochrome P-450 exist, differing with respect to overall substrate specificity and even regio- or stereoselectivity for the metabolism of individual substrates. The relative proportions and total concentrations of various cytochrome P-450 isozymes in liver microsomes may be significantly altered following exposure of animals to foreign chemicals. One supported laboratory (37) has demonstrated that cytochrome P-450e is selectively expressed in livers of untreated rats and plays a substantial role in the metabolism of 7,12-dimethylbenz(a)anthracene (DMBA), despite its relatively low level of expression ($\leq$3% of the total spectrally detectable cytochrome P-450). The levels of expression of cytochromes P-450b and P-450e (both inducible by phenobarbital (PB) and differing by only 14 of 491 amino acids) in liver microsomes from untreated male rats were separately quantitated by Western blotting using a polyclonal antibody raised against cytochrome P-450b that is equally effective against cytochrome P-450e (anti P-450b/e). Microsomes from uninduced livers of individual male rats from five different strains exhibited only minor interstrain and interindividual variability in the expression of cytochrome P-450e (17 ± 5 pmol P-450e/mg microsomal protein) with the exception of the Brown Norway strain (8.5 ± 0.5 pmol P-450e/mg). Expression of cytochrome P-450b varied widely from undetectable levels (< 2 pmol/mg) in most Sprague-Dawley rats to about 50% of cytochrome P-450e levels in Fischer and Brown Norway strains. Anti cytochrome P-450b/e inhibited the total metabolism of DMBA by uninduced microsomes, to an extent that was dependent on rat strain (15-30%), predominantly through the inhibition of formation of 12-hydroxymethyl-7-methyl benzo(a)anthracene (BA) (65-85%), the major metabolite formed by using, purified cytochrome P-450e. A specific activity for cytochrome P-450e-dependent DMBA metabolism was calculated from four sets of microsomes where the cytochrome P-450b content was either undetectable or very low (0.7-1.0 nmol/nmol P-450e/min^{-1}). Comparable calculated activities were, however, obtained from other untreated rat liver microsomes where cytochrome P-450b levels were significant. Polymorphism in cytochrome P-450b was detected but did not affect total cytochrome P-450b expression or the sensitivity of DMBA metabolism to anti P-450b/e. A monoclonal antibody (MAb) against cytochrome P-450b (2-66-3) recognized cytochrome P-450b, b_2, and e on Western blots. MAb 2-66-3 and two other MAbs produced against cytochrome P-450b inhibited 12-methylhydroxylation of DMBA by untreated rat liver microsomes to the same extent as anti P-450b/e. Following phenolbarbital (PB) induction, cytochrome P-450b was induced to about double the level of cytochrome P-450e in most rat strains examined.

Enzymes that increase or decrease the levels of epoxides in the cell should have a major effect on epoxide-mediated carcinogenicity. One such enzyme that affects the intracellular concentration of epoxides is epoxide hydrolase, which converts epoxides to dihydrodiols. This conversion is normally regarded as a deactivation step. For example, epoxide hydrolase has been shown to deactivate the mutagenic epoxide, benzo(a)pyrene-4,5-oxide (BPO). This deactivation has been demonstrated both as a decrease in the mutagenicity of this compound in Salmonella tester strains and in the epoxide hydrolase-dependent decrease in the covalent binding of this metabolite to DNA. However, in some cases dihydrodiols can be further metabolized to other potent carcinogenic intermediates. Benzo(a)pyrene-7,8-oxide can be converted via epoxide hydrolase to benzo(a)pyrene-7,8-diol, which can, in turn, be oxidized once again to form the very potent carcinogen, benzo(a)pyrene-7,8-diol-9,10-oxide. Because epoxides are involved as intermediates in the bioactivation of such a large number of toxins and carcinogens, the levels and substrate specificities of epoxide hydrolases can be regarded as important factors in cellular mechanisms of defense against these chemicals.

The biochemical properties of the epoxide hydrolases have been the subject of many studies, and it has been determined that several different epoxide hydrolases exist (25). A procedure has been developed for the purification of an epoxide hydrolase from human liver cytosol. This epoxide hydrolase is highly active towards trans-stilbene oxide, but not towards benzo(a)pyrene 4,5-oxide, and appears to be analogous to the cytosolic epoxide hydrolase previously demonstrated in, and purified from, mouse liver. The enzyme has been isolated to high purity, and has a molecular weight of 122 kD, as judged by gel exclusion chromatography. The electrophoretogram showed one major band corresponding to a molecular weight of 61 kD, suggesting that the intact enzyme comprises two subunits. Several faint bands representing possible contaminants are also seen. Antiserum to the isolated enzyme was raised. Western blotting of the isolated enzyme and of intact human cytosol reveals two bands, corresponding to the enzyme and to a minor contaminant. Western blotting of guinea pig cytosol shows very little cross-immunoreactivity. Amino acid analysis suggests that there are structural differences between this human enzyme and mouse cytosolic epoxide hydrolase.

The observed association between catalase (CA) activity and cytosolic epoxide hydrolase (CEH) activity during enzyme purification led to the examination of several CA inhibitors as inhibitors of CEH and microsomal epoxide hydrolase (MEH) activities (25). CA and CEH are inhibited by hydroxylated metabolites of diphenylthiazole (DPT). 4-Hydroxy diphenylthiazole (4-OH-DPT) and 4,5-diOH-DPT are potent inhibitors; 5-OH-DPT is less potent. Unmetabolized DPT has no effect. 4-OH-DPT inhibits, but 5-OH-DPT enhances MEH activity. 4,5-diOH-DPT and DPT have no effect. Other compounds that inhibit CA and CEH activities, but not MEH activity, are dihydroguaiaretic acid and 2-aminothiazole. MEH activity is enhanced by 2-aminothiazole and levamisole. The selective CEH inhibitors 4- and 4'-phenylchalcone oxide inhibit CA activity, but not MEH activity at the concentrations used. Trichloropropene oxide, an MEH inhibitor, does not inhibit CA. These results suggest that CEH and CA share inhibitor binding characteristics, perhaps reflecting catalytic site similarities, that are not shared by CEH and MEH.

Dihydrodiol dehydrogenase has been implicated in the detoxification of trans-dihydrodiols of PAH. It has been suggested that this enzyme will oxidize these proximate carcinogens to innocuous catechols and thereby prevent the formation of the ultimate carcinogens, the anti-diol epoxides. Homogeneous dihydrodiol

dehydrogenase was purified from rat liver cytosol (69). 3-Alpha-hydroxysteroid/dihydrodiol dehydrogenase was purified from rat liver cytosol and subjected to electrophoretic and immunochemical characterization to provide further evidence of homogeneity. Non-denaturing gels of the dehydrogenase stained for protein and enzyme activity confirm that a single protein catalyzes the oxidation of androsterone and benzene dihydrodiol. Sodium dodecyl sulphate (SDS)-polyacrylamide gels of the same enzyme preparation revealed a single band (Mr = 34,000) and 2-dimensional gels showed one predominant protein with a pI of 5.9. High-titer polyclonal antibody raised against the homogeneous 3-alpha-hydroxysteroid/dihydrodiol dehydrogenase was found to detect 250 ng of purified enzyme at a dilution of 1 in 30,000. Western blots of cytosolic proteins prepared from male and female Sprague-Dawley rat liver indicated the presence of a single band with a molecular weight of 34,000 in both sexes. Immunotitration of the 3-alpha-hydroxysteroid dehydrogenase activity catalyzed by either rat liver cytosol or the purified enzyme gave sigmoidal curves that could be superimposed. In both instances all the enzyme activity was inhibited. These data indicate that contrary to previous reports, only one form of the dehydrogenase exists in Sprague-Dawley rat liver cytosol. Although 3-alpha-hydroxysteroid dehydrogenase activity is known to be widely distributed in male rat tissues, Western blots indicate that only the liver, lung, testis, and small intestine contain immunoreactive protein with a molecular weight of 34,000. The levels of immunoreactive protein in these tissues resemble the distribution of dihydrodiol dehydrogenase in rat tissues.

To assess the contribution of dihydrodiol dehydrogenase to cellular PAH metabolism, this group has characterized a cell line which contains this enzyme. Using benzene dihydrodiol (1,2-trans-dihydroxy-3,5-cyclohexadiene) as a substrate, the enzyme can be detected in lysates of rat hepatoma H-4IIe cells spectrophotometrically. The specific activity in lysates is similar to that observed in rat liver cytosol. The hepatoma cell enzyme has many properties in common with the homogeneous enzyme prepared from rat liver cytosol which include mol. wt., pI, immunoreactivity, co-purification with 3-alpha-hydroxysteroid dehydrogenase and sensitivity to inhibition by indomethacin and 6-medroxyprogesterone acetate, (IC_{50} values = 3.0 microM and 40nM respectively). This cell line would also appear to be a good choice in which to study the regulation of the enzyme.

This group also reports that the homogeneous 3-alpha-hydroxysteroid-dihydrodiol dehydrogenase of rat liver cytosol catalyzes the nicotinamide adenosine dinucleotide phosphate (NADP)-dependent oxidation of a wide variety of polycyclic aromatic trans-dihydrodiols and has been implicated in their detoxification. The racemic trans-3,4-dihydrodiols of benz(a)anthracene (BA) and 7-methylbenz(a)anthracene (7-MBA) were completely consumed by the purified dehydrogenase, indicating that both stereoisomers are substrates. However, 50% of the (±)-trans-3,4-dihydrodiols of 12-methylbenz(a)anthracene (12-MBA) and DMBA were oxidized, suggesting that only one stereoisomer is utilized in each case. At low substrate concentrations, enzymatic oxidation of the trans-3,4-dihydrodiols of BA, 12-MBA, and DMBA followed simple first-order kinetics. By contrast, oxidation of the 3,4-dihydrodiol of 7-MBA was of higher order, due to differences in the rate of oxidation of each stereoisomer. Rate constants estimated for each reaction indicate that the non-bay region methyl group at the 7-position has a greater enhancing effect on the rate of oxidation than the bay-region methyl group at the 12-position (10- vs fourfold respectively). The 3,4-dihydrodiol of DMBA, which possesses both non-bay and bay-region methyl groups, is oxidized more than 30 times faster than the unmethylated parent hydrocarbon. The absolute stereochemistry of the preferentially oxidized dihydrodiols was assigned by circular dichroism (CD)

spectrometry. For the 3,4-dihydrodiols of DMBA and 12-MBA, the stereoisomer oxidized has the 3S,4S configuration. A large negative Cotton effect was observed in the CD spectrum of the 7-MBA 3,4-dihydrodiol which remained at the end of the rapid phase of oxidation of this racemic substrate, indicating that the dehydrogenase displays partial stereochemical preference for the 3S,4S enantiomer. These results suggest that methylation of BA at C-7 greatly enhances the oxidation of the 3S,4S-dihydrodiol, while the presence of a bay-region methyl group at C-12 completely blocks the oxidation of the 3R,4R-stereoisomer. Rapid, stereoselecitve oxidation of methylated polycyclic aromatic trans-dihydrodiols by this route in vivo may significantly influence their carcinogenicity.

Although the bay region theory predicts ultimate carcinogens of PAHs, it does not, by itself, explain the exceptional activity of certain methylated PAHs. Studies have shown that 5-methylchrysene (5-MeC), a strong carcinogen, and 6-methylchrysene (6-MeC), a weak carcinogen, are metabolized in mouse skin to similar amounts of bay region 1,2-diol-3,4-epoxides, suggesting that a methyl group in the same bay region as the epoxide ring, as in 5-MeC-1,2-diol-3,4-epoxide, is a key factor in expression of biological activity. However, another possible explanation for the different tumorigenic activities of 5-MeC and 6-MeC is differing stereoselectivity in their metabolism to dihydrodiols and diol epoxides. The stereoselectivity of mouse skin metabolic activation to dihydrodiols of the strong carcinogen, 5-MeC, and the weak carcinogen, 6-MeC, was investigated (30). Synthetic 1,2-dihydro-1,2-dihydroxy-5-methylchrysene (5-MeC-1,2-diol), 5-MeC-7,8-diol, and 6-MeC-1,2-diol were resolved into their R,R- and S,S-enantiomers by chiral stationary phase high performance liquid chromatography. The absolute configurations of the enantiomers were assigned by their circular dichroism spectra. Using these enantiomers as standards, the metabolism of 5-MeC and 6-MeC in vitro in rat and mouse liver and in vivo in mouse epidermis was investigated. Only the R,R-enantiomers of each dihydrodiol predominated (>90%). The dihydrodiol enantiomers were tested for tumor initiating activity on mouse skin. In each case, the R,R-dihydrodiol enantiomer was significantly more tumorigenic than the S,S-enantiomer. The most tumorigenic compound was 5-MeC-1R,2R-diol; it was significantly more active than either 5-MeC-7R,8R-diol or 6-MeC-1R,2R-diol. The results of this study demonstrate that there is a high degree of stereoselectivity in the metabolic activation of 5-MeC and 6-MeC to proximate tumorigenic dihydrodiols in mouse skin. The bay region methyl group has no effect on the stereoselectivity of activation to 1,2-dihydrodiol metabolites in the chrysene system.

The stereochemistry of diol epoxide formation in mouse epidermis upon topical application of [^{3}H]-1R,2R-dihydroxy-1,2-dihydro-5-methylchrysene ([^{3}H]-5-MeC-1R,2R-diol) and [^{3}H]-6-MeC-1R,2R-diol, and the tumorigenicity in mouse skin and in newborn mice of the R,S,S,R and S,R,R,S enantiomers of 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydro-5-methylchrysene (5-MeC-1,2-diol-3,4-epoxide), 5-MeC-7,8-diol-9,10-epoxide, and 6-MeC-1,2-diol-3,4-epoxide were also examined by this group (30). Analysis of tetraols and their derived tetraacetates present in mouse epidermis, 2 h after application of [^{3}H]-5-MeC-1R,2R-diol or [^{3}H]-6-MeC-1R,2R-diol, demonstrated greater than 90% stereoselectivity in formation of 5-MeC-1R,2S-diol-3S,4R-epoxide and 6-MeC-1R,2S-diol-3S,4R-epoxide. Taken together with previous data, these results demonstrate that there is a high degree of stereoselectivity for formation of R,S,S,R enantiomers of 5-MeC- and 6-Mec-1,2-diol-3,4-epoxides in mouse skin. The results of the tumorigenicity studies in mouse skin and in newborn mice clearly demonstrated that 5-MeC-1R,2S-diol-3S,4R-epoxide was the most tumorigenic of the diol epoxide enantiomers tested; 6-MeC-1R,2S-diol-3S,4R-epoxide was inactive. The results of this study show that the high

tumorigenicity of 5-MeC, compared to 6-MeC, is due to the remarkable tumorigenic activity of 5-MeC-1R,2S-diol-3S,4R-epoxide which, in contrast to 6-MeC-1R,2S-diol-3S,4R-epoxide, has a methyl group in the same bay region as the epoxide ring. It has been proposed that such methyl bay region diol epoxides of other carcinogenic methylated polynuclear aromatic hydrocarbons will also show unique tumorigenic properties.

Arene epoxides are the most abundant products formed in the metabolism of PAHs. The formation of epoxides is catalyzed by cytochrome P-450 isozymes in the drug-metabolizing enzyme complex. Depending on its stability, an enzymatically formed epoxide is hydrated to form a trans-dihydrodiol and/or is nonenzymatically rearranged to form one or both of the two possible phenolic products. When epoxide hydrolase is inhibited by using an inhibitor such as 3,3,3-trichloropropylene 1,2-oxide (TCPO), the enzymatically formed epoxide is not hydrated to a trans-dihydrodiol and may remain as the epoxide if sufficiently stable. K-region epoxides of PAHs are generally more stable than non-K-region epoxides. The absolute configurations of K-region epoxide enantiomers of 3-methylcholanthrene, benz(a)anthracene, and benzo(a)pyrene have been determined via their monomethyl ether derivatives (89). Methoxylation of each racemic or enantiomeric epoxide by sodium methoxide resulted in a pair of monomethyl ether derivatives, which were separated by normal-phase high-performance liquid chromatography (HPLC). The position of the methoxy group was determined by products formed by acid-catalyzed dehydration and/or demethanolization of each monomethyl ether derivative. Enantiomers of each epoxide and its methoxylated derivatives were resolved by at least two of the four Pirkle chiral stationary phase HPLC columns utilized in this study. The absolute stereochemistries of enantiomeric monomethyl ether derivatives were established by comparing their circular dichroism spectra with those of enantiomeric monomethyl ether derivatives derived from trans-dihydrodiol enantiomers of known absolute configurations. The absolute configuration of each epoxide enantiomer was deduced from the location of the methoxy group and the absolute configuration of enantiomeric monomethyl ether derivatives. The results indicate that the method described is useful in general for the determination of absolute configurations of K-region epoxide enantiomers of polycyclic aromatic hydrocarbons.

The PAH benzo(c)phenanthrene (B(c)Ph) is a widespread environmental contaminant with weak tumorigenic activity. B(c)Ph is metabolized in rat liver microsomal incubations principally to its K-region dihydrodiol. Metabolism of B(c)Ph on the benzo ring occurs almost exclusively at the 3,4 position; the highly sterically hindered bay-region 1,2-dihydrodiol is not detected. In accordance with predictions of the bay-region theory of PAH metabolic activation, B(c)Ph 3,4-dihydrodiol, the precursor to the bay-region diol-epoxide of B(c)Ph, is more mutagenic than B(c)Ph and its 1,2- and 5,6-dihydrodiols upon metabolic activation in Salmonella typhimurium strains, TA 98 and TA 100. The 3,4-dihydrodiol is also a more potent tumor initiator than B(c)Ph on mouse skin. The sterically hindered bay-region benzo(c)phenanthrene 3,4-diol-1,2-epoxides (B(c)PhDEs) are highly mutagenic to bacteria and mammalian cells and are the most potent tumor initiators of all of the PAH diol-epoxides tested in the mouse skin initiation-promotion assay. B(c)PhDE exists as a pair of diastereomers: B(c)PhDE-1 (benzylic 4-hydroxyl group and epoxide oxygen are cis) and B(c)PhDE-2 (benzylic 4-hydroxyl group and epoxide oxygen are trans). Each diastereomer consists of a pair of enantiomers. Both B(c)PhDE-1 and B(c)PhDE-2 have high tumor-initiating activity on mouse skin. This may be related to the fact that both diastereomers prefer the conformation in which their hydroxyl groups are pseudodiequatorial.

Ultimate carcinogenic metabolites of PAH have been found covalently bound to DNA upon metabolism of the parent hydrocarbons in cells. To determine which B(c)Ph metabolites are bound to DNA in cells from several species, one of the MERIT awardees has analyzed, using immobilized boronate chromatography and reverse-phase HPLC, the B(c)Ph-deoxyribonucleoside adducts formed in Sencar mouse, Syrian hamster, and Wistar rat embryo cell cultures (4). To determine whether B(c)Ph is metabolically activated to bay-region diol-epoxides that bind to DNA in cells, Sencar mouse, Syrian hamster, and Wistar rat embryo cell cultures were exposed to [5-^{3}H]-B(c)Ph, and the B(c)Ph-deoxyribonucleoside adducts formed were analyzed by immobilized boronate chromatography and reverse-phase high-performance liquid chromatography. Greater than 74% of the B(c)Ph-deoxyribonucleoside adducts formed in all three species resulted from reaction of (4R,3S)-dihydroxy-(2S,1R)-epoxy-1,2,3,4-tetrahydro-B(c)Ph [(-)-B(c)PhDE-2] with DNA to yield deoxyadenosine and deoxyguanosine adducts in a ratio of 3:1. A much smaller proportion of B(c)Ph-deoxyribonucleoside adducts were formed by reaction of (4S,3R)-dihydroxy-(2S,1R)-epoxy-1,2,3,4-tetrahydro-B(c)Ph [(+)-B(c)PhDE-1] with deoxyadenosine. No B(c)Ph-deoxyribonucleoside adducts arising from either (+)-B(c)PhDE-2 or (-)-B(c)PhDE-1 were detected. The absence of adducts from these isomers of B(c)PhDE was not due to failure of these isomers to react with DNA in cells, for reaction of (±)-B(c)PhDE-1 or (±)-B(c)PhDE-2 with DNA in solution or in hamster embryo cell cultures resulted in the formation of DNA adducts from both the (+)- and (-)-enantiomers of each B(c)PhDE. These results indicate that both the (+)- and (-)-3,4-dihydrodiols of B(c)Ph are formed and that their metabolic activation to diol-epoxides occurs with high stereospecificity in cells from all three species of rodents. The finding that the major DNA-binding metabolite is (-)-B(c)PhDE-2, the diol-epoxide with the (R,S)diol-(S,R)-epoxide absolute configuration that is associated with high carcinogenic activity of diol-epoxides of other hydrocarbons, demonstrates that these cells are able to activate B(c)Ph to an ultimate carcinogenic metabolite. The fact that a high proportion of the B(c)Ph-DNA adducts are deoxyadenosine adducts suggests that B(c)Ph has DNA-binding properties similar to those of the potent carcinogen DMBA. The stereospecificity observed in the metabolic activation of B(c)Ph to DNA-binding metabolites and the reaction of these metabolites with both deoxyguanosine and deoxyadenosine suggest that studies of the interactions of B(c)Ph with DNA in vivo may be a valuable approach for establishing the role of specific activation pathways and DNA adducts in tumor induction.

Alkylating Agents: Current thinking on the etiology of gastric carcinoma supposes that genetic and environmental factors determine the microenvironment of the gastric cavity and that this, in turn, provides the abnormal stimuli associated with chronic gastritis--a precursor of atrophy, metaplasia, dysplasia, and finally carcinoma. The microenvironment conducive to this chain of events is complex and poorly understood. It is in great measure determined by type of diet and by abnormal gastric secretion. An important characteristic is the presence of nitrite in gastric juice in above-normal concentrations, probably as a result of the reduction of nitrate in the food and water by bacteria in the gastric juice and saliva. Bacteria may be abnormally present in the gastric juice as a consequence of an elevated pH, which, in turn, is the result of decreased secretion of acid in atrophied mucosa. Nitrite is a relatively reactive ion and may react with nitrogen-containing compounds abundantly present in food to yield N-nitroso compounds. Some of these nitroso compounds have been found to be highly mutagenic.

N-nitrosodimethylamine (NDMA) is a potent carcinogen in a wide variety of animal species. In experimental animals, dimethylamine and nitrite, precursors of NDMA, are found in gastric fluid where the acidic conditions are suitable for formation of nitrosamines. The concentrations of mono-, di-, and trimethylamine (MMA, DMA, and TMA) in gastric fluid from humans, rats, dogs, and ferrets, as well as in saliva, blood, and urine from humans were measured (78). Human gastric fluid contained 3.7 ± 0.4 nmol/ml MMA, 12.6 ± 1.4 nmol/ml DMA and 2.0 ± 0.4 nmol/ml TMA. MMA, DMA, and TMA concentrations in human gastric fluid were similar to those present in human saliva and blood, but were much lower than those present in human urine. The concentrations of these amines in human gastric fluid were lower than those measured in gastric fluid from experimental animals. When sodium nitrite was added to human gastric fluid, NDMA was formed. DMA is normally present in human gastric fluid, and can be nitrosated to form NDMA. This study showed that human gastric fluid contains MMA, DMA, and TMA and also showed that the acidic conditions present in gastric fluid are suitable for nitrosation of this endogenous DMA. The data suggest that NDMA could be formed within human gastric fluid from substrates (DMA and nitrite) which are normally present.

The carcinogenic nitrosamines and nitrosamides differ mainly in their chemical stability and mechanism of biological activity. Nitrosamides are unstable in aqueous solution and rapidly decompose under physiological conditions to chemically reactive alkylating agents. They are considered to be direct-acting carcinogens requiring no metabolic activation to exert their biological effects. In contrast, the nitrosamines are quite stable under physiological conditions and require metabolic transformation before they are active as carcinogens. A widely proposed pathway for the activation of dialkylnitrosamines involves oxidation at an alpha-carbon by the cytochrome P-450 monooxygenase system to an unstable intermediate that decomposes through a series of steps to yield an aldehyde, molecular nitrogen, and a reactive carbonium ion. Several observations, however, have raised doubts as to the significance of this oxidative demethylation mechanism as the sole pathway for nitrosamine activation. For example, microsomes, as well as specific isozymes of cytochrome P-450 metabolize nitrosamines by a denitrosation mechanism in addition to the demethylation reaction. Nitrosamine metabolism also occurs under anaerobic conditions and by reductive denitrosation initiated by cytochrome P-450 or superoxide, whereas demethylation is an oxidative aerobic reaction. Reductive denitrosation is also prominent in the metabolism of N-nitrosourea compounds. Cytochrome P-450 reductase has been shown to be the microsomal enzyme responsible for this reductive metabolism. These denitrosation reactions indicate cleavage of the N-N bond in the N-nitroso compounds and suggests the generation of reactive free radical intermediates. A model system has been used to study the types of radicals formed upon the denitrosation of N-nitroso compounds (70). Free radicals were formed at room temperature (22°-23°C) and neutral pH by photolytic cleavage of N-nitroso bonds and were partially characterized following their addition to the spin traps 5,5-dimethyl-1-pyrroline-N-oxide and N-tert-butyl-alpha-phenyl-nitrone (PBN). Carbon-centered radical adducts were obtained during nitrosamine photolysis and nitrogen-centered radical adducts during nitrosamide photolysis. Since both the nitrosamines and nitrosamides initially form nitrogen-centered radicals on photolysis, a secondary reaction or rearrangement must occur after initial N-nitroso bond cleavage in the nitrosamines. The results indicate that photolytic denitrosation of nitrosamines and nitrosamides leads ultimately to the formation of different reactive radical species--nitrogen-centered radicals for the nitrosamides and carbon-centered radicals for the nitrosamines.

This group has continued to examine the role free radicals may have in determining differences in the mode of action of the direct-acting nitrosamide versus the indirect-acting nitrosamine carcinogens. Photolysis (350 nm) at room temperature and neutral pH was used as a model system for the denitrosation reaction which results in the formation of free radicals of N-nitroso compounds. The presence of phosphate esters (inorganic phosphate or organic mono- or diesters) during the photolysis of nitrosamines have been found to result not only in an increase in free radical generation, but also in the formation of a stable mutagen. For the nitrosamides, e.g., N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), neither free radical formation nor mutagenesis is enhanced when photolysis is carried out in the presence of phosphate esters. Free radical quantitation is by electron spin resonance (ESR) spectroscopy of PBN spin trapped adducts and mutagenesis by the TA-100 salmonella reversion assay.

Another apparent difference between the nitrosamines and the nitrosamides concerns the ability of the radicals generated during photolysis to "directly" initiate mutagenesis. To examine any "direct" mutagenic effect of these radicals, photolysis of the nitroso-compounds was carried out in the presence of TA-100 and the number of reversions obtained compared with photolysis done separately followed by addition of TA-100. Results with the nitrosamine, nitrosomorpholine, indicated that there was more than a doubling of the reversion number following photolysis in the presence of TA-100, while there was essentially no difference in the reversion number obtained for the nitrosamide, MNNG, with or without photolysis. These results suggest that for nitrosamines, activation by photolysis results not only in mutagenesis from a stable mutagen formed in the presence of phosphate, but also by direct radical modification of DNA. The involvement of free radicals in nitrosamine mutagenesis following photolysis was further confirmed in studies with the antioxidants, ascorbic acid and thiourea, and the spin trap, PBN. All of these compounds effectively trapped the radicals generated during nitrosamine photolysis and inhibited mutagenesis in a dose-dependent fashion. Similar studies with the nitrosamides showed no significant effects.

In an attempt to elucidate the molecular basis for the decrease in rat liver carcinogenicity and DNA-alkylating ability that accompanies deuteration of N-nitrosodimethylamine (NDMA), NDMA and its fully deuterated analogue, ($[^2H_6]$NDMA), were incubated with acetone-induced rat liver microsomes (87). Rates for the competing metabolic routes, denitrosation and demethylation, were determined from colorimetric data on nitrite and formaldehyde generation, respectively. The V_{max} calculated for demethylation of NDMA was 7.9 nmol/min/mg, while that for denitrosation was 0.83 nmol/min/mg. Deuteration of NDMA did not significantly change the V_{max} for either pathway, but it did increase the K_m for demethylation from 0.06 to 0.3 mM. The K_m for denitrosation was also increased from 0.06 to 0.3 mM on deuteration, as determined by incubating an equimolar mixture of amino-^{15}N-labeled NDMA with $[^2H_6]$NDMA and measuring the methyl-$[^{15}N]$amine:$[^2H_3]$methylamine ratio by derivatization-gas chromatography-mass spectrometry. The fact that the K_m values for denitrosation were so similar to those for demethylation suggested that the two pathways were catalyzed by the same enzyme. The isotope effects calculated from these data $[V_{max}{}^H/V_{max}{}^D$ Ca. 1 and $(V_{max}/K_m)^H/(V_{max}/K_m)^D$ Ca. 5] show that microsomal metabolism of NDMA is not significantly shifted from demethylation to denitrosation on deuteration of substrate and may indicate a low commitment to catalysis for the enzyme. The results are consistent with the view that the metabolism of NDMA is initiated by formation of an alpha-nitrosamino radical which either combines with a hydroxyl radical to form the alpha-hydroxynitrosamine as the initial product of the

demethylation pathway or fragments to nitric oxide and N-methylformaldimine as the first products of denitrosation.

The metabolism of NDMA, N-nitrosodiethylamine, N-nitrosobenzylmethylamine, and N-nitrosobutylmethylamine was also investigated in incubations with human liver microsomes by this group. All of the 16 microsomal samples studied were able to oxidize NDMA to both formaldehyde and nitrite at NDMA concentrations as low as 0.2 mM; the rates of product formation of the samples ranged from 0.18 to 2.99 nmol formaldehyde/min/mg microsomal protein (median, 0.53 nmol). At a concentration of 0.2 mM NDMA, the rates of denitrosation (nitrite formation) were 5 to 10% (median, 6.3%) those of demethylation (formaldehyde formation); the ratio of denitrosation to demethylation increased with increases in NDMA concentration, in a manner similar to rat liver microsomes. Immunoblot analysis with antibodies prepared against rat cytochrome P-450_{ac} (an acetone-inducible form of cytochrome P-450) indicated that the cytochrome P-450_{ac}[P-450_j (isoniazid-inducible form)] orthologue in human liver microsomes had a slightly higher molecular weight than rat cytochrome P-450_{ac}, and the amounts of cytochrome P-450_{ac} orthologue in human liver microsomes were highly correlated with NDMA demethylase activities ($r = 0.971$;$P < 0.001$). Analysis of four selected microsomal samples showed that human liver microsomes exhibited at least three apparent K_m and corresponding V_{max} values for NDMA demethylase. This result, suggesting the metabolism of NDMA by different cytochrome P-450 enzymes, is similar to that obtained with rat liver microsomes, even though most of the human samples had lower activities than did the rat liver microsomes. The high affinity K_m values of the four human samples ranged from 27 to 48 microM (median, 35 microM), which were similar to or slightly lower than those observed in rat liver microsomes, indicating that human liver microsomes are as efficient as rat liver microsomes in the metabolism of NDMA. The human liver microsomes also catalyzed the dealkylation and denitrosation of other nitrosamines examined. The rates of product formation and the ratios of denitrosation to dealkylation varied with the structures and concentrations of the substrates as well as with the microsomal samples tested. The results indicate that human liver microsomes are capable of metabolizing N-nitrosodialkylamines via the pathways that have been established with rat liver microsomes.

NDMA and aflatoxin B_1 (AFB_1) have been investigated extensively during the past decade and shown to be carcinogenic to a number of species. Since environmental exposure to these compounds through a variety of sources is highly possible, they pose a high risk to humans. NDMA and AFB_1 require cytochrome P-450-dependent metabolic activation for expression of their carcinogenicity in several species. Scientists in many laboratories have searched for agents that inhibit their carcinogenic effects. Since several plant flavonoids, particularly catechin, inhibit the metabolic activation of several polycyclic hydrocarbons, they are potential anticarcinogens. Effects of catechin, a plant phenolic flavonoid, and of the commonly used organic solvents, dimethyl sulfoxide (DMSO) and ethanol (EtOH), on the microsome-mediated metabolism of two hepatocarcinogens, NDMA and AFB_1, were studied (55). Using hamster liver microsomes as a source of mixed-function oxidases, it was shown that catechin at 0.1-0.2 mM levels had no effect on the oxidation of either carcinogen. However, at 1-5 mM levels it caused a concentration-dependent inhibition (38-70%) of the formation of formaldehyde from NDMA, and at the 5 mM level it caused a 40% inhibition of AFB_1-DNA binding. DMSO and EtOH totally inhibited NDMA demethylase activity but had little effect on the binding of AFB_1 to DNA. These observations indicate that the mixed-function oxidases (cytochrome P-450) essential for the metabolic activation of these carcinogens exhibit different sensitivities to different inhibitors.

Extrapolation of carcinogenicity data from animals to humans is fraught with difficulty. The inherent susceptibility of tissues to the carcinogenic action of NDMA, the efficiency and fidelity of repair processes, quantitative and qualitative metabolic aspects, and the pharmacokinetics of the compound may be very different in humans. Some of these problems can be studied in isolation. For instance, the availability of a suitable data base may allow extrapolation of the pharmacokinetic data from animals to humans. Currently, the only species for which good pharmacokinetic data exist are the rat and the rabbit. These studies in rats have shown that NDMA, when administered p.o. at low doses, is well absorbed from the gastrointestinal tract, but only a very small fraction, about 10%, of the dose passes through the liver into the general circulation. This was shown directly by measuring the concentration of NDMA in blood after p.o. or i.v. administration and indirectly by measuring the extent of DNA alkylation in kidney relative to liver after p.o. and i.v. dosing. The extent of first pass metabolism in species other than the rat is not known. Interspecies scaling of pharmacokinetic data is difficult, especially when the compound is cleared primarily by metabolism, and extrapolation is virtually impossible when data are available for only one or two species. If several species are studied, allometric analysis may allow a reasonable interspecies extrapolation.

The purpose of this study by one of the MERIT awardees (56) is to collect detailed information on the pharmacokinetics of NDMA in mammalian species other than the rat to eventually allow interspecies comparisons to be made and, if possible, to extrapolate the data to describe the pharmacokinetics of NDMA in humans. The pharmacokinetics of NDMA has been studied in beagles. Four male beagles were given 0.5- and 1.0-mg/kg doses of NDMA i.v. and 1.0- and 5.0-mg/kg doses p.o., and at appropriate times after dosing, blood samples were drawn and the concentration of NDMA was measured. The experiments were separated by at least 1 week. Following a bolus i.v. dose, the concentration of NDMA in blood declined biphasically with a mean distribution half-life of 19 minutes and a mean elimination half-life of 73 minutes. The areas under the blood concentration versus time curves were proportional to the dose indicating that the pharmacokinetics in this dose range were first order. The mean systemic clearance was 43.3 ml/min/kg, the volume of distribution at steady state was 1.9 liters/kg and the mean residence time was 45 minutes. The clearance of NDMA in the dog was entirely metabolic because no NDMA could be detected in urine after i.v. dosing. The areas under the curve and maximum concentration in blood after the two p.o. doses were not proportional to dose. The evidence suggests that the pharmacokinetics of the 1.0-mg/kg dose were first order, but at the 5.0-mg/kg dose the metabolism of NDMA was saturated. The bioavailability of the lower p.o. dose (i.e., the fraction of the dose that reached the systemic circulation) averaged 93%. The high bioavailability was unexpected since, in the rat, the bioavailability of NDMA is only about 10%, and the systemic clearance in the dog exceeds hepatic blood flow. These data suggest that a substantial fraction of the systemic clearance is extrahepatic and that the pharmacokinetics of NDMA in higher species may be quite different from that observed in rodents.

The pharmacokinetics of NDMA has also been studied in swine by this group. The pharmacokinetics were studied following i.v. administration of 0.1, 0.5, and 1.0 mg/kg, and following oral doses of 1.0 and 5.0 mg/kg of NDMA. Following a bolus i.v. dose, the concentration of NDMA in blood declined biphasically with a mean distribution half-life of 7 minutes and a mean elimination half-life of 28 minutes. The areas under the blood concentration versus time curves (AUC) were roughly proportional to dose, indicating that the pharmacokinetics in this dose

range were first order. The mean systemic clearance from blood was 65.8 ml/min/kg, the steady state volume of distribution was 1.4 liters/kg, and the mean residence time was 20 minutes. Following the oral doses, the AUC and peak concentration in blood were not proportional to the dose. It is likely that the pharmacokinetics at the lower dose were first order, but at the higher dose the pharmacokinetics were no longer first order because metabolism was saturated. The bioavailability of the 1.0 mg/kg dose was 67%. Since the clearance was probably due to metabolism and the clearance from blood exceeded hepatic blood flow, the high bioavailability suggests that extrahepatic metabolism plays an important role in the systemic clearance of NDMA in swine.

Hormones: Hormonal carcinogenesis is another important area of research included under the carcinogenesis mechanism studies. Over a decade ago, a causal association of vaginal cancer in young women with in utero exposure to diethyl stilbestrol (DES) was reported. Since then, correlations have been made between oral contraceptives and other prescribed estrogens and endometrial, hepatic, cervical, ovarian, and breast tumors. Because of these observations, there is now a growing awareness of the carcinogenic potential of both natural and synthetic hormones.

Several investigators supported by the program have made significant findings that suggest fundamental mechanisms of action in hormonal carcinogenesis by estrogens. Estrogen-induced tumors in the hamster, particularly in the kidney, have become one of the most intensively investigated models. Estrogen-dependent renal adenocarcinomas are known to be induced with 100% incidence in gonadectomized male Syrian hamsters following chronic exposure to DES. During the past year, the effect of estrogens on the growth of renal proximal tubular cells in culture was examined (49). Ultrastructural analysis of cells in tissue explant outgrowths exhibited features typical of proximal tubules, when grown on basement membrane substrate in serum free medium. At 7-14 days of culture, the number of cells was two- to threefold greater in medium containing either 17-beta-estradiol(E_2) or DES. When added concomitantly with E_2, tamoxifen at threefold excess inhibited this increase in cell number. Progesterone, 5-alpha-dihydrotesterone, and the inactive estrogen metabolite, beta-dienestrol, did not cause an increase in cell number when added in culture. The effect of estrogen on proximal tubular cell growth appears to be species specific, since E_2 was without effect on cultured rat or guinea pig proximal tubule cells. Additionally, more mitoses were observed in the presence of added estrogen, which suggests that this hormone has a direct mitotic effect. These results represent the first in vitro demonstration that estrogens can directly induce primary epithelial cell proliferation in target tissue.

Various laboratories have provided evidence that nonhormonal events may also be involved in the estrogen induction of renal carcinomas in the hamster. A role for metabolism of estrogens to reactive intermediates has been suggested by studies showing that estrogen-induced renal tumorigenesis is partially inhibited by concomitant administration of alpha-naphthoflavone (ANF) (49) or ascorbic acid (52). In Syrian hamster kidney carcinogenesis, estrogen-induced covalent DNA modification has been observed to precede malignancy specifically in the kidney (52). It was postulated that these DNA adducts were generated by an endogenous electrophile(s) induced by exposure of the animal to estradiol. Recently it was reported that administration of testosterone or deoxycorticosteron acetate to estrogen-treated hamsters decreased estrogen-induced DNA adduct levels and prevented renal carcinoma. Since co-administration of progesterone and tamoxiphen

to estrogenized animals did not influence DNA adduction, tumor prevention by these hormones may proceed by a different yet unknown mechanism.

The study of the mechanisms involved in the induction of hepatocellular carcinomas in hamsters and rats by synthetic estrogens and the study of the role which ANF plays in the tumorigenic process is the goal of another investigator (50). A high percent incidence of multinodular hepatocellular carcinomas was observed in castrated male hamsters and two strains of rats treated with ethyl estradiol (EE_2) if the animals were maintained on a chow diet containing ANF. During this reporting period, the influence of ANF on the metabolism and binding of EE_2 in hepatic microsomal proteins in castrated hamsters was studied. Hamsters exposed to ANF in the diet for 2 months exhibited inhibition of EE_2 metabolites. However, after 4 months, ANF stimulated the oxidative metabolism of EE_2. Consistent with this observation was a decline in covalent binding of EE_2 metabolites to hamster liver microsomal proteins after 2 months of treatment and its elevation after 4 months exposure to ANF in comparison to control levels. These alterations in the EE_2 metabolism in the presence of ANF may be related to the induction of hepatocellular carcinomas by this hormone.

Primary liver cancer occurs more frequently in men than in women. Following administration of several different carcinogens, sex differences in tendency to form malignant hepatic tumors have also been noted in various strains of rats and mice. Studies were conducted to explore the basic aspects of the sex difference in response to hepatocarcinogens and to distinguish effects on carcinogen metabolism that might affect different stages of carcinogenesis in the resistant rat hepatocyte model (28). Data obtained in this laboratory indicated that the hypothalamo-pituitary-liver axis, previously postulated to control the sex differential steroid metabolism in adult rats, was also responsible for the sex differentiation of several xenobiotic metabolizing enzyme activities including the oxidative metabolism of 2-acetylaminofluorene (2-AAF). Furthermore, the results support the concept that multiple cytochrome P-450 species with different and/or overlapping substrate specificities are involved in xenobiotic and steroid metabolism in the liver. In view of these results, it is suggested that the concept of the hypothalamo-pituitary-liver axis should be taken into consideration when investigating mechanisms mediating the sex differences in chemical carcinogenesis of the rat liver. Sex difference in response to hepatocarcinogens was also observed in the newborn and infant mice by another investigator (84). Newborn and infant mice were shown to be extremely susceptible to the development of hepatic neoplasms even after a single dose of a variety of carcinogens, including diethylnitrosamine (DEN). Hepatocellular basophilic foci were the earliest morphologically distinct cell populations which could be found following single DEN treatment of the infant mouse. These tinctorial foci, which represented the earliest putative progenitors of hepatocellular carcinomas, were characterized by a decrease in glucose-6-phosphatase and an increase in DNA synthesis, in RNA, glucose-6-phosphate, and glyceraldehyde-3-phosphate dehydrogenase. The hepatocarcinogenesis proceeded faster in male than in female mice. Orchidectomy and estrogen treatment of sham-orchidectomized males delayed the onset and the course of morphologic expression and neoplastic progression of hepatocarcinogenesis. Thus, the presence of endogenous androgens advanced hepatocarcinogenesis.

Adenocarcinoma of the prostate is almost exclusively a disease of older men. Most evidence suggests that aging in human males is accompanied by a significant decrease in the mean levels of free testosterone (T), which is considered to be

biologically active. On the other hand, the mean level of free estrogen (E) does not decline with age resulting in a significantly higher free E to free T ratio in men over the age of 50. The Noble rat was chosen to acquire a comprehensive understanding of the role sex hormones may play in human prostatic carcinogenesis (45). This animal model is highly susceptible to sex hormone-induced carcinogenesis in the prostate. The resulting neoplastic lesions resemble those found in the malignant human gland. Results showed that simultaneous implantation of Noble rats with T and E_2 filled-silestic capsules for 16 weeks caused atypical dysplasia exclusively in the dorsolateral prostate of all animals. This dysplasia was considered to be a preneoplastic lesion since long-term administration of these steroids to Noble rats induced adenocarcinoma in the dorsolateral prostate. Treatment of rats with T alone for 16 weeks caused hyperplasia but not dysplasia, implicating estrogen as a key factor in the genesis of the lesion. Results from these experiments indicate that these effects result from chronic hyperstimulation of the target organ by the abnormal levels of hormones and their normal metabolites produced in abnormal amounts.

Additional studies at the molecular level have contributed to understanding the mechanism of hormonal carcinogenesis. To study the regulation and structure of the casein multigene family in the normal mammary gland and to identify how these regulatory mechanisms have deviated in hormone-dependent breast cancer was the goal of another investigator (74). The rat beta-casein gene is a member of a small gene family, encoding the principal milk proteins. Using cDNA probes corresponding to these proteins, the levels of different rat casein mRNAs during normal mammary gland development were found to increase in response to both peptide and steroid hormones. In order to elucidate the mechanisms by which expression of the rat beta-casein gene is regulated in a tissue-and stage-specific fashion, initially, the corresponding genomic sequences were isolated and characterized. To complement gene transfer studies in cell culture, experiments were initiated in transgenic mice in which these constructs could be introduced as linear DNA fragments free of any viral enhancer or vector sequences by direct microinjection. This approach permitted germline transmission and the assembly of these integrated DNA sequences into appropriate higher order chromatin structures during mammary development. Several lines of transgenic mice were generated containing the entire rat beta-casein promoter-chloramphenicol acetyltransferase (CAT) reporter constructs. Studies with both the entire beta-casein gene and beta-casein-CAT constructs have demonstrated tissue-specific and developmental expression in the mammary gland. Explant cultures derived from transgenic mice during midpregnancy were developed in serum-free medium to study the effects of individual peptide and steroid hormones. These lines of mice carrying the rat beta-casein transgene that was generated will provide useful models for studying the developmental and hormonal regulation of milk protein gene expression.

Other Agents: When tryptophan-rich foods are pyrolyzed, as in cooking, several carboline derivatives are formed. The gamma-carbolines, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) which are strongly mutagenic, have been characterized in such pyrolysates. It was noted that the mutagenicities of these two compounds were greatly increased by two other compounds, beta-carbolines, that were also found to be present in the tryptophan pyrolysate; these compounds, harman, 1-methyl-9H-pyrido[3,4-b]indole, and norharman, 9H-pyrido[3,4-b]indole, are themselves not mutagenic; however, they appear to be able to enhance or cause the mutagenicities of certain other compounds, particularly nonmutagenic aromatic amines such as aniline or o-toluidine. The term "comutagenicity" was coined to describe this phenomenon.

Harman and norharman have been identified as components of cooked foods and cigarette smoke condensate.

The biological activities of these two compounds are complicated; generally norharman is more active than harman. They can be considered to have modifying effects on the activities of five different groups of chemicals. These effects are generally observed only in the presence of metabolizing enzymes unless the compound is already an activated metabolite. Harman and norharman may cause certain aromatic amines (that are not themselves mutagenic) to become mutagenic. They may enhance the mutagenicity of certain mildly mutagenic compounds. The mutagenicities of certain polycyclic hydrocarbons such as benzo(a)pyrene may be enhanced by comutagens if large amounts of microsomal enzymes are present; if small amounts of enzyme are present, the opposite effect may be seen. On the other hand, the mutagenicities of certain other compounds, such as dimethylbenz(a)anthracene, are not affected by these comutagens. Some other compounds, such as the flavonoid kaempferol, are less mutagenic in the presence of norharman, regardless of the concentration of microsomal enzymes. In each case norharman is more effective than harman.

The mechanism of this comutagenic effect has been studied extensively. Both harman and norharman interact with DNA, presumably by intercalation, with an unwinding of 17 ± 3° per base pair. Harman binds more efficiently in this case. It appears that when these comutagens interact with DNA they do not affect the affinity of proven mutagens for DNA; however, they do inhibit covalent binding of the mutagens to DNA.

One site of activity of harman and norharman with polycyclic aromatic hydrocarbons appears to be cytochrome P-450. Both compounds influence the distribution of metabolites obtained by the action of this enzyme; the formation of polar (less mutagenic) metabolites is inhibited by norharman. It has been concluded that norharman binds to cytochrome P-450 on the sixth ligand position of iron and near the active site area where polycyclic aromatic hydrocarbons, such as benzo(a)-pyrene, bind and are activated. Thus, norharman is more effective as a comutagen than harman, but binds less tightly to DNA than does harman. Harman has been crystallized and studied by X-ray diffraction techniques (20). The molecule is shown to be flat, apart from the hydrogen atoms of the methyl group. Crystals are orthorhombic, space group $P2_12_12_1$. The hydrogen bonding between molecules leads to a hypothesis on the relationship to mutagens such as proflavine, particularly when harman is complexed with an aromatic amine so that the pair of molecules can then interact with biological macromolecules in a manner similar to that of certain known mutagens.

N-acetylation is an important step in the detoxification and activation of many drugs and xenobiotics. A genetic polymorphism in N-acetylation occurs in humans, which is due to variation in the activity of the hepatic enzyme N-acetyltransferase (NAT), controlled by two alleles at a single autosomal locus. Acetylator phenotype is involved in determining susceptibility to toxicity from arylamine and hydrazine drugs, and has been implicated in determining susceptibility to bladder cancer following exposure to industrial arylamines.

In addition to N-acetylation, two other hepatic acetylation activities are involved in arylamine activation. Arylhydroxamic acid N,O-acyltransferase (AHAT) catalyzes the metabolic activation of arylhydroxamic acids by intramolecular

transfer of the acetyl group from N to O, forming an unstable N-acetoxyarylamine. The reactions catalyzed by NAT and AHAT both proceed by an ordered ping-pong mechanism. NAT and AHAT activities were shown to be catalyzed by the same enzyme in rabbit liver, and they are both subject to wide genetic variation between rapid and slow acetylator rabbits. A hepatic O-acetyltransferase (OAT) activity was also identified recently, which catalyzes the activation of N-OH-3,2'-dimethyl-4-aminobiphenyl (N-OH-DMABP) by direct O-acetylation, forming a reactive N-acetoxyarylamine. The O- and N-acetylation activities have been suggested to be catalyzed by a single enzyme in hamster liver.

Hepatic cytosolic arylamine NAT was purified from rapid (C57BL/6J) and slow (A/J) acetylator mice using anion-exchange, gel filtration, hydrophobic interaction, and methotrexate gel chromatography (40). Identical purification procedures were used with rapid and slow NAT, and the rapid enzyme was purified to homogeneity, while the slow enzyme was purified to about 50% homogeneity, as judged by silver staining of 20% SDS-polyacrylamide gels. AHAT and OAT activities were detected in pooled fractions from each step of the purification procedure, as well as in the homogeneous NAT preparation from C57BL/6J liver. Additionally, NAT, AHAT, and OAT had identical elution profiles from the final (gel filtration) step of the C57BL/6J purification procedure. All three acetylation activities corresponded with the presence of a single 31,000 Dalton protein. These data are consistent with the conclusion that AHAT, OAT, and NAT activities are catalyzed by a common protein in mouse liver.

A substantial proportion of newly synthesized hepatic heme is incorporated into cytochrome P-450. Many chemicals which induce cytochrome P-450 also induce 5-aminolaevulinate synthase, the first and rate-controlling enzyme of the heme-biosynthetic pathway. It has been assumed that increases in this enzyme result in increased production of heme for incorporation into newly synthesized induced apoprotein. The effects of inducers of cytochrome P-450 on heme biosynthesis from 5-aminolaevulinate were examined by using cultured chick-embryo hepatocytes (76). Cultures treated with either 2-propyl-2-isopropylacetamide or 3-methylcholanthrene contained increased amounts of cytochrome P-450 and heme. After treatment for 3 h with 5-amino [4-^{14}C] laevulinate, the relative amounts of radioactivity accumulating as heme corresponded to the relative amounts of total cellular heme, but not to increases in the amounts of cytochrome P-450. Treatment with 5-aminolaevulinate did not alter cellular heme or ctyochrome P-450 concentrations in either control or drug-treated cultures. The mechanism of the enhanced accumulation of radioactivity in heme was investigated. Although 2-propyl-2-isopropylacetamide enhanced the uptake of 5-aminolaevulinate and increased the cellular concentration of porphobilinogen 1.5-fold, these changes did not account for the increases in heme radioactivity. The inducing drugs had no effect on the rates of degradation of radioactive heme, but appeared to enhance conversion of protoporphyrin into heme. This latter effect was shown by 1) a decreased accumulation of protoporphyrin from 5-aminolaevulinate in cells treated with inducers, and 2) complete prevention of this decrease if the iron chelator desferrioxamine was present. The data show that inducers of cytochrome P-450 may increase heme synthesis not only by increasing activity of 5-aminolaevulinate synthase, but also by increasing conversion of protoporyphrin into heme.

CARCINOGENESIS MECHANISMS

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. ABUL-HAJJ, Yusuf J. University of Minnesota of Mnpls-St Paul 1 R01 CA41269-01A2	Macromolecular Binding of Estrogens and Carcinogenesis
2. ARCHER, Michael C. Ontario Cancer Institute 5 R01 CA26651-08	Mechanism of Nitrosamine Alkylation of DNA and RNA
3. AUST, Steven D. Gordon Research Conferences 1 R13 CA45554-01	1987 Gordon Research Conference Mechanisms of Toxicity
4. BAIRD, William M. Purdue University West Lafayette 5 R37 CA28825-08	Modifiers of Chemical Carcinogenesis in Cell Culture
5. BRESNICK, Edward University of Nebraska Lincoln 1 R13 CA47350-01	Biology and Chemistry of N-Nitroso and Related Compounds
6. BUHLER, Donald R. Oregon State University 2 R01 CA22524-09	Pyrrolizidine Alkaloid Toxicity, Metabolism, and Binding
7. COLBY, Howard D. College of Medicine at Rockford 5 R01 CA43604-02	Adrenal Carcinogen Metabolism
8. CONNEY, Allan H. Rutgers the State Univ New Brunswick 1 R13 CA47210-01	Microsomes and Drug Oxidations
9. CUCHENS, Marvin A. University of Mississippi Medical Center 2 R01 CA33111-04A1	Carcinogenesis of B-Lymphocytes in Rat Peyer's Patches
10. DUFFEL, Michael W. University of Iowa 2 R01 CA38683-04	Aryl Sulfotransferase in Drug and Xenobiotic Metabolism
11. EL-BAYOUMY, Karam E. American Health Foundation 2 R01 CA35519-04	Nitroaromatics: Carcinogenicity and Metabolism
12. ELSTON, Ralph A. Battelle Pacific Northwest Division 5 R01 CA44269-02	Biology of Hemic Neoplasia in the Marine Mussell

13.	ENSLEIN, Kurt Health Designs, Inc. 2 R44 CA37494-02A2	SAR Estimation of Carcinogenesis Bioassay Results
14.	FIALA, Emerich S. American Health Foundation 5 R01 CA31012-06	Disposition of Hydrazines: Species and Strain Effects
15.	FLESHER, James W. University of Kentucky 1 R01 CA45823-01	Bioalkylation in Chemical Carcinogenesis
16.	FLOSS, Heinz G. Ohio State University 5 R01 CA37661-03	Biochemical Mechanisms of Nitrosamine Carcinogenesis
17.	FORD, George P. Southern Methodist University 5 R01 CA38473-03	Prediction of Nucleoside-Carcinogen Reactivity
18.	GESSNER, Teresa Roswell Park Memorial Institute 5 R01 CA24127-07	Conjugations and Carcinogen Metabolism
19.	GIBSON, David T. University of Texas Austin 5 R01 CA19078-11	Microbial Degradation of Carcinogenic Hydrocarbons
20.	GLUSKER, Jenny P. Institute for Cancer Research 5 R01 CA10925-38	Application of Crystallographic Techniques
21.	GOLD, Barry I. University of Nebraska Medical Center 3 R01 CA38976-03S1	Metabolism and Genetoxicity of Nitrosamines
22.	GOLDMAN, Peter Harvard University 5 R01 CA34957-05	Carcinogen Metabolism by Host Intestinal Bacteria
23.	GREENE, Geoffrey L. University of Chicago 5 R01 CA02897-31	Steroids and Growth
24.	GROVER, Philip L. University of London 5 R01 CA21959-11	Mechanisms of Activation of Polycyclic Hydrocarbons
25.	GUENTHNER, Thomas M. University of Illinois at Chicago 5 R01 CA34455-05	Toxicologic Implications of Multiple Epoxide Hydrolases

26. GUENTHNER, Thomas M.
University of Illinois at Chicago
1 R01 CA46129-01

Xenobiotic-Metabolizing Enzymes in Human Lung

27. GURTOO, Hira L.
Roswell Park Memorial Institute
2 R01 CA25362-07A1

Genetics of Aflatoxin Metabolism Role in Carcinogenesis

28. GUSTAFSSON, Jan-ake
Caroline Institute
1 R01 CA42054-01A1

Hormonal Regulation of Liver Carcinogenesis

29. HARVEY, Ronald G.
University of Chicago
2 R01 CA36097-04

Mechanism of Carcinogenesis of Polycyclic Hydrocarbons

30. HECHT, Stephen S.
American Health Foundation
5 R01 CA32242-05

Carcinogenic Methylated PAH: Structural Requirements

31. HECHT, Stephen S.
American Health Foundation
1 R35 CA44377-01

Metabolic Activation of Carcinogens

32. HENDRICKS, Jerry D.
Oregon State University
5 R01 CA44317-02

Neoplasia--Tumor and Mechanism Studies

33. HINTON, David E.
University of California Davis
5 R01 CA45131-02

Cell and Martix Biology in Fish Liver Carcinogenesis

34. HOLLENBERG, Paul F.
Wayne State University
7 R01 CA16954-12

Hemoprotein-Catalyzed Oxygenations of Carcinogens

35. HYLEMON, Phillip B.
Virginia Commonwealth University
5 R01 CA17747-12

Bile Acids and Large Bowel Carcinogenesis

36. JAMES, Margaret O.
University of Florida
5 R01 CA44297-02

Carcinogen Biotransformation by Aquatic Invertebrates

37. JEFCOATE, Colin R.
University of Wisconsin Madison
5 R01 CA16265-13

Metabolism of Polycyclic Hybrocarbons and Carcinogenesis

38. JENSEN, David E.
Temple University
2 R01 CA31503-06A1

N-Nitroso Compound Detoxification

	Investigator / Institution / Grant	Title
39.	KAUFFMAN, Frederick C. University of Maryland School of Medicine 2 R01 CA20807-09A2	Pharmacology of Carcinogen Activation in Intact Cells
40.	KING, Charles M. Michigan Cancer Foundation 5 R01 CA23386-10	Mechanistic Approaches to Carcinogenesis
41.	KING, Charles M. Michigan Cancer Foundation 1 R13 CA45889-01	3rd International Conference on N-Substituted Aryl Compounds
42.	KOKKINAKIS, Demetri M. Northwestern University 1 R01 CA42983-01A1	DNA Damage Induced by Pancreatropic Nitrosamines
43.	KOREEDA, Masato University of Michigan at Ann Arbor 5 R01 CA25185-09	Bioorganic Chemistry of Arene Oxides and Related Epoxide
44.	LAWSON, Terence A. University of Nebraska Medical Center 1 R01 CA43646-01A2	Alkylation and Mutagenesis by Pancreas Carcinogens
45.	LEAV, Irwin Tufts University 2 R01 CA15776-10A1	Prostatic Differentiation and Sex Hormone Metabolism
46.	LEE, Mei-Sie Michigan Cancer Foundation 5 R01 CA37885-03	Metabolic Activation of Unsubstituted Hydroxamic Acid
47.	LEHR, Roland E. University of Oklahoma Norman 5 R01 CA22985-11	Diol Epoxide and Other Derivatives of PAH:SAR's
48.	LEVINE, Walter G. Yeshiva University 5 R01 CA14231-14	Role of Metabolism in the Biliary Excretion of Drug
49.	LI, Jonathan J. University of Minnesota of Mnpls-St Paul 5 R01 CA22008-09	Estrogen Carcinogenicity and Hormone Dependent Tumors
50.	LI, Jonathan J. University of Minnesota of Mnpls-St Paul 5 R01 CA41387-02	Sex Hormones and Hepatocellular Carcinomas
51.	LI, Jonathan J. Gordon Research Conferences 1 R13 CA46780-01	Gordon Research Conference on Hormonal Carcinogenesis

52. LIEHR, Joachim G.
University of Texas Med Br Galveston
5 R01 CA43232-02
Prevention of Estrogen-Induced Tumors by Chemical Means

53. LISTOWSKY, Irving
Yeshiva University
5 R01 CA42448-02
High Affinity, High Capacity Steroid, and Carcinogen Binding

54. LOEPPKY, Richard N.
University of Missouri Columbia
2 R37 CA26914-07A1
Carcinogenesis: Nitrosamine Formation and Inhibition

55. LOTLIKAR, Prabhakar D.
Temple University
5 R01 CA31641-05
Modulation of Mycotoxin Carcinogenesis by Glutathione

56. MAGEE, Peter N.
Temple University
1 R37 CA43342-01
Formation and Metabolism of Nitrosamines

57. MALEJKA-GIGANTI, Danuta
University of Minnesota of Mnpls-St Paul
5 R01 CA28000-08
Mammary Carcinogenesis by N-Substituted Aryl Compounds

58. MARCHOK, Ann C.
Oak Ridge National Laboratory
5 R01 CA43857-02
Effects of HCHO and Benzopyrene in a New Tracheal Model

59. MC ELROY, Anne E.
University of Massachusetts Amherst
5 R01 CA44289-02
Trophic Transfer of PAH and Metabolites

60. MELIKIAN, Assieh
American Health Foundation
1 R01 CA43910-01
Mechanism of Catechol Co-carcinogenesis With BAP

61. MIRVISH, Sidney S.
University of Nebraska Medical Center
5 R01 CA32192-03
N-Nitroso Compounds Formed In Vivo From Nitrogen Dioxide

62. MIRVISH, Sidney S.
University of Nebraska Medical Center
2 R01 CA35628-04
Nitrosamine Metabolism in the Esophagus

63. MUKHTAR, Hasan
Case Western Reserve University
5 R01 CA38028-03
Goeckerman Therapy of Psoriasis--Oncogenic Mechanisms

64. NAGEL, Donald L.
University of Nebraska Medical Center
5 R01 CA31016-06
An In Vitro Model for Alkylation by Pancreas Carcinogens

65. NAIRN, Rodney S.
University of Texas System Cancer Center
5 R01 CA44303-02

Genetic Determinants of Carcinogenesis

66. PAQUETTE, Leo A.
Ohio State University
5 R01 CA12115-17

Cytotoxic, Cocarcinogenic, and Antileukemic Agents

67. PARTHASARATHY, Rengachary
Roswell Park Memorial Institute
5 R01 CA23704-09

Stereochemistry of Thiol-Disulfide Interchanges

68. PAULI, Bendicht U.
Cornell University Ithaca
1 R01 CA48642-01

Carcinogenesis Testing of Sintered Porous Cocrmo Implants

69. PENNING, Trevor M.
University of Pennsylvania
5 R01 CA39504-02

Dihydrodiol Dehydrogenase and the Aspirin-like Drugs

70. PIETTE, Lawrence H.
Utah State University
5 R01 CA42013-03

ESR Studies of Biological Free Radical Mechanisms

71. POUR, Parviz M.
University of Nebraska Medical Center
1 R01 CA42133-01A1

Transplacental Induction of Pancreatic Cancer

72. PURDY, Robert H.
Southwest Foundation for Biomedical Res
5 R01 CA41569-02

Role of Catechol Formation in Estrogen-Mediated Cancer

73. REINISCH, Carol L.
Tufts University
1 R01 CA44307-01A1

Unique Antigens on Neoplastic Cells of *Mya arenaria*

74. ROSEN, Jeffrey M.
Baylor College of Medicine
5 R01 CA16303-12

Hormonal Regulation of Breast Cancer

75. SCHATZ, Robert A.
Northeastern University
1 R01 CA47671-01

Solvent and Ethanol Interaction on BAP Metabolism

76. SINCLAIR, Peter R.
Dartmouth College
2 R01 CA25012-09

Liver Cell Cultures for Study of Carcinogen Activation

	Investigator / Institution / Grant	Title
77.	STEGEMAN, John J. Woods Hole Oceanographic Institution 5 R01 CA44306-02	Environmental Tumorigenesis
78.	TANNENBAUM, Steven R. Massachusetts Institute of Technology 5 P01 CA26731-08	Endogenous Nitrite Carcinogenesis in Man
79.	TAYLOR, John-Stephen A. Washington University 5 R01 CA40463-03	DNA Photolesion Structure-Activity Relationships
80.	TOLBERT, Laren M. Georgia Institute of Technology 1 R01 CA43806-01A1	Bio-Oxidation of Arylalkyl Hydrocarbons
81.	TOTH, Bela University of Nebraska Medical Center 5 R01 CA31611-05	Carcinogenesis and Chemistry of Cultivated Mushrooms
82.	UNDERWOOD, Graham R. New York University 1 R01 CA47599-01	Mechanistic Studies of Arylamide Carcinogens
83.	VESSELINOVITCH, Stan D. University of Chicago 5 R01 CA25522-09	Role of Sex Hormones in Hepatocarcinogenesis
84.	VOLLHARDT, K. Peter C. University of California Berkeley 5 R01 CA20713-10	Activated Mutagenic and Aromatic Hydrocarbons
85.	WALKER, Bruce E. Michigan State University 5 R01 CA41599-02	Mechanism of DES Transplacental Carcinogenesis
86.	WHALEN, Dale L. University of Maryland Balt Co Campus 5 R01 CA26086-06	Kinetics of Nucleic Acid-Catalyzed Epoxide Hydrolyses
87.	YANG, Chung S. University of Medicine & Dentistry of NJ 2 R37 CA37037-04	Metabolic Activation of N-Nitrosamines
88.	YANG, Shen K. U.S. Uniformed Services Univ of Hlth Sci 5 R01 CA29133-07	Metabolic Activation of Monomethylbenz Anthracenes

SUMMARY REPORT

DIET AND NUTRITION

The Diet and Nutrition component within the Chemical and Physical Carcinogenesis Branch contains 42 grants with FY 88 funding of $3.98 million. The component supports laboratory investigations searching for cancer etiologic factors related to diet and nutrition. These investigations include mechanism studies of cancer induction by a variety of dietary constituents (i.e., fats of varying sources and saturation levels; proteins of various types and levels; fiber; nitroso compounds; mycotoxins and other naturally occurring carcinogens; inhibitors of carcinogenesis; compounds associated with the gut including bile acids, fecal steroids, and the influence of microflora). In addition, support is given to studies which focus on specific dietary factors; i.e., nutrients or micronutrients, host factors involved in pathogenesis, and the development of methods or refinements of techniques for identifying putative carcinogens in foods, body fluids or feces, as well as the influence of various methods of food processing and cooking.

The mechanisms whereby diet influences the incidence of human cancers are not understood. Experiments in laboratory animals and evidence from epidemiological studies have suggested a large number of possibilities such as the ingestion of direct-acting carcinogens or their precursors, inhibition of the formation of carcinogens, or the activation or detoxication of pre-carcinogens by components of the diet.

One group of investigators (renewal applications under review at this time) has an interest in 1,2-dicarbonyls because of their presence (microgram to miligram amounts) as Maillard "browning" reaction intermediates in heat processed foods and in certain beverages (e.g., coffee and tea), coupled with their known chemical reactivity with DNA-bases and their direct base-substitution mutagenic activity in Ames/*Salmonella* assays. Coffee is a widely consumed beverage for which there is some epidemiological data suggesting that it is a human bladder and colon carcinogen. Its cytotoxic and Ames/*Salmonella* mutagenic properties result from the dry roasting of the coffee beans, as does its dicarbonyl content and its H_2O_2-generating system. Data on the mammalian genotoxic activities of 1,2 dicarbonyls and coffee are needed to evaluate their likely contributions to food-associated carcinogenesis which is currently thought to represent about one-third of the cancer cases in the U.S. Work by this group at the Lawrence Livermore National Laboratories has concentrated on the 1,2 dicarbonyl and coffee-induced mutation. Sister chromatid exchange (SCE) and an unusual and abnormal process of cell replication involving two rounds of DNA replication without an intervening cytokinesis (ER) have been studied. Dose response curves have been completed in chinese hamster cells (CHO AUXB1) for four 1,2,-dicarbonyls (methylglyoxal, glyoxal, diacetyl, and kethoxal) and five separate coffee preparations (three major brands of drip-brewed regular coffee, one brand of drip-brewed decaffeinated coffee, and one brand of instant regular coffee) with respect to induced reversion mutations at specific loci, induction of SCEs, and the induction of ERs. Following 20-22 h exposures, the induced GAT^+ revertant frequency/micromolar concentration increment was kethoxal $65 \times 10^{-8} >$ diacetyl $4.0 \times 10^{-8} >$ methylglyoxal $3.7 \times 10^{-8} >$ glyoxal 1.0×10^{-8}. Treatments for 20-22 h with the above diluted (0.2-1.0 mg/ml), freshly made coffee preparations yield dose-dependent GAT^+ revertant frequencies up to 15- to 20-fold greater than the spontaneous controls ($0.5\text{-}1.0 \times 10^{-6}$). Bisulfite protects

against the FPGS locus mutagenic activities of both the 1,2-dicarbonyls and the various coffee preparations. Using similar treatments, dose-dependent two-to threefold increases above the baseline SCE frequency (9.8-11.4 SCEs/cell) are induced by 0.3-1.3 micromolar concentrations of the four 1,2-dicarbonyls and 0.2-1.0 mg/ml of the five diluted coffees. Moreover, against a baseline of <0.1% ERs each of the 1,2-dicarbonyls and each of the coffee preparations induce 3-11% ERs. It has been suggested from studies with aphidocolin in V79 cells that DNA polymerase beta is required for the expression of ERs. At 41° C CHO AUXB1 cells grow, but polymerase beta is inactivated. These workers found that the 37° C induction of ERs by methylglyoxal, glyoxal, kethoxal, and one drip-brewed regular coffee likewise did not occur in CHO AUXB1 cells at 41° C. This observation is consistent with, but is not conclusive proof for, an essential role of polymerase beta in the accumulation of ERs.

Using 90 minute exposures and the technique of alkaline/filter elution, the above four 1,2-dicarbonyls and five coffees were systematically compared with respect to their promotion of DNA-protein cross-linking and DNA single-strand breakage. Only methylglyoxal and glyoxal (weak response) produce cross-links, while all the 1,2-dicarbonyls caused single-strand breakage in CHO AUXB1. All five diluted coffee preparations (1-6 mg/ml) resulted in a dramatic dose-dependent increase in single-strand breakage, but no detectable cross-linking. At concentrations greater than 100 micromolar, H_2O_2 was also a potent DNA strand-breaking agent. Bisulfite co-treatment completely negated all the DNA damage observed for both the 1,2-dicarbonyls and the diluted coffee preparations.

In another laboratory (11), a duplex *Escherichia coli* bacteriophage M13 genome was constructed containing a single *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ intrastrand cross-link, the major DNA adduct of the anticancer drug, *cis*-diaminedichloroplatinum. The duplex dodecamer, d(AGAAGGCCTAGA)-d(TCTAGGCCTTCT), was ligated into the *Hinc II* site of M13mp18 to produce an insertion mutant containing a unique *Stu I* restriction enzyme cleavage site. A genome with a 12-base gap in the minus strand was created by hybridizing *Hinc II* linearized M13mp18 duplex DNA with the single-stranded circular DNA of the 12-base insertion mutant. The dodecamer, d(TCTAGGCCTTCT), was synthesized by the solid-phase phosphotriester method platinated by reaction with *cis*-$[Pt(NH_3)_2(H_2O_2)_2]^{2+}$ (yield, 39%). Characterization by pH-dependent ^1H nuclear magnetic resonance (NMR) spectroscopy established that platinum binds to the N7 positions of the adjacent guanosines. The platinated oligonucleotide was phosphorylated in the presence of gamma-[^{32}P]-ATP with bacteriophage T4 polynucleotide kinase and incorporated into the 12-base gap of heteroduplex, thus situating the adduct specifically with a *Stu I* site in the minus strand of the genome. Approximately 80% of the gapped duplexes incorporated a dodecanucleotide in the ligation reaction. Of these, approximately half did so with the dodecanucleotide covalently joined to the genome at both 5' and 3' termini. The site of incorporation of the dodecamer was mapped to the expected 36-base region delimited by the recognition sites of *Xba I* and *Hind III*. The *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ cross-link completely inhibited *Stu I* cleavage, which was fully restored following incubation of the platinated genome with cyanide to remove platinum as $[Pt(CN)_4]^{2-}$. Gradient denaturing gel electrophoresis of a 289 base-pair fragment encompassing the site of adduction revealed that the presence of the *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ cross-link induces localized weakening of the DNA double helix. In addition, double- and single-stranded genomes, in which the *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ cross-link resides specifically in the plus strand, were constructed. Comparative studies revealed no difference in survival between platinated and unmodified double-stranded

genomes. In contrast, survival of the single-stranded platinated genome was only 10-12% that of the corresponding unmodified single-stranded genome, indicating that the solitary cis-[$Pt(NH_3)_2${d(GpG)}] cross-link is lethal to the single-stranded bacteriophage.

A laboratory at the Royal Postgraduate Medical School, London, England (10) has established a sensitive and specific assay for 2-amino-3,8-dimethylimidazo[4,5-7] quinoxaline (MeIQx) in cooked meat and shown it to be present at a level of 2.4 ng/g. In addition, carbon-14-labeled MeIQx has been synthesized. The next experiments will determine the physiologic disposition and metabolic fate of MeIQx in animals and man. In animals, mainly rats, carbon-14-labeled MeIQx will be administered by various routes, including the oral route, to identify whether the excretion of unchanged amine or a major metabolite in urine can be used to assess the body burden of the chemical in humans eating fried beef. Preliminary studies in animals suggest that measurement of unchanged MeIQx in urine is a reliable measure of the systematic availability of the amine when taken orally. Human volunteers, who nominally consume beef, will be asked to eat fried ground beef, a portion of which will be analyzed for MeIQx. Urine will be collected at 12 hour intervals for the following 72 hours and the excretion of MeIQx will be measured and used to estimate the systemic level of the amine. At the same time work will be carried out to identify an excretory product of the mutagenic metabolite of MeIQx (assumed to be N-hydroxyMeIQx) in order to assess the degree of mutagenic activation of the amine.

Whole body autoradiography will be conducted on animals dosed with radio-labeled MeIQx to attempt to identify the "hot spots" for covalent binding of MeIQx. These studies will be followed up with specific assays on "hot spot" tissues. Studies will continue to determine the isozyme specificity of the conversions of MeIQx to a mutagen by the cytochrome P-450 system in animal and human tissues. Activation of MeIQx to a mutagen will be studied using freshly isolated human hepatocytes to determine whether the mutagenic metabolite can diffuse from hepatocytes to targets in the surrounding medium. Studies are continuing at Cornell University (17) on the stability of N-nitrosamides in porcine gastric fluid. Previous workers have pointed out that most N-nitrosamides have short half-lives, even at an acid pH. This laboratory has investigated the half-life of some nitrosamides in porcine gastric fluid using the HPLC-chemiluminescence detector. N-nitrosomethylurea (NMU) was the original choice as a marker for the endogenous formation of N-nitrosamides in gastric fluid, however, a half-life of less than 30 minutes was found. This means that the observed rate of MNU formation is the actual rate minus the rate of decay. Even non-aqueous standards are not stable for more than 24 hours. This makes it difficult to quantify the amount of nitrosamide formed because samples cannot be stabilized fast enough to allow low micrograms/L analyses. It was found that the N-nitroso derivative of trimethylurea (NTMU) is considerably more stable. The half-life of this N-nitrosourea is greater than seven days in dry solvent and several hours in gastric fluid. This group of scientists now has developed an analytical protocol for this compound in porcine gastric fluid. The HPLC-chemiluminescence detector is used to quantify the NTMU. Sensitivity is on the order of one microgram/L of gastric fluid. The significance is that now there is a compound of sufficient stability to use as a marker for the endogenous formation of N-nitrosamides. There are also studies on the kinetic rates of loss of other N-nitrosamides from porcine gastric fluid and the effect that pH has on the stability of each compound. Results should show whether there are factors which stabilize or destabilize carcinogenic N-nitrosamides in gastric fluid.

The laboratory has obtained 20 Kg of the dried fish which is most commonly consumed in Hong Kong. These types of fish are strongly associated with certain types of human cancer. An in vitro gastric digestion system was developed in which the fish is enzymically digested at pH=2 for two hours. The affects that the amount of fish, nitrite, thiocyanate, and ascorbic acid have on the formation of N-nitrosodimethylamine (NDMA) and other nitroso compounds have been determined. Detectable amounts (0.5 micrograms/L) of nitrosodimethylurea (NDMA) are formed with as little as 40 micrograms/L of nitrite. Mathematical equations which relate the amount of nitroso compound formed with the other variables are being constructed from these data. As expected, increasing the nitrite and thiocyanate concentration increases the formation of nitroso compounds. What is surprising is that ascorbic acid is much less capable of inhibiting this formation than the simple in vitro chemistry would predict. This work demonstrates that dried salted fish are capable of forming nitrosamines under conditions similar to digestion. Two questions remain: Is the amount of nitrosamine formed sufficient to account for the mutagenicity associated with the samples, and will the results be similar when the fish is "fed" to the fistulated pig?

Work is continuing on the pig as a model for gastric nitrosation. A rigid teflon cannula, which can be increased in size as the animal grows larger, has been designed. This allows one to keep the animal chronically fistulated for periods of up to 6 months and use the same animals for several studies. The gastric residence time in each animal has been determined using a marker. When 200 ml of artificial gastric fluid is introduced, with the marker, the emptying time is 35 to 45 minutes. By consecutive additions of the marker, it was determined that the volume decreases from 250 to 25 ml during this period. More importantly, the change in volume of the stomach over time, after giving a "meal" through the fistula, is known. Knowing this volume change will allow the investigators to integrate over time the total amount of nitrosotrimethylurea (NTMU) formed in the stomach. The significance is that one is able to estimate a total exposure to N-nitroso compounds due to endogenous formation under given circumstances.

Studies are continuing on the anaerobic metabolism of mutagens in human food (20). The major metabolite of 2-amino-3-methyl-3H-imidazo[4,5]quinoline (IQ) by human fecal bacteria has been identified as the oxidized product "hydroxy-IQ" (HOIQ). A synthesis route has been developed for this compound and reasonable overall yield has been achieved.

Grant-supported research is continuing in other areas of the Diet and Nutrition component of the Branch. A study being conducted at the University of Illinois at Chicago is designed to determine the role of zinc and ethanol in esophogeal carcinogenesis (4).

Epidemiological studies in China and the United States have demonstrated that dietary zinc deficiency, environmental exposure to methylbenzylnitrosamine (MBN) and chronic consumption of ethanol are factors associated with an increased incidence of esophageal carcinoma in man. Rats treated with MBN also develop squamous cell carcinoma of the esophagus, and rats raised on zinc- deficient diets exhibit an increased incidence of MBN-induced esophageal carcinoma as compared with controls. Chronic ethanol consumption in the zinc- deficient rat model further increases the incidence of MBN-induced esophageal carcinoma.

The proposed mechanism of MBN-induced carcinogenesis is viewed as a complex process involving cytochrome P-450 dependent activation of the carcinogen, detoxification of the carcinogen and activated metabolites, chemical modification of cellular constituents including DNA, and repair of critical cellular damage. MBN is activated by cytochrome P-450 to form benzaldehyde and a carbonium ion which methylates DNA. MBN is known to methylate DNA in vivo forming O^6-methylguanine (O^6MeG) adducts and the highest concentration of these adducts is found in the esophageal DNA of the rat, the target tissue of this carcinogen. Misreading of O^6MeG adducts can lead to guanine to adenine point mutations and such guanine to adenine point mutations are responsible for certain carcinogen-induced tumors.

These workers examined the kinetics of the esophageal and hepatic metabolism of MBN and the hepatic metabolism of the related hepatic carcinogen, dimethylnitrosamine. In examining the effects of dietary zinc deficiency, rats were divided into three groups: the first group was fed the zinc-deficient diet containing 2.3 ppm zinc ad libitum (zinc deficient), the second group was fed the zinc control diet containing 50 ppm zinc ad libitum (zinc control), and the third group was pair-fed the control diet to match the daily caloric intake of the zinc-deficient group (pair-fed zinc control). To examine the effect of chronic ethanol consumption, rats were fed a nutritionally complete liquid ethanol diet (ethanol fed) or pair-fed the liquid control diet to match the caloric intake of the ethanol-fed animals (ethanol, pair-fed control). These studies demonstrated a family of enzymes involved in the esophageal and hepatic microsomal metabolism of these nitrosamines and that these enzymes were noncompetitively inhibited by zinc in vitro.

The initial hypothesis was that dietary zinc deficiency and chronic ethanol consumption, treatments associated with an increased incidence of MBN-induced esophageal carcinoma, would show an increased microsomal metabolism of MBN and an increased formation of esophageal O^6MeG, while the caloric restriction of pair-feeding, associated with a decreased incidence of MBN-induced esophageal carcinoma, would show a decreased rate of MBN metabolism and a decreased formation of MBN-induced esophageal O^6MeG. To date, results have demonstrated that dietary zinc deficiency and chronic ethanol consumption do increase the esophageal microsomal metabolism of MBN, but that caloric restriction of pair-feeding also increases MBN metabolism. These results suggested that some of the enzymes involved in the microsomal metabolism of MBN activated the carcinogen to a methylating agent, while other enzymes simply degraded the carcinogen without activation to a methylating agent.

Continuing work is designed to further examine the enzymes involved in the microsomal metabolism of MBN and to initiate experiments on the role of dietary zinc deficiency and ethanol in MBN-induced methylation of esophageal DNA.

In a second phase of this grant, studies were undertaken to determine whether the observed differences in microsomal metabolism were associated with activation or degradation of MBN. The MBN-induced formation of O^6MeG in the esophageal DNA of rats in the zinc-deficient, zinc control, and pair-fed zinc control groups was examined. Following a single dose of MBN, levels of esophageal O^6MeG were highest at one hour in all three groups. O^6MeG levels then declined significantly ($p<.002$) over the next 5 hours with the 06MeG levels in the zinc-deficient group consistently higher than controls at all time points. Comparisons of the

treatment groups demonstrated that the levels of esophageal O^6MeG in the zinc-deficient animals were significantly higher than controls ($p<.05$) and that the levels of esophageal O^6MeG in the pair-fed animals was consistantly lower than in the ad libitum controls. Esophageal levels of 7-methylguanine (7MeG) were also measured to determine if alterations in the esophageal O^6MeG were due to specific changes in the formation and repair of O^6MeG, or were due to a nonspecific effect on total methylation. Calculation of the O^6MeG/7MeG ratio allowed a comparison of the clearance rates of these two methylated bases. Following a single injection of MBN, levels of esophageal 7MeG revealed a pattern similar to that of O^6MeG adducts. Levels of 7MeG were highest in all groups at one hour and declined significantly with time ($p<.05$), similar to the pattern seen with the O^6MeG adducts. Levels of 7MeG in the zinc-deficient animals were significantly greater than the ad libitum and pair-fed controls ($<.05$) and levels of 7MeG were lower in the pair-fed controls than in the ad libitum controls.

Examination of the ratio of the levels of O^6MeG to 7MeG revealed no significant differences in this ratio with respect to time or dietary treatment. Thus, the differences in the levels of esophageal O^6MeG between the treatment groups appears to be a result of alterations in total methylation and not due to selective methylation of guanine to form one adduct as opposed to another. That there was no difference in the clearance of O^6MeG as compared to 7MeG suggests that the esophageal O^6methyltransferase repair system was consumed by the initial methylation injury and that additional O^6methyltransferase repair did not appear during the 24 hour period of observation.

These results support the hypothesis that dietary zinc deficiency increases the activity of certain cytochrome P-450 enzymes which activate MBN to a methylating agent and that caloric restriction and chronic ethanol consumption increase enzymes which metabolize MBN without activation of the carcinogen to a methylating agent. Work is continuing on characterizing the enzyme involved in the degradation of MBN and it appears to be the ethanol inducible form of cytochrome P-450 (P-450J). Both ethanol and caloric restriction included hepatic cytochrome P-450J. Ethanol induction of P450J is associated with an increased hepatic microsomal metabolism and activation of the related hepatic carcinogen, dimethylnitrosamine, but the data suggests that the ethanol inducible form of cytochrome P-450 does not activate MBN. Ethanol is a competitive inhibitor of P-450J, and acute ethanol intoxication of rats immediately prior to dimethylnitrosamine administration does reduce dimethylnitrosamine-induced hepatotoxicity and carcinogenesis. In the esophagus, chronic ethanol consumption also induces cytochrome P-450J, but this increase in cytochrome P-450J is not associated with an increase in the MBN-induced formation of O^6MeG.

In the microsomal assays, the addition of ethanol in vitro inhibited the microsomal metabolism of MBN, while acute ethanol intoxication of zinc control and zinc-deficient rats did not reduce the MBN-induced esophageal formation of O^6MeG.

Work continues on the quantitation of the specific levels of cytochrome P-450J in the esophageal microsomal protein of rats raised on the various zinc and ethanol diets. Enzyme concentrations are being determined using a radiolabled antibody specific for cytochrome P-450J after separation of the microsomal protein on polyacrylamide gels. This antibody is also a blocking antibody and may be used in future studies to selectively inhibit the activity of cytochrome P-450J in the microsomal assays of MBN metabolism in vitro.

DIET AND NUTRITION

GRANTS ACTIVE DURING FY88

	Investigator/Institution/Grant Number	Title
1.	ABRAHAM, Samuel Children's Hospital Med Ctr N CA-Oakland 2 R01 CA29767-04A3	Effect of Dietary Fat on Mammary Neoplasia
2.	AUSMAN, Lynne M. Ctr for Molecular Medicine & Immunology 5 R01 CA42352-02	Nutritional Influences on Colon Cancer in the Tamarin
3.	AYLSWORTH, Charles F. Michigan State University 5 R23 CA36364-03	Dietary Fat--Cell Communication and Breast Tumorigenesis
4.	BARCH, David H. University of Illinois at Chicago 5 R23 CA40487-03	Role of Zinc and Ethanol in Esophageal Carcinogenesis
5.	BENNETT, Alice S. Ball State University 1 R15 CA41690-01A1	Fatty Acids and Murine Mammary Tumorigenesis
6.	BIRT, Diane F. University of Nebraska Medical Center 5 R01 CA31655-03	Role of Dietary Fat in Experimental Pancreas Cancer
7.	BIRT, Diane F. University of Nebraska Medical Center 5 R01 CA42986-02	Dietary Fat, Calories, and Two-Stage Carcinogenesis
8.	BOKKENHEUSER, Victor D. St. Luke's-Roosevelt Inst for Hlth Sci 5 R01 CA25763-12	Bacterial Metabolism of Flavonoid Glycosides/Aglycones
9.	CAMPBELL, T. Colin Cornell University Ithaca 5 R01 CA34205-04	Dietary Protein and Chemical Carcinogenesis
10.	CHATTORAJ, Sati C. Boston University 5 R01 CA39381-02	Catechol Estrogens, Diet and Breast Cancer
11.	DAVIES, Donald S. University of London 5 R01 CA40895-03	The Metabolic Fate of Mutagenic Amines in Animals and Man
12.	ESSIGMANN, John M. Massachusetts Institute of Technology 5 R01 CA40817-03	Biological Effects of Cyclic Nucleic Acid Adducts Formed

13. FLEMING, Sharon E.
University of California Berkeley
5 R01 CA40845-03
Fiber Volatile Fatty Acids and Colonic Cell Biology

14. GALIVAN, John H.
New York State Dept of Health
5 R01 CA34314-03
Vitamin Function in Liver Studied In Vitro

15. GALLAHER, Daniel D.
North Dakota State University
5 R01 CA40843-02
Dietary Fiber and Fat in Bile Acid Excretion

16. GHOSHAL, Amiya K.
University of Toronto
5 R01 CA41537-02
Diet and Cancer: Choline and Methionine in Liver Cancer

17. GRUBBS, Clinton J.
University of Alabama at Birmingham
5 R01 CA41994-02
Effect of Alcohol on Chemically-Induced Cancers

18. HOTCHKISS, Joseph H.
Cornell University Ithaca
5 R01 CA40833-03
Gastric Formation of N-Nitroso Compounds in the Pig

19. ISSENBERG, Phillip
University of Nebraska Medical Center
1 R01 CA43589-01A1
Cruciferous Vegetables/ Endogenous Nitrosamine Forms

20. JANGHORBANI, Morteza
Boston University
5 R01 CA38943-03
Dietary Biovailability of Selenium in Man

21. KINGSTON, David G.
Virginia Polytechnic Inst and St Univ
5 R01 CA40821-03
Anaerobic Metabolism of Mutagens in Human Foods

22. LEA, Michael A.
University of Medicine & Dentistry of NJ
1 R01 CA46442-01
Nucleotide Metabolism and Promotion of Carcinogenesis

23. LONGNECKER, Daniel S.
Dartmouth College
1 R01 CA47327-01
Transgenic Mouse Models of Pancreatic Carcinogenesis

24. NEWBERNE, Paul M.
Boston City Hospital
5 R01 CA40080-03
Zinc, Nitrosamine and Esophageal Cancer

25. PETERS, John H.
SRI International
5 R01 CA40918-03
Fecapentaenes: Mechanistic Studies

26. RAPOPORT, Henry
University of California Berkeley
5 R01 CA40984-03
Metabolism and Bioavailability of MeIQx from Fried Beef

27. REDDY, Bandaru S.
American Health Foundation
5 R01 CA37663-03
Mechanisms of Dietary Fat Effects in Colon Cancer

28. SARKAR, Nurul H.
Medical College of Georgia
2 R01 CA45127-02
Effect of Diet on Murine Mammary Tumorigenesis

29. SCANLAN, Richard A.
Oregon State University
5 R01 CA25002-17
N-Nitrosamines in Foods

30. SCHUT, Herman A.
Medical College of Ohio at Toledo
1 R01 CA47484-01
Carcinogenesis Studies on Heterocyclic Amines

31. SELIVONCHICK, Daniel P.
Oregon State University
5 R01 CA30087-06
Membrane Protein Composition: Cyclopropenoid Fatty Acids

32. SINHA, Dilip K.
Roswell Park Memorial Institute
5 R01 CA42853-02
Prevention of Mammary Cancer By Caloric Restriction

33. TAYLOR, Robert T.
University of California
Lawrence Livermore National Laboratory
5 R01 CA40816-03
Genotoxicity of Food Related 1,2-dicarbonyl Compounds

34. TOTH, Bela
University of Nebraska Medical Center
5 R01 CA40989-03
Capsaicin: Chemistry, Carcinogenesis, and Mode of Action

35. TOTH, Bela
University of Nebraska Medical Center
1 R01 CA44075-01
False Morel Hydrazines: Carcinogenesis and Chemistry

36. VISEK, Willard J.
College of Medicine at Peoria
5 R01 CA41707-02
Calorie Consumption and Experimental Colon Cancer

37. WAINFAN, Elsie
New York Blood Center
1 R01 CA47600-01
Diet Cancer and Oncogene Expression in Inbred Mice

38. WEISBURGER, John H.
American Health Foundation
5 R01 CA24217-09
Food Mutagens: Bioassay for Carcinogenicity

39. WEISBURGER, John H.
American Health Foundation
5 R01 CA42381-02

Metabolism of the Carcinogen Aminomethylimidazoquinoline

40. WEISBURGER, John H.
American Health Foundation
1 R01 CA45720-01

Identification of Carcinogens

41. WELSCH, Clifford W.
Michigan State University
5 R01 CA37613-03

Caffeine and Experimental Mammary Gland Tumorigenesis

42. WELSCH, Clifford W.
Michigan State University
5 R01 CA42876-02

Can Dietary Fat Affect Mammary Gland Growth Processes

SUMMARY REPORT

MOLECULAR CARCINOGENESIS

The Molecular Carcinogenesis component of the Branch includes 276 grants with FY 88 funding of approximately $40.03 million. There are no contracts in this area. The currently active grants consist of 233 R01 (Research Project) grants, 3 R23 (Young Investigator) grants, 11 R29 "FIRST" (First Independent Research Support & Transition) award, 1 R15 "AREA" (Academic Research Enhancement Award) grant, 3 R43 (Small Business) grants, 7 P01 (Program Project) grants, and 4 R35 Outstanding Investigator grants. In addition, 10 grants have been approved as R37 "MERIT" (Method to Extend Research In Time) Awards (3 additional from R01s in FY 88). Also included are 7 R13 conference grant awards. Research supported by this component focuses on the role of exocyclic DNA adducts in carcinogenesis (3 grants); the characterization of carcinogen-macromolecular interactions (31 grants); changes in biological macromolecules and cell functions as a result of carcinogen or cocarcinogen exposure (24 grants); the identification of biochemical and molecular markers and properties of cells transformed by carcinogens (35 grants); the genetics and mechanisms of cell transformation (52 grants); the development of carcinogenicity/mutagenicity testing procedures and the development of organ and cell culture systems and whole animal biological models for use in carcinogenesis studies (14 grants); the mechanisms of carcinogen-induced mutagenesis and genetic damage (26 grants); the identification and properties of tumor promoters and mechanisms of tumor promotion (52 grants); interspecies comparisons in carcinogenesis (3 grants); the genetics and regulation of enzymes associated with carcinogenesis induced by chemical and physical carcinogens (12 grants); development of analytical methodology for detecting chemical carcinogens in body fluids and environmental samples (1 grant); and the role of DNA repair in carcinogenesis (24 grants). Expanded descriptions of individual subject areas, along with examples of research accomplishments, are provided below.

Grants Activity Summary

Exocyclic Adducts in Carcinogenesis

In January 1986 a Program Announcement entitled "Biological Role of Exocyclic Nucleic Acid Derivatives in Carcinogenesis" was issued. The purpose of this Announcement was to stimulate basic research on a class of important compounds which have the capability of forming exocyclic nucleic acid adducts. It was expected that studies relevant to many of the above-listed subject areas would be stimulated by this program initiative. Two applications directly relevant to the focus of the Program Announcement were submitted prior to its publication. These have both been funded. One grant supports a project to develop monoclonal antibodies to various exocyclic nucleic acid adducts. The second grant supports studies on the reaction of crotonaldehyde and 4-hydroxynonenal with guanine nucleotides and with DNA, both in vitro and in vivo. Between January 1986 and January 1987, five applications (including one Small Business grant application) were submitted in response to the Program Announcement. One grant, which is focused on a study of acrolein as a possible causative agent in human bladder cancer, was funded and the Small Business grant, whose goal is to develop monoclonal antibodies specific for the major nucleic acid adducts of bifunctional carbonyl compounds (i.e., malonaldehyde, acrolein, methyl glyoxal) in human DNA, was also funded. In early 1987 the proceedings of the meeting which led to the generation of the Program Announcement were published by the International Agency

for Research on Cancer (IARC) Scientific Publications. The availability of this publication and the relatively low number of responses received up to that time led to the decision, in April 1987, to reissue the Program Announcement. Five additional responses have resulted since this reissuance. Three of these grant applications were funded in FY88. One grant supports a study to test the hypothesis that N-nitroso-2-pyrrolidone is the proximate carcinogen derived from two successive metabolic oxidative transformations of N-nitrosopyrrolidine and that N-nitrosopyrrolidine genotoxicity is derived from the formation of transient DNA adducts. The second grant supports studies on the in vitro mutagenic potential of N^2,3-ethenoguanine, the evaluation of the effects of site-specific 1,N^6-ethenoadenine and 3,N^4-ethenocytosine lesions on duplex DNA structure and replication fidelity, as well as the investigation of whether adducts other than the known lesions are formed by metabolites of vinyl chloride. Previous support for the above studies had been included in one large grant which has now been divided into two more focused grants, one of which represents this response to the Program Announcement. The third grant supports studies on the mechanisms of mutagenesis of ethenocytidine and ethenoadenine adducts induced in DNA by metabolites of the carcinogen, vinyl chloride.

Trans-4-hydroxy-2-nonenal is a major alpha,beta-unsaturated aldehyde released during lipid peroxidation. It has been shown to be a direct-acting mutagen in bacterial and mammalian cell mutagenesis assays and has been suggested to play a major role in liver toxicity associated with lipid peroxidation. Its reaction with DNA under physiological conditions was investigated in order to assess its DNA damaging potential. When dissolved in tetrahydrofuran (THF) that was unprotected from light, reactions of trans-4-hydroxy-2-nonenal yielded 6 separable adducts. Adduct 1 was characterized as 1,N^2-ethenodeoxyguanosine and adduct 4 as a substituted 1,N^2-etheno derivative. Adducts 2, 3, 5, and 6 were tentatively identified as N^2-substituted deoxyguanosine diastereomers which could be converted quantitatively to a single product at pH 10.5 at room temperature. This product was shown to be identical to 1,N^2-ethenodeoxyguanosine. Using THF that had been protected from light, however, the reactions gave three other major adducts, 7, 8, and 9. These adducts were shown to possess UV spectra similar to that of 1,N^2-propanodeoxyguanosine and were not converted to 1,N^2-ethenodeoxyguanosine upon base treatment. These and other results obtained demonstrated that trans-4-hydroxy-2-nonenal readily forms adducts with deoxyguanosine either by direct Michael addition or via its epoxide formation. The facile conversion of some of these adducts to a single adduct suggested that 1,N^2-ethenodeoxyguanosine may provide a simple and useful marker for assessing potential DNA damage by trans-4-hydroxy-2-nonenal and related alkenals associated with lipid peroxidation. This, of course, would require the development of a ^{32}P-postlabeling method using synthetic 1,N^2-ethenodeoxyguanosine 3'-monophosphate or a monoclonal antibody-based immunoassay (37).

Vinyl chloride has been shown to be a human carcinogen by several epidemiological studies. The mechanism for its carcinogenicity, however, has not yet been determined despite intensive studies on this compound. A battery of mutation studies has confirmed that vinyl chloride and its major metabolites, chloroethylene oxide and chloroacetaldehyde, are potent mutagens. DNA adducts that had been previously identified following vinyl chloride exposure in vivo consisted of 1,N^6-ethenoadenine, 3,N^4-ethenocytosine and 7-(2-oxoethyl)guanine. Neither of the etheno derivatives has been shown to be markedly mutagenic in in vitro replication and transcription studies. The primary derivative identified in DNA after inhalation experiments with vinyl chloride, 7-(2-oxoethyl)guanine, has been shown

to be not mutagenic in vitro. Recently, another highly fluorescent derivative, N^2,3-ethenoguanine, was detected in liver DNA of rats given vinyl chloride by inhalation. The formation of N^2,3-ethenoguanine in DNA was unexpected since it, unlike 1,N^2-ethenoguanine, is not formed by the direct chloroacetaldehyde modification of guanine. Because of the strong possibility that N^2,3-ethenoguanine could be mutagenic by hydrogen bonding to thymine, the modified nucleoside was synthesized, converted to the 5'-diphosphate and polymerized with CDP. The deoxypolynucleotide complement, synthesized by AMV reverse transcriptase was shown to contain dC and dT, in addition to dG. dT incorporation, which represents a mutagenic event, occurred with an approximate 20% frequency. On the basis of this mutagenicity, it is proposed that N^2,3-ethenoguanine is involved in the initiation of vinyl halide induced carcinogenesis (230).

Carcinogen-Macromolecular Interactions: The projects in this subject area focus on studies on the identification, quantitation and characterization of carcinogen-nucleic acid adducts. Interest in the identification and characterization of DNA adducts stems from the role that alterations in DNA play in the initiation of carcinogenesis. Most of the carcinogens used in these studies are ones which are metabolized by cellular xenobiotic metabolizing enzymes to a variety of metabolites of which one or a few are reactive and bind to nucleic acids and/or proteins. The identification and quantitation of the binding species are generally determined by chromatographic and radioisotopic techniques. A technique using fluorescence line-narrowing spectrometry, which has a detection level of about five adducts per 10^6 bases, is being developed for the purpose of analyzing complex mixtures of DNA adducts. In experiments to evaluate this technique, intact DNA-polycyclic aromatic hydrocarbon (PAH) and globin-PAH adducts, as well as polar PAH metabolites were examined. A detection limit of about three modified bases in 10^8 for a DNA adduct formed with a diol epoxide of benzo(a)pyrene has been reported. The methodology employed avoids or minimizes spectral degradation and loss of sensitivity due to photooxidation and nonphotochemical hole burning (262). In addition, the development of monoclonal antibodies to various carcinogen-nucleic acid adducts has led to the increasing use of radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) as a sensitive means of adduct detection. For derivatives such as exocyclic adducts, highly sensitive immunoassays need to be developed in order to provide the necessary sensitivity for the detection of those adducts in the DNA of exposed cells. Other methods which are being developed for detecting and analyzing low levels of adducts such as the exocyclic nucleic acid derivatives involves the coupling of systems such as liquid chromatography-mass spectrometry.

In one laboratory, a monoclonal antibody to N-2-acetylaminofluorene (AAF)-modified DNA has been developed from BALB/c mice immunized with the modified DNA complexed with methylated bovine serum albumin. This laboratory had previously developed monoclonal antibodies to DNA modified by benzo(a)pyrene diol epoxide, 1-aminopyrene, and 8-methoxypsoralen plus ultraviolet light A (UVA). One stable hybridoma line was characterized by ELISA and found to produce antibody that recognized AAF-DNA but not unmodified DNA or free AAF. The antibody was shown to cross-react with aminofluorene or AAF-modified deoxyguanosine, but not with 8-methoxypsoralen or benzo(a)pyrene diol epoxide I-modified DNA. However, significant cross-reactivity was detected with 1-aminopyrene, N-methyl-4-aminoazobenzene and 4-aminobiphenyl DNA adducts. In addition, a monoclonal antibody which recognizes the AAF-induced Z-DNA conformation was developed. It has been shown to recognize epitopes unique to AAF induced-Z-DNA. These

antibodies should be useful in studies on adduct formation as well as structural changes induced by carcinogen binding. These antibodies can also be used to monitor humans for exposure to environmental carcinogens (262).

A method known as the ^{32}P-postlabeling technique, which has been developed for the detection of the in vivo formation of carcinogen-DNA adducts where the use of radiolabeled test compounds is not required, has come into increasingly widespread use as the technique has become more refined and standardized. The method involves the reaction of DNA with chemicals in vitro or in vivo and the purification and enzymatic digestion of DNA to deoxyribonucleoside 3'-monophosphates. These are converted to ^{32}P-labeled deoxyribonucleoside 3',5'-bisphosphates after incubation with (gamma-^{32}P) adenosine triphosphate (ATP) and T4 polynucleotide kinase. The ^{32}P-labeled digests are then fingerprinted by using reversed-phase liquid chromatography and anion-exchange thin-layer chromatography on polyethyleneimine-cellulose followed by detection by autoradiography and quantitation by scintillation counting. One refinement to the procedure involves the chromatographic removal of normal DNA nucleotides prior to ^{32}P-labeling. A further minor modification of this procedure has been recently reported which entails the postincubation of DNA digests with Penicillium citrinum nuclease P1 before ^{32}P-labeling. Nuclease P1 was found to cleave deoxyribonucleoside 3'-monophosphates of normal nucleotides to deoxyribonucleosides which do not serve as substrates for polynucleotide kinase, while most adducted nucleotides were found to be totally or partially resistant to the 3'-dephosphorylating action of nuclease P1. These refinements and modifications have enhanced the technique's sensitivity of adduct detection to about 1 adduct in 10^{10} nucleotides for a 10 microgram DNA sample. The new procedure was found to be simple, highly reproducible, and applicable to the detection and measurement of aromatic or bulky non-aromatic DNA adducts formed with many structurally diverse carcinogens. This method has been applied to answer questions about the tissue specificity of carcinogen adduct formation, the degree of persistence of DNA adducts in cells, and to detect adducts in cells of humans exposed to carcinogenic chemicals.

The heterocyclic aromatic carcinogen, 7H-dibenzo(c,g)carbazole (DBC), is formed during the combustion of tobacco products and in the production of carbon black and is found in synfuel products and sediments of industrially polluted rivers. The carcinogenic activity of DBC has been shown to be different from that of other polycyclic aromatics in that it is both a local and systemic carcinogen. When metabolized by 3-methylcholanthrene-induced rat and mouse liver microsomes, several hydroxylated metabolites were shown to be formed. Since the metabolism of DBC in the intact animal is presently unknown, the laboratory of Dr. Randerath has investigated whether some of the metabolites formed in vitro are involved in the in vivo activation of DBC to DNA-binding compounds. The DNA adduction pattern of DBC and the chemically synthesized 2-hydroxy, 3-hydroxy, and 4-hydroxy metabolites of DBC was investigated in liver and skin of female CD-1 mice using the ^{32}P-postlabeling method. The adduct pattern in liver produced by 3-hydroxy-DBC was shown to be qualitatively similar to the DBC adduct pattern. This was not the case for the 2-hydroxy or 4-hydroxy metabolite. In skin, adduct patterns elicited by all the DBC phenols tested were shown to be different from the DBC-induced pattern and were significantly lower quantitatively. A comparison of the skin and liver DBC-DNA adduct patterns after topical application of DBC showed that only one of the four major chromatographically resolved skin adducts corresponded to a major liver adduct, and that total adduction in liver was 13.5-fold higher than in skin. Conclusions drawn from the results presented were the following: 1) activation of DBC to DNA-binding compounds in liver occurs through at least two

pathways with 3-hydroxy-DBC being a proximate carcinogen involved in the formation of most of the adducts; 2) 3-hydroxy-DBC and the other two phenolic metabolites investigated play a minor role in the formation of DBC-DNA adducts in skin; 3) metabolic activation of DBC to DNA-binding compounds in liver and skin appears to follow pathways that are different in terms of both the chemical nature and the amount of the adducts formed; and 4) DBC and 3-hydroxy-DBC exhibit a strong preference for liver versus skin DNA. In a subsequent study it was shown that the N-methylation of DBC reduces the DNA-binding activity of DBC about 300-fold in liver but only about twofold in skin. Topically applied N-methyl-DBC was shown to bind preferentially to skin versus liver DNA by a factor of 10, while the opposite was true for DBC. This is in agreement with the carcinogenicity reported for DBC and N-methyl-DBC and supports the hypothesis that the extent of covalent DNA modification by these compounds is associated with their biological activity. It is concluded from these results that an unsubstituted nitrogen is essential for the genotoxic activity of DBC in liver but not in skin. The results also demonstrate the potential of the ^{32}P-postlabeling assay in predicting the organotropism of closely related carcinogens (201).

Estrogens have been shown to induce tumors in laboratory animals and are increasingly associated with genital tract malignancies in humans. The mechanism of carcinogenesis is currently unknown. Evidence is accumulating from several laboratories that estrogen-induced neoplastic cell transformation or carcinogenic activity cannot be correlated with hormonal potencies of the substances tested. As a result, it has been postulated that estrogen metabolism plays a key role in hormonal carcinogenesis. Several enzyme systems have been shown to oxidize or co-oxidize estrogens and are thus associated with hormonal carcinogenesis. At present, though, it is not clear which of these enzyme systems is involved in the induction of hamster renal carcinoma by estrogen, which is known to arise in the cortex at the cortico-medullary junction. The role of metabolism in the formation of estrogen-induced covalent DNA adducts in the hamster kidney has also not been elucidated. Estrogens were previously shown to induce covalently modified nucleotides of unknown structure which did not contain the estrogen moiety specifically in the target organ. From these results, the role of metabolism in estrogen-induced DNA damage and subsequent tumor formation is thought to have unique features, since the activation of unknown endogenous genotoxic factors is elicited by the estrogens. In order to ascertain a role of metabolism and adduct formation in estrogen-induced carcinogenesis, the intrarenal distribution of estrogen-induced DNA modification, as measured by ^{32}P-postlabeling analysis, and the estrogen metabolizing cytochrome P-450 and prostaglandin endoperoxide synthase enzymes were investigated in male Syrian hamsters. The results obtained show that microsomal cytochrome P-450 levels and estrogen-induced DNA adduct formation were highest in hamster kidney cortex, the origin of renal tumorigenesis. From these data it is postulated that estrogen metabolism by cytochrome P-450 enzymes leading to covalent DNA modification plays a role in hormonal carcinogenesis in the hamster kidney (150, 201).

The levels and persistence of specific DNA adducts are often related to the organ specificity of the carcinogen and may indicate which of the adducts are biologically relevant. For many carcinogens, such as the polycyclic aromatic hydrocarbons, alkyl nitrosamines, N-2-acetylaminofluorene, and aflatoxin B_1, the reactive metabolites and the identity of the various nucleoside adducts are known. The chemical nature and physical conformation of the adducts is thought to determine the biological effect of the adduct. For this reason, several investigators are focusing on the chemical and biophysical characterization of carcinogen-DNA

adducts and on the resultant conformational changes the adducts may introduce into the DNA molecule. In many of the studies, defined polydeoxynucleotide sequences containing a modified base are synthesized for analysis. Several different techniques have been utilized for the characterization of carcinogen-nucleic acid adducts. These include high pressure liquid chromatography, absorption and fluorescence spectroscopy, nuclear magnetic resonance, optically detected magnetic resonance, linear and circular dichroism spectroscopy and x-ray crystallography. Another determinant of the biological effect of carcinogen DNA adducts is their potential site or sequence-specific interaction on the DNA molecule. The examination of this possibility for aromatic amine, polycyclic aromatic hydrocarbon and metal carcinogens is the focus of several studies. The development and use of sophisticated molecular biological techniques to analyze site-specific interactions of carcinogens has made this a growing area of interest. In addition, computer analysis of possible carcinogen-DNA adduct conformations in a defined DNA sequence has allowed the building of molecular models for the most likely conformations. The results of these studies give information as to the possible mechanisms by which a carcinogen may cause a mutation or other alteration in the DNA structure.

A critical event in the initiation stage of chemical carcinogenesis is the covalent binding of chemicals to DNA that affect its structure and function. Since it has been shown that DNA can adopt a variety of conformations depending on its sequence and physicochemical environment, it is important to determine how the binding of some carcinogens to DNA may affect its conformation and subsequently its function. In one such study, poly(dG-dC) was modified to different extents by the carcinogens, 4-aminobiphenyl (ABP) and N-methyl-4-aminoazobenzene (MAB). More than 90% of ABP and 81% of MAB modification was shown to occur at the C8 position of guanine by high performance liquid chromatography (HPLC) analysis of the enzymatically hydrolyzed modified polymers. Circular dichroism (CD) was used to study the conformational changes of the unmodified and modified polymers as a function of ethanol and magnesium ion concentrations. The modified polymers were shown to have a CD inversion pattern similar to that of the salt-induced B to Z transition of poly(dG-dC). The data obtained demonstrate that ABP and MAB can enhance the conversion from B to Z conformation in alternating purine-pyrimidine sequences. ABP has been shown to be better than MAB at inducing the B to Z transition in poly(dG-dC). This conversion to Z-DNA might result in the inhibition of DNA adduct repair, since ABP-C8-guanine adducts have been shown by others to be more persistent and less readily repaired in vivo than the MAB-C8-guanine adducts (262).

Changes in Cellular Macromolecules and in Cell Functions: The types of research activities in this subject area include studies on alterations in the composition and amounts of various proteins and small molecules, and changes in the pattern of DNA methylation in cells induced by carcinogens to the preneoplastic or neoplastic state. Biochemical and immunochemical methods have been used to isolate, identify and characterize nonhistone chromosomal proteins, phosphoproteins, and cytosolic proteins which are either altered or specifically appear in chemically induced hepatocarcinogenesis models. Neoplastic cells have been shown to manifest a variety of morphological and biochemical phenotypes, different from their normal cell counterparts. This generation of heterogeneity and phenotypic instability in cancer is presumed to be due, in part, to changes in the control of gene expression during the transformation of normal cells to neoplastic cells. One possible manner in which the derepression and repression of genes could occur is by alterations in nuclear DNA-nuclear protein complexes. There is also much evidence

showing that the state of DNA methylation regulates gene expression and is also involved in the control of cell differentiation. Thus, a greater understanding of the effects of chemical carcinogens and other oncogenic agents on production of aberrant DNA methylation patterns during carcinogenesis is warranted. Several studies are being supported which seek to define the role of altered chromosomal protein-DNA complexes in carcinogenesis and to understand the role of DNA methylation in the control of gene expression and carcinogenesis. Some of the latter studies are focused on elucidating the properties and regulation of DNA methyltransferase, the enzyme responsible for the postreplication methylation of cytosine residues in DNA. Other studies are focused on the state of methylation of specific DNA sequences or genes as a result of carcinogen exposure. The biological effects of DNA hypomethylation, i.e., altered cell differentiation or induction of cell transformation, are being studied by using compounds such as 5-azacytidine, which are known to affect the transfer of methyl groups to DNA.

Approximately 3 to 6% of cytosine residues in the DNA of all vertebrates is modified to 5-methylcytosine, which is predominately found in the dinucleoside sequence 5-CpG. Methylation patterns appear to be tissue-specific and the hypomethylation of many genes is correlated with their active expression. For example, the inhibition of genomic methylation by 5-azacytidine can result in the reactivation of genes on the transcriptionally inactive X chromosome, the induction of tissue-specific gene expression and the expression of differentiated phenotypes in cultured cells. Hypermethylation of gene sequences has been shown to preclude gene expression. Despite such correlations, the relationship between methylation patterns and expression of some genes remains unclear and it is not known how changes in the methylation patterns of certain genes alter expression, while the activities of other genes remain unaffected. Some studies have provided evidence that cytosine methylation may be involved in the structural ordering of chromatin. There are observations which suggest that positioning of structural nuclear proteins may be involved in transducing the methylation signal. One remaining question is whether methylation also alters the direct binding of specific transcription factors to defined promoter elements. The transcription factor, Sp1, and the human metallothionein IIA promoter region were selected for one study. Sp1 was shown to bind to a decanucleotide consensus sequence designated the "GC box." GC box sequences have been identified in the 5' region of several eukaryotic genes, including the human metallothionein gene. This gene was chosen as a substrate for Sp1 binding because the promoter region contains at least five distinct control elements in the 5' flanking region. Synthetic oligonucleotides were used to investigate the effect of cytosine methylation on the binding of Sp1 to its target GC box. It was shown that the presence of 5-methylcytosine in the CpG sequence of the GC box did not influence Sp1 binding. This result was confirmed using double-stranded 20-mers containing 16 base pairs of a complementary sequence. The formation of DNA-protein complexes was demonstrated using electrophoretic gel retardation analysis. The formation of these complexes was not inhibited when an oligomer without a GC box was used as a competitor. The results obtained therefore preclude a direct effect of cytosine methylation on Sp1-DNA interactions. Proteins have been identified that preferentially bind to methylated sequences. Thus, while the binding and functional activity of positive-acting transcription factors, such as Sp1, may be indifferent to changes in methylation patterns, the presence of proteins that preferentially bind to methylated sequences may prevent binding of the specific regulatory molecules. Further in vivo studies employing methylated GC-box constructs will be necessary to determine whether the functional activity of Sp1 is retained, whether this

change is sufficient to direct the transfected DNA into an inactive conformation and whether potential protein-protein interactions with other required trans-acting factors are affected (122).

Mounting experimental evidence has indicated that nickel and chromium compounds are human carcinogens. The carcinogenic potency of nickel compounds differs in that the crystalline nickel sulfide compounds exhibit the most potent activity. Hexavalent chromium, which exists as an oxyanion at physiological pH is suspected as being the more potent carcinogen compared with the trivalent form of chromium. The carcinogenic compounds of chromium and nickel have been shown to induce transformation in cell cultures, induce chromosomal aberrations and increase the incidence of sister chromatid exchanges. The DNA lesions induced by carcinogenic chromium and nickel compounds have been shown to include single strand breaks and DNA-protein cross-links. In studies on the localization of chromosome damage, nickel ions were shown to preferentially damage heterochromatic regions of chromosomes and to frequently induce specific decondensation/fragmentation of the heterochromatic long arm of the X chromosome in Chinese hamster ovary cells. On the other hand, chromium compounds did not selectively damage heterochromatin. Recently, magnesium was shown to be a potent inhibitor of nickel-induced carcinogenesis in vivo. It was thought that this divalent cation should also reduce the DNA damage induced by nickel. To test this hypothesis the effect of varying the extracellular levels of magnesium ions on nickel- and chromate-induced DNA damage and cell transformation were examined. DNA damage was assessed by alkaline elution analysis and by examining the incidence of chromosome damage and sister chromatid exchanges. The results obtained show that magnesium protects against nickel-induced but not chromate-induced DNA damage. The elevation of extracellular magnesium levels prevented the effects of nickel on heterochromatin and inhibited cell transformation, but did not substantially reduce the DNA damage induced by nickel in euchromatic regions. These results suggest that heterochromatic DNA damage may be important to the nickel-induced neoplastic transformation process (44).

Poly(ADP-ribose) is synthesized in the nucleus of eukaryotic cells from nicotinamide adenine dinucleotide. Although the function of poly(ADP-ribose) metabolism is poorly understood, inhibitors of poly(ADP-ribose) synthetase have been shown to alter many chromatin-associated processes including sister chromatid exchanges, DNA repair replication, de novo DNA replication, cellular recovery from DNA damage and cellular differentiation. A wide spectrum of DNA damage results in a rapid alteration of poly(ADP-ribose) metabolism due to the activation of poly(ADP-ribose) polymerase by DNA strand breaks. The distribution of endogenous ADP-ribose polymers in the nucleus or within different fractions of chromatin had not previously been studied. By use of a procedure that allows for the radiolabeling and detection of endogenous polymers of ADP-ribose, the nuclear distribution of these polymers in untreated cells and in cells subjected to hyperthermia, N-methyl-N'-nitro-N-nitrosoguanidine, or both were analyzed. The data obtained following nuclease digestion and salt extraction of isolated nuclei suggested that the nuclear matrix may be a major site of poly(ADP-ribose) metabolism. In view of the short-lived nature of polymers of ADP-ribose, the working model generated is that the coordinated synthesis and turnover of these polymers mediates topological changes unique to matrix-associated chromatin (118).

The exposure of cells to carcinogens directly affects DNA replication, RNA transcription and RNA transport from the nucleus to the cytoplasm. Several investigators are studying the mechanism of DNA replication following carcinogen-induced

DNA damage. Other studies are focused on the characterization of the effects of carcinogen-modified DNA on RNA transcription and the mechanism of altered gene transcription and translation. A possible effect of carcinogen exposure is to alter the fidelity of DNA replication. The identification of cellular factors which control the fidelity of DNA synthesis, such as altered DNA polymerases, is being explored, as well as the relationship between tumor progression and the fidelity of DNA replication.

It is generally accepted that mutagens which react with DNA to produce "bulky"-type lesions result in structures in the DNA template which act as blocks to DNA synthesis. This has been demonstrated for lesions such as thymine dimers and benzo(a)pyrene adducts. Synthesis on templates containing such lesions has been shown to terminate at the lesion site or one or two nucleotides before the adduct, and to rarely take place past the adduct. However, there is some evidence that one of the bulky-type adducts produced upon exposure to N-2-acetylaminofluorene (AAF) may be an exception to this situation. The major adducts produced by exposure to AAF are both bound through the arylamine of the fluorene ring to the C8 position of guanine, but differ by the presence (AAF adduct) or absence (AF adduct) of a nitrogen-linked acetyl group. Although both of these adducts have been reported to block DNA synthesis in vitro, the extent of translesion synthesis has only been measured for the AAF adduct. Structural analyses have shown that AF adducts cause less DNA distortion; thus, it was thought that translesion synthesis could occur on templates containing this adduct. To study this possibility a site-specific modified DNA was constructed that contained a single AF lesion. DNA synthesis was measured on this template using purified E. coli DNA polymerase I (large fragment) or T7 DNA polymerase. The results, whether measured by radiolabel incorporation or by polyacrylamide gel analysis, demonstrated that the majority of the DNA synthesized by either enzyme bypassed the AF lesion. The substitution of Mn^{2+} for Mg^{2+} as the divalent cation resulted in even higher levels of translesion synthesis. Further analysis by cloning and DNA sequencing procedures has shown that the large majority of synthesis past this adduct is accurate (210).

N-nitroso alkylating agents have been shown to be potent mutagens and carcinogens that mediate at least some of their biologic effects by covalent modification of DNA. The mutagenicity of these agents is thought to be due largely to the formation of O^6-alkyl-deoxyguanosine (dG) adducts which are effective miscoding lesions. Several recent studies, however, also implicate O^4-alkyl-deoxythymidine (dT) residues as causative lesions in mutagenesis as well as in carcinogenesis. O^2-alkyl-dT adducts have been implicated as weak mutagenic lesions. Molecular structure studies of O^4-alkyl-dTs suggest that these adducts will pair with dA as well as dG residues in DNA. One direct approach to the evaluation of the mutagenic effect of alkylnucleotides in vivo is to assay mutations in extra-chromosomal DNA containing single, structurally defined adducts. To this end, an enzymatic procedure was developed for the site-specific incorporation of purified alkyl-deoxynucleoside triphosphates (dNTPs) into biologically active DNA. Coupled with a reversion assay for mutagenesis, this procedure permits a highly sensitive evaluation of the mutagenicity of alkyl-dT adducts during DNA replication in E. coli. By use of this system, the relative mutagenicities of O^4-methyl-, O^4-ethyl-, O^4-isopropyl-, and O^2-methyl-dTs site-specifically incorporated into phiX174 am3 DNA was compared. DNAs containing O^4-methyl- and O^4-ethyl-dT lesions were shown to be the most mutagenic and to contain dG residues opposite the original site of the alkyl-dTs. The data obtained are consistent with a mechanism of mutagenesis involving the formation of O^4-alkyl-dT·dG base pairs during DNA

replication in E. coli and suggest that the formation of A·T to G·C transition mutations is characteristic of mutagenesis by O^4-methyl- and O^4-ethyl-dTs in vivo (153, 230).

Measurement of spontaneous mutation frequencies in animal cells suggests that the average frequency of errors in DNA replication is in the range of 10^{-9} to 10^{-11}/ base pairs replicated. This high fidelity is thought to be achieved via a multistep process involving nucleotide selection as well as error correction. DNA polymerase-alpha is thought to play the major role in this process and thus to be an important determinant of the overall accuracy of DNA replication. Several laboratories have reported, however, that the error rate of DNA polymerase-alpha purified from eukaryotic cells is about 1 in 30,000 nucleotides incorporated when assayed on a natural DNA template. The difference of 10^{-5} to 10^{-7} in fidelity from in vivo to in vitro synthesis suggests that methods traditionally employed to purify these enzymes may remove subunits or cofactors which facilitate accurate DNA replication in vivo. The introduction of immunoaffinity chromatography has allowed the rapid purification of high molecular weight multiprotein DNA polymerase-alpha complexes from a variety of sources. By use of such techniques, DNA polymerase-alpha was isolated from calf thymus, Chinese hamster V-79 cells and a human lymphoblast TK-6 cell line. These DNA polymerase-primase complexes were shown to be 12- to 20-fold more accurate in DNA synthesis than conventionally purified calf thymus DNA polymerase-alpha with estimated error rates of 1 in 460,000 to 1 in 830,000. The polymerase-primase complex from calf thymus was shown to have no detectable 3' to 5' exonuclease activity. The high fidelity of the immunoaffinity-purified complexes in the absence of a proofreading exonuclease suggests that the processes of base selection and incorporation are more efficient in these eukaryotic enzymes than in their prokaryotic counterparts. The high fidelity of the complex, however, is still several orders of magnitude lower than suggested by spontaneous mutation rates in vivo, which suggests that a proofreading and/or mismatch repair system may be operative in vivo (153).

Proliferation of cells after exposure to chemical carcinogens is important to the initiation of malignant transformation. This has been shown for carcinogenesis in vivo and for the transformation of cells in culture. The maximal sensitivity to transformation of mouse fibroblasts was observed when cells were exposed to the carcinogenic agent during the early portion of the S phase. In order to investigate this sensitivity, the effect of exposure to benzo(a)pyrene diol epoxide I (BPDE-I) on C3H10T1/2 cells actively synthesizing DNA was studied. BPDE-I has been shown to block DNA synthesis in in vitro systems. A direct analysis of the effect of BPDE-I adducts on DNA replication was used in one study. The approach taken uses antibodies with high affinity for BPDE-I adducts attached to the exocyclic nitrogen of dG in DNA and uses electron microscopy to visualize and quantitate antibodies bound to BPDE-I DNA adducts. The frequency of DNA replication forks containing carcinogen adducts was found to be eightfold higher than expected for an average distribution. The proportion of replication forks that were apparently blocked at the site of the DNA damage was shown to increase when replication was allowed to occur after carcinogen exposure. These results support the conclusions that the fork junction is particularly vulnerable to adduction by BPDE-I and that BPDE-I adducts block the displacement of replication forks during DNA synthesis in intact cells (124).

Markers and Properties of Transformed Cells: Research included in this subject area involves studies on the documentation of various growth and functional properties of initiated cells, preneoplastic cells and fully transformed cells,

and the identification of biochemical and molecular markers for distinguishing these altered cell types from normal cells. The evidence obtained to date strengthens the supposition that the development of most cancers involves a multi-step process in which cells progress from normal to initiated, preneoplastic, and premalignant stages to the end point of malignant neoplasia. In order to characterize cells at each stage, a detailed analysis and knowledge of the sequence of relevant biochemical and biological alterations associated with the development of chemically induced carcinogenesis is needed. To achieve this purpose, a variety of model systems, both in vivo in animals and in cells in culture, are being used. Of the animal model systems, a predominant one currently in use is the rat chemically induced hepatocarcinogenesis model. Although this model was established some time ago, the treatment regimens being employed have undergone a variety of changes depending on the purpose of the experiment and on the end point desired. Chronic or intermittent exposure regimens have been used, along with initiation-promotion type regimens in which various initiating carcinogens and promoting stimuli are used. The sequential appearance of foci of altered hepatocytes, nodules, and hepatocellular carcinomas can be observed and analyzed. In addition, cell lines can be derived from either liver tumors or from normal tissues for further study. These cells and cell lines which have been treated with a carcinogen in vitro have been tested for the expression of various phenotypic markers which may be correlated with tumorigenicity.

It has been previously reported that epidermal growth factor (EGF) can induce or enhance the colony-forming ability in soft agar of chemically treated or transformed rat liver epithelial cells. The emergence of EGF-induced anchorage-independence was shown to be the earliest and possibly the most reliable phenotype to indicate the imminent tumorigenic transformation of cultured rat liver epithelial cells. In contrast, transforming growth factor-beta (TGF-beta) has been shown to inhibit DNA synthesis and proliferation of normal cultured rat liver epithelial cells, but not chemically transformed cells. The differential sensitivity of normal versus malignant cells to the inhibitory effect of TGF-beta has been proposed to be one of the mechanisms by which TGF-beta exerts a role in tumorigenesis. In one study the production of TGFs by normal rat liver epithelial cells lines, by clones of chemically transformed rat liver epithelial cells and by cell lines derived from tumors produced by the transformed epithelial cells was analyzed. Also, the roles of these growth factors in the autocrine regulation of the growth of these cells was examined. The normal cultured rat liver epithelial cell line, WB-F344, was shown to not secrete an EGF-like (putatively TGF-alpha) activity, but several clonal cell strains derived from WB-F344 cells which had been treated with N-methyl-N'-nitro-N-nitrosoguanidine were shown to secrete TGF-alpha-like activity into their conditioned medium. This is especially true for those cells that expressed high levels of gamma glutamyl transpeptidase (GGT) activity. Cell lines obtained from tumors which were produced by these cell strains were shown to vary in their abilities to secrete TGF-alpha, even though all expressed high levels of GGT activity. In contrast to TGF-alpha-like activity, all cell lines/strains tested released TGF-beta activity into their conditioned media. The tumor cell lines produced activated TGF-beta de novo, while both normal or chemically transformed cell strains typically produced the inactive form. TGF-beta was shown to either stimulate, inhibit or to not affect the anchorage-independent growth of cell lines that produced active TGF-beta. Cell lines that were inhibited by added TGF-beta were shown to concurrently produce TGF-alpha which was usually able to overcome the negative autocrine effect of TGF-beta. It was thus concluded that both TGF-alpha and TGF-beta, singly or in combination, are variously involved in the growth of transformed rat liver

epithelial cells. TGF-alpha was shown to have a predominantly positive autocrine action on the growth of rat liver epithelial tumor cell lines. The results also suggest that the paracrine effect of TGF-beta may be at least as important as its autocrine effect in the growth of these transformed epithelial cell lines (91).

There are other interesting model systems which are being established and analyzed by one or more laboratories. For example, one interesting experimental system involves the establishment and sequential analysis of stages of oral carcinogenesis using hamster buccal pouch epithelium. The buccal pouch consists of a flat epithelium which has no glandular elements and normally lacks histochemical evidence of gamma glutamyltranspeptidase (GGT) activity. Whole mounts of this epithelium can be prepared for analysis. Also, with this system it appears that it will be possible to relate the cells displaying altered growth in vitro to populations of presumptive initiation sites in vivo. This is not possible with other existing models. In an earlier study it was shown that the topical application of chemical carcinogens to Syrian hamster buccal pouch epithelium was associated with the development in culture of a population of morphologically altered keratinocytes. The development of these cells, termed type II keratinocytes, in vivo was shown to precede, by several weeks, the formation of grossly detectable squamous epithelial neoplasms. In an attempt to elucidate further the significance of these type II keratinocytes in the process of hamster buccal pouch carcinogenesis, whole cell dissociates of hamster buccal pouch epithelium initiated in vivo with 7,12-dimethylbenz(a)anthracene (DMBA), as well as populations of type II keratinocytes were assayed for the expression of the angiogenic phenotype. It is well established that this property is acquired early during the progression of normal cells to the neoplastic state. It was observed that single-cell suspensions of DMBA-initiated hamster buccal pouch epithelium are angiogenic in the rat corneal bioassay of neovascularization and that the potency of the neovascular response parallels the frequency of type II colonies observed in culture. These results demonstrate that angiogenic activity is an early manifestation of hamster pouch carcinogenesis and suggests that type II keratinocytes, presumptive preneoplastic cells in this model, are the principal source of this activity (237).

Research relevant to respiratory carcinogenesis is being conducted using a rat tracheal implant system. The properties of carcinogen-initiated cells can be studied in short-term organ culture where normal tissue interactions can be preserved. The cells can also be studied while growing in cell culture and also in vivo by allowing the cells to repopulate denuded trachea which are implanted into nude mice. Recently, it has been shown that normal and carcinogen-altered rat tracheal epithelial cells have markedly different in vitro growth requirements for pyruvate as a supplement to tissue culture medium. While normal tracheal cells stringently require exogenous pyruvate for survival and growth, the altered tracheal cells do not. This growth advantage of altered cells can be used to quantitatively select altered cell populations from carcinogen-exposed tracheal implants. Other studies show that the growth advantage appears to be a property acquired by transformed cells early in neoplastic progression and could be due to a metabolic change which is fundamental to the development of neoplasia in these cells. Recently, a study was completed in which the progression of neoplasia in cell populations isolated from tracheal implants at increasing lengths of time after exposure to different doses of DMBA was tracked. Both classical morphological markers and changes in the in vitro growth behavior of carcinogen-altered cell populations isolated from the same tracheas were followed. This allowed direct correlations between in vivo and in vitro markers of cancer progression to be made. The results showed that progressive changes take place in the

carcinogen-exposed tracheal epithelium in vivo that can be detected subsequently as quantitative increases in growth parameters in vitro. Another aim of the studies was to determine whether cytochalasin B-induced multinucleation occurs in the tracheal cell lines and whether there is any correlation with anchorage-independent growth and/or tumorigenicity. The results obtained indicate that anchorage independence precedes the other markers of growth autonomy and that uncontrolled nuclear division appears as a separate property after anchorage independence and before tumorigenicity. The results also showed that tumorigenicity appears preferentially in the cell populations that exhibit anchorage independence and uncontrolled nuclear division and that progression in growth autonomy occurs in the tracheal implants in vivo which can be detected in vitro as an increase in cell lines positive for the three phenotypic markers (164).

Properties of normal and carcinogen-treated human respiratory epithelium can also be studied by using denuded rat tracheal implants in nude mice. This in vivo culture system permits carcinogen-induced changes in normal tracheobronchial epithelium to be studied. In one study using human tracheobronchial cells obtained from intermediate autopsies, the growth of thin stratified epithelium with keratinization, but without atypia, was observed. Exposure of this epithelium to DMBA resulted in the observation of a thick epidermoid metaplasia with mild to moderate atypia. In a further study the repopulation and growth of human lung-derived cell lines was examined with the objective of detecting tumor latency periods between cells inoculated s.c. and intratracheally and assessing the possible advantage of intratracheal innoculation for the rapid detection of initial preneoplastic and neoplastic growth. The results showed that the intratracheal inoculation and xenotransplantation of human-derived cell lines offers a time-saving alternative to the s.c. inoculation assay for tumorigenicity. It was also shown that this could be a valuable approach to studying preneoplastic and neoplastic progression with human cell subpopulations. These types of studies are ongoing and represent exciting new approaches to studying respiratory neoplasia and human respiratory neoplasia in particular. It should allow us to better extrapolate animal carcinogenesis results to their human counterpart. Research using other animal model systems, i.e., breast, colon, pancreas, bladder, and prostate, is also being conducted. These studies augment similar studies supported by the Organ Systems Program which will now be integrated with those currently being supported in our program.

In addition to the utilization of animal systems, the in vitro transformation of cells in culture occupies the focus of several other research groups. The use of cell cultures which are derived from in vivo carcinogenic lesions allows investigators to analyze more easily the properties of the cells in question. The ability to transform cells in culture allows for the study of mechanistic questions regarding chemically induced transformation. For some of this research, standard rodent fibroblast or epithelial cell lines have been used. With the increasing success in transforming human fibroblast and epithelial cells following the pioneering work of Kakunaga, Milo, and DiPaolo, several groups of investigators are increasingly turning to the use of human cell cultures in their research. This focus on the use of cultured human cells will continue to be vigorously supported by this program.

A significant proportion of human tumors from various organ sites have been shown to contain activated oncogenes from the ras family. This finding has prompted investigations into the role ras oncogenes play in bringing about transformation

of cells in culture. In several of the studies the level of expression of the ras oncogene is shown to be an important factor governing the degree of transformation by ras oncogenes and that high expression of the transfected ras oncogene is required for the induction of the tumorigenic phenotype in such fibroblasts. Diploid human fibroblasts, however, have proven to be more resistant to transformation by ras oncogenes. One explanation for the negative results seen so far with human fibroblasts is that the transfected ras oncogene was not expressed or that the level of expression of the ras oncogene was not high enough to cause measurable effects. In an attempt to determine how normal human fibroblasts respond to high expression of the T24 H-ras oncogene, such cells were transfected with the plasmid vector, pHO6T1, containing the T24 H-ras oncogene with 5' and 3' enhancer sequences, and the aminoglycoside phosphotransferase gene which confers resistance to the drug, G418. Approximately 1.5 percent of the G418-resistant colonies obtained after transfection and selection were shown to consist of cells exhibiting obvious morphological transformation. These morphologically transformed cells exhibited anchorage independence at a frequency at least 60 times higher than that of cells that had been transfected with the control plasmid containing the normal cellular H-ras gene. Also, transformation was shown to be correlated with the expression of the T24 H-ras oncogene protein product p21 in the human fibroblasts. Further studies to determine if diploid human fibroblasts that continue to express the T24 H-ras oncogene are tumorigenic and whether this gene cooperates with other oncogenes in causing malignant transformation of human fibroblasts are in progress (169).

Upon transformation by chemicals, most cells acquire altered growth properties which allow them to proliferate under selective growth conditions. This can involve the ability to grow in soft agar (anchorage-independent growth), the loss of contact inhibition of growth, or the ability to grow in medium containing low calcium or other nutrients. Several biochemical and molecular markers have been used to identify transformed, preneoplastic and neoplastic cells. The histochemical expression of GGT activity and the loss of histochemically determined glucose-6-phosphatase (G-6-Pase) and adenosinetriphosphatase (ATPase) activity are common markers used to identify carcinogen-altered liver cells and other epithelial cells. Other enzyme markers, such as the presence of epoxide hydrolase, alkaline phosphatase isozymes, aldehyde dehydrogenase isozymes, and more recently, the placental form of glutathione S-transferase (PGST), are being evaluated. Reduced levels of enzymes of oxidative xenobiotic metabolism have been shown to occur in many preneoplastic lesions during the stage of promotion, but this pattern is not ubiquitous. The results generated to date supports the theses that no single biologic marker or set of markers is uniquely characteristic of the preneoplastic and/or neoplastic phenotype and that marker or phenotypic heterogeneity is by far the rule rather than the exception in hepatocarcinogenesis in the rodent and quite possibly in all histogenetic types of neoplasms in mammals. Functional markers for liver cells being utilized currently include the production of albumin, alpha-fetoprotein, transferrin, and fibrinogen. An increasing need is being seen for the development of genetic markers of neoplasia. The development of chromosomal abnormalities and aneuploidy in transformed cells are now being evaluated.

Many models of multistage hepatocarcinogenesis in the rat involve the quantitation of altered hepatic foci for the characterization of effects of putative initiating and promoting agents. In view of the different distribution of phenotypes in the Solt-Farber, Peraino and Pitot models of multistage hepatocarcinogenesis, the efficiency of PGST as a marker of altered hepatic foci was studied in two of these

models. Quantitative stereologic methods were applied for the numerical quantitation of altered hepatic foci in stained liver sections from rats treated with various tumor-promoting agents in the Pitot model of multistage hepatocarcinogenesis. These agents included phenobarbital, WY 14,643 (a peroxisome proliferator) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The value of PGST as a marker, along with GGT, G-6-Pase and ATPase, was also assessed in the Solt-Farber model. PGST was shown to score more foci in all groups than GGT and ATPase. Greater focal volume was marked by PGST than with GGT or ATPase; and PGST marked focal volume equal to or greater than G-6-Pase in rats treated with phenobarbital, TCDD or the Solt-Farber protocol. After treatment with WY 14,643, however, GGT and PGST were shown to mark less focal volume than ATPase or G-6-Pase, and PGST scored fewer foci than G-6-Pase. Numerical estimations of foci scored by those markers on the basis of area of the entire tissue section were shown to be relatively different from those values determined by quantitative stereology. The results obtained demonstrate the importance of quantitative stereologic analysis of altered hepatic foci during multistage hepatocarcinogenesis and also shows that PGST is a useful marker in the quantitation of promotion (174).

Previous work has shown that a tumor-associated aldehyde dehydrogenase (tumor ALDH) can be induced by a number of different chemical carcinogens during rat hepatocarcinogenesis. This isozyme has been shown to preferentially oxidize aromatic aldehyde substrates using nicotinamide-adenine dinucleotide phosphate ($NADP^+$) as a coenzyme and differs from normal liver ALDH isozymes in a number of physical and functional properties. The appearance of the tumor ALDH isozyme during hepatocarcinogenesis has been previously shown not to be due to the derepression of a fetal ALDH gene. This gene product is also not found in regenerating liver after partial hepatectomy. In order to study the regulation of tumor ALDH gene expression, the cloning of the tumor ALDH gene was undertaken using the well-established rat hepatoma cell line, HTC, as a source of mRNA. The identification and complete nucleotide sequence of a cDNA encoding the tumor ALDH monomer has been described. This immunologically detected cDNA clone has been shown to direct the synthesis of very high levels of functional tumor ALDH and will be useful in studying the expression and the regulation of the tumor ALDH isozyme during rat hepatocarcinogenesis (152).

In the laboratory of one of the MERIT awardees, models of colon carcinoma utilizing procarcinogens and carcinogens are being studied in order to identify changes in lipid biochemistry which might be identifiable at a stage prior to malignant transformation. A primary model system being used is the dimethylhydrazine (DMH)-induced colon carcinoma model in which the neoplasms induced have been found to closely parallel human colon cancer with respect to clinical and pathological features. Considerable attention has been focused on membrane lipid fluidity and its relationship to the malignant transformation process. Alterations in the fluidity of cancer cells have been reported by several laboratories. Prior studies have suggested a direct correlation between lipid fluidity and the sodium-hydrogen ion (Na^+-H^+) exchange process in rat colonic brush-border membrane vesicles. The maintenance of normal electrolyte and water balance is an important function of the large intestine. Since dexamethasone had previously been shown to influence membrane lipid fluidity in rat small intestinal and hepatic membranes, the effect of this steroid on both the Na^+-H^+ exchange process and lipid fluidity of rat colonic brush-border membrane vesicles was examined. The results obtained demonstrated that the Na^+-H^+ exchange activity and the lipid fluidity of rat distal colonic brush-border membrane vesicles was increased by dexamethasone. The increase in lipid fluidity was shown to be due, at least in part, to a decrease in

the cholesterol/phospholipid content in membranes prepared from dexamethasone-treated rats. Since various cations, especially protons, have been reported to be involved in the mitogenic response elicited by growth factors, it was also of interest to determine the involvement of Na^+-H^+ exchange in the malignant transformation process. As a control, the effect of 1,2-dimethylhydrazine treatment on Na^+-H^+ exchange activity in renal cortex brush-border membrane vesicles was examined, since 1,2-dimethylhydrazine is not known to cause kidney tumors. 1,2-Dimethylhydrazine treatment was shown to result in an increase in the proton-stimulated sodium influx across treated-membrane vesicles without significantly influencing Na^+ permeability or proton conductance. The results also showed that the kinetic parameters of Na^+-H^+ exchange in renal cortex brush-border membrane vesicles was not significantly affected. This data suggested that alterations in Na^+-H^+ exchange in rat colonic brush-border membranes may be involved in the malignant transformation process induced by this carcinogen in the large intestine (19).

Genetics and Mechanisms of Cell Transformation: In the subject area of genetics and mechanisms of cell transformation are studies designed to test the somatic cell mutation hypothesis of cell transformation and to attempt to identify those specific genes which are responsible or have an influence on cell transformation. There is a large body of data demonstrating a high correlation between the mutagenicity and carcinogenicity of various chemicals. This evidence supports the hypothesis that somatic mutations are involved in the process leading to neoplasia. Also, efforts are being made to develop animal models for hereditary cancer which would allow the examination of genetic mechanisms of carcinogenesis. A rat strain which develops hereditary renal carcinoma is being studied in one laboratory. This may yield a model analogous to human retinoblastoma and Wilms' tumor. In another laboratory, a mouse model for susceptibility to hepatocarcinogenesis is being developed. It has been shown that C3H/HeJ male mice are 20- to 50-fold more susceptible to the induction of liver tumors than are male C57BL/6J mice and that about 85% of this difference in susceptibility to liver tumor induction results from an allelic difference at a single locus. Further studies are planned to characterize the model and the susceptibility gene.

While activated nuclear oncogenes are found in many carcinogen-induced tumors, there is considerable uncertainty as to whether the tumorigenic state can be attributed solely to oncogene activation. A mitochondrial role in chemical carcinogenesis has been proposed because many diverse lipophilic carcinogens have been shown to modify mitochondrial DNA (where they do not appear to be as readily repaired) more effectively than nuclear DNA in the same cell and to induce mutations in the mitochondrial genome. Mitochondria of tumor cells have been reported to be different in functional and structural aspects from those of normal cells. There is also evidence that mitochondrial genomes which have undergone a mutagenic event may be involved in the process of carcinogenesis. It is suggested that mutated mitochondria provide the cell with a growth advantage. The techniques of somatic cell genetics have provided a tool for investigating whether extrachromosomal elements, such as the mitochondrial genomes, play a role in the activation and/or suppression of the phenotypic expression of tumorigenicity by allowing the transmission of these mitochondrial genomes from one cell to another cell. Several investigators have shown that tumorigenicity can be suppressed by fusing tumor cells with cytoplasts from nontumorigenic cells, and it has been hypothesized that a mitochondrial factor, in combination with a nuclear gene, codes for a suppressor of tumorigenicity. More definitive evidence for cytoplasmic suppression has been sought by using reconstructed cells in which the dilution of

cytoplasmic suppressors is minimized and by not using mutagens or carcinogens to introduce genetic markers into the nucleus or cytoplasm. The methods used involved the examination of tumorigenicity in reconstructed cells made from tumorigenic karyoplasts and nontumorigenic cytoplasts and by using a ricin-antiricin selection method to obtain the reconstructed cells. Tumorigenic karyoplasts were derived from a clone of NIH/3T3 cells that had survived exposure to anti-benzo(a)pyrene diol epoxide. Ten clones which survived ricin selection were found to be nontumorigenic in nude mice. The data obtained are said to offer support for the presence of cytoplasmic factors in nontumorigenic mouse cells that suppress benzo(a)pyrene diol epoxide-induced tumorigenicity. Other studies have revealed the presence of nuclear suppressors of tumorigenicity. To ascertain whether the factor(s) responsible for cytoplasmic suppression are different from those that mediate nuclear suppression will require the isolation and identification of both kinds of suppressors (224).

Several studies on the role of specific genes and gene products in chemically induced cell transformation have been initiated and are ongoing. Recombinant DNA, gene cloning, and DNA sequencing techniques have been employed in this research which has resulted in a veritable explosion of publications demonstrating the isolation and characterization of genes responsible for the transformation of cells to malignancy. To date, several different transforming genes have been isolated from different human tumor cells and their homology to various viral oncogenes has been established. Studies involving the identification of specific transforming genes or the activation of known, previously identified oncogenes are currently ongoing or have just been initiated in several laboratories. Various chemically induced animal or cell model systems are being utilized, which include rat hepatocellular, mouse thymic lymphoma, rat nasal carcinoma, skin carcinoma, mouse bladder carcinoma, human pancreas and in vitro hamster and human fibroblast and epithelial cell transformation systems. In other studies the mouse two-stage skin carcinogenesis model is being used to determine whether the altered expression of specific genes (several oncogenes, murine leukemia virus proviral sequences, and long terminal repeat sequences) coincides with particular stages of carcinogenesis and/or tumor development. The interaction of chemicals and viruses such as Epstein-Barr virus, murine mammary tumor virus, and adenovirus type 5 are being examined by several laboratories to determine their role in carcinogenesis.

In a large majority of human tumors of mesenchymal origin, a common observed phenotype is the ability of the tumor cells to grow in an anchorage-independent fashion when suspended in soft agar. Elevated levels of c-sis mRNA, derived from the human cell homolog of v-sis which has been shown to code for a protein nearly identical to human platelet-derived growth factor (PDGF), have been detected in several of these tumors. In order to determine whether acquisition of this common tumor phenotype was related to overproduction of PDGF resulting from overexpression of the c-sis gene, multiple copies of an expression vector containing a human c-sis cDNA clone was introduced into human diploid fibroblasts using either transfection or electroporation. Acquisition of anchorage-independence was shown to have a linear, positive correlation with the level of expression of the c-sis coding sequence in human fibroblasts. Protein products generated from the transfected c-sis construct in two overexpressing clones were immunoprecipitated with anti-human PDGF antibodies. One clone was shown to contain an apparent PDGF dimer of 21 kilodaltons; the second clone contained only an apparent monomer of 12 kilodaltons, which was shown to account for all of the mitogenic activity present

in the cells. The results obtained demonstrate a clear link between sis overexpression and acquisition of a partially transformed, anchorage-independent phenotype, and implicates sis overexpression as a genetic mechanism which contributes to human cell transformation (64).

The appearance of preneoplastic focal lesions in rat liver following the administration of a hepatocarcinogen is believed to reflect one or more of the early stages in multistage hepatocarcinogenesis. One important finding potentially related to the basis of neoplasia is the altered expression of specific proto-oncogenes in different liver tumors induced with various protocols of hepatocarcinogenesis. However, since oncogenic alterations are not always observed in preneoplastic foci, it has been suggested that the elevated expression of proto-oncogenes in some tumors may be a nonessential or secondary alteration which occurs during multistage hepatocarcinogenesis. In an attempt to further characterize the role of altered proto-oncogene expression during the early stages of hepatocarcinogenesis, it was assumed that proto-oncogenes that show consistent alterations in primary liver tumors are those most likely to play a key role in the progression of altered hepatic foci to malignancy and that because only relatively few such lesions progress to neoplasia, altered hepatic foci must be examined as individual entities. The c-raf proto-oncogene was chosen since many primary liver tumors have been observed to contain elevated expression of the mRNA for this proto-oncogene. For comparative purposes, the expression of GGT mRNA and the mRNA for the major rat liver gap junction protein was also measured. Increased c-raf mRNA was demonstrated in 11 of 13 primary lesions, consisting of either neoplastic nodules or hepatocellular carcinomas. This increased level appeared to be unrelated to their cellular proliferative status. Decreased expression of the major rat liver gap junction protein mRNA was observed in all of the primary tumors. All preneoplastic foci having positive GGT enzyme staining also exhibited a marked increase in GGT mRNA as determined by in situ hybridization. Due to the important role gap junctions play in cell-cell communication and in affecting the regulation of cell proliferation and specific gene expression, the reduction in the expression of this gene may play a role in tumor promotion and in the early stages of the neoplastic process and may also be related to the progression of some altered hepatic foci to malignancy (174).

In previous studies from a different laboratory, the increased expression of the c-myc oncogene and two endogenous retrovirus-like DNA sequences (rat leukemia virus and 30S sequences) and decreased expression of the EGF receptor gene were found in rat liver tumors induced by the combination of diethylnitrosamine and phenobarbital. Enhanced expression of endogenous Moloney murine leukemia virus-like and intracisternal A particle sequences have also been observed in both carcinogen-induced and spontaneous mouse liver tumors. The mechanisms responsible for expression of these sequences in rodent liver tumors are not known at the present time. In order to test whether the expression of these genes is merely related to cell proliferation, the expression of c-myc and c-Ha-ras oncogenes, the EGF receptor gene and rat leukemia virus and 30S sequences was examined in control (nonregenerating) rat livers and at various times after partial hepatectomy, liver regeneration after partial hepatectomy being one of the best in vivo phenomena of compensatory growth in mammals. The results demonstrate that the expression of endogenous rat leukemia virus-related sequences and also the c-myc oncogene are markedly increased during normal liver cell proliferation, while expression of the EGF receptor gene is decreased. The expression of 30S sequences, which was shown to increase in rat liver tumors, did not increase in the regenerating livers. Further studies are required to determine whether the altered levels of RNAs found

in the present study reflect changes in de novo transcription of RNA stability and the relevance of these changes to growth control in normal and neoplastic liver cells (262).

In spite of many studies on the role of the ras gene family in transformation and carcinogenesis, the effects of activated ras expression on the regulation of cellular genes have remained largely unexplored. Expression of activated ras genes results in many complex phenotypic changes that include anchorage-independent growth in vitro, tumorigenicity in vivo, specific morphologic alterations, and increased glucose uptake--these changes depending to some extent on the cell of origin. It is thought that the observed phenotypic changes are mediated through the activation or inactivation of specific cellular genes by ras-dependent pathways. The precise cellular targets, however, have not been identified, with the exception of the glucose transport protein and transin, the steady-state levels of which have been shown to be increased by activated ras. The availability of cloned cDNA probes for GGT and glutathione transferase-P provided useful marker probes for studies using cultured rat liver epithelial cells. To facilitate studies of the effects of ras on gene expression, a metallothionein-ras fusion gene, MTrasT24, that allows regulation of mutant ras expression in cultured cells by $ZnSO_4$, was constructed. The expression of GGT, glutathione transferase-P, and alpha-tubulin was examined in rat liver epithelial cells and Rat-1 fibroblast cells transfected with MTrasT24. Zinc induction studies indicated that GGT and glutathione transferase-P RNA levels were directly dependent on MTrasT24 mRNA levels. Alpha-tubulin mRNA levels, in contrast, were shown to be unchanged or to fall slightly. The results obtained suggest that expression of GGT and glutathione transferase-P expression are part of a reorientation of cellular gene expression during carcinogenesis and that activated ras expression, like chemical carcinogens, can bring about this change (148).

Another possible mechanism of cell transformation by chemicals could involve the induction of DNA sequence rearrangements, chromosome alterations, mitochondrial genes and metabolism, free radical intermediates, or specific proteases. These changes could result in the altered cell growth and other properties characteristic of transformed cells. In one study the occurrence of DNA sequence rearrangements during hepatocarcinogenesis in rats and whether such rearrangements involve transforming genes is being examined using gene cloning, restriction endonuclease analysis and DNA transfection technologies. The success in developing a reliable method for obtaining chromosome preparations from fresh solid tumors has allowed a study to determine whether chromosomal alterations are mechanistically related to the origin and/or progression of chemically induced mouse skin tumors. In an initial study it has been shown that aneuploidy is an early event in mouse skin tumor development (42). In another project the role of DNA recombinational events, free radical intermediates, cell growth modification, patterns of cell differentiation, and the induction of specific proteases is being examined in the mouse embryo C3H 10T1/2 cell line and in a human diploid cell line transformed by chemicals and radiation. In addition, there is increasing interest from a number of laboratories in investigating the role of active oxygen or different oxygen radicals in the initiation of carcinogenesis.

Evidence has been presented which suggests that free radicals may be important in the induction of malignant transformation in vitro. Several of the agents which have been shown to have suppressive effects on malignant transformation in vitro are known to interact in some way with free radicals. One of the free-radical scavenging agents which can suppress radiation-induced transformation in vitro is

dimethylsulfoxide (DMSO). The available data suggest that another factor besides the ability to scavenge free radicals, such as unique solvent properties, might be important in the anticarcinogenic activity of DMSO. One study was undertaken to determine whether some compounds with solvent properties similar to those of DMSO (such as dimethylformamide and dimethylacetamide) have the same ability as DMSO to suppress transformation in vitro. DMSO was demonstrated to suppress radiation-induced transformation in vitro, even when DMSO treatment began as late as 10 days post-irradiation. DMSO was also shown to inhibit the 12-O-tetradecanoylphorbol-13-acetate (TPA) enhancement of radiation-induced transformation in vitro and not to effect the expression of transformed cells as foci. The results show that DMSO may be affecting radiation-induced transformation through its solvent properties (i.e., the 'Water Structure' theory), while its effects on the TPA enhancement of radiation transformation may be mediated by its free radical scavenging abilities (129).

Protease inhibitors from a variety of sources have been reported to have the ability to inhibit carcinogenesis in vitro and in vivo. Earlier studies have indicated that the soybean-derived Bowman Birk inhibitor will inhibit carcinogen-induced tranformation of C3H10T1/2 cells in vitro, dimethylhydrazine-induced colon carcinogenesis in mice and dimethylbenzanthracene-induced cheek pouch carcinogenesis in hamsters. In vitro studies have revealed that on a molar basis, inhibitors of chymotrypsin are more potent suppressors of radiation-induced transformation than are other types of protease inhibitors which are capable of suppressing transformation. In one study a protease inhibitor obtained from potatoes (chymotrypsin inhibitor 1; CI-1) was investigated as to whether it would inhibit carcinogen-induced transformation of C3H10T1/2 cells. It was shown to be as effective as the soybean-derived Bowman-Birk inhibitor at suppressing radiation-induced transformation of C3H10T1/2 cells and that this inhibitor does not reversibly bind to specific receptor proteins on the surface of these cells. The mechanism by which protease inhibitors suppress malignant transformation is currently unknown. It is believed that they act by inhibiting one or more proteases that are critical for the induction and/or expression of the transformed phenotype. In a further study, a particular cellular proteolytic activity in C3H10T1/2 cells that is strongly inhibited by the anticarcinogenic protease inhibitors and not by soybean trypsin inhibitor, which lacks anticarcinogenic activity, was isolated. It was shown to have a mass of about 70 kilodaltons, to contain a single subunit and to exhibit maximal activity at pH 7.0. The enzyme was shown to be a serine protease due to its inhibition by diisopropyl fluorophosphate. The evidence suggests that this endopeptidase activity may be a cellular target of the anticarcinogenic protease inhibitors (129).

Another focus of projects in this subject area are studies designed to test the cell cycle specificity of the induction of cytotoxicity, mutagenesis, and neoplastic transformation by chemical carcinogens. Also, the quantitative relationship between the level, persistence, and species of carcinogen-nucleotide adducts and the concomitant cell transformation frequency are being determined. There is a substantial amount of information supporting the hypothesis of cell cycle specificity of carcinogenesis. It has been shown that in mouse embryo C3H10T1/2 cells, G_1 and S phase cells are susceptible to cytotoxicity and mutation, while only S phase cells (in synchronized cultures) are susceptible to neoplastic transformation by exposure to alkylating agents. In adult rat liver, the hepatocytes are generally resistant to carcinogenesis by a single exposure to agents capable of inducing cancer in other tissues. Hepatocyte susceptibility to carcinogenesis is

increased by certain treatments which stimulate the proliferation of carcinogen-damaged cells. Additional work is in progress to determine more specifically in rat liver the phase of the cell cycle which is most susceptible to the initiating effect of carcinogenic chemicals.

Development of Carcinogenicity Test Systems and Mechanisms of Mutagenesis: The development of carcinogenicity test systems in this subject area includes projects in which epithelial and fibroblast cell culture systems, specially constructed bacterial strains, and erythroid cells are being used to monitor the effects of exposure to known and potential carcinogens. The end points being measured include cell transformation, mutagenesis, or the induction of sister chromatid exchanges. Most tumor cells have the capability to proliferate in medium containing low calcium concentrations, while normal cells do not. Mouse epidermal cells can be subcultured in the absence of feeder layers in low calcium medium. In the presence of high calcium medium, these cells cease to grow and terminally differentiate. Epidermal cells altered by chemical carcinogens, however, continue to grow in high calcium medium and do not terminally differentiate. This difference in growth response to high calcium is being used to select cells transformed by carcinogens. An additional alteration in some clones is the loss of dependence on epidermal growth factor (EGF) for continued growth. The mechanism for this loss of dependence is being pursued.

The bacterial mutagenesis systems currently in use have proven to be reasonably successful in predicting the carcinogenicity of most types of organic compounds. In the laboratory of an Outstanding Investigator Awardee, a major goal of research efforts is to improve both the detection and analysis of mutagens by developing a set of bacterial strains, each of which reverts by only one specific base-pair change so that the set of strains will be diagnostic for the spectrum of mutations generated by any mutagen and be much more sensitive for detecting mutagens. Bacterial mutagenesis systems have been much less successful in predicting the carcinogenicity of metal compounds. For this reason mammalian cell systems are being developed. Chinese hamster V79 cells have the known capability to phagocytize insoluble metal compounds. This should allow the study of particulate metal compounds which generally do not enter bacteria. For the detection of mutations, a plasmid containing a specific gene locus (gpt gene) will be used and mutation frequencies can be compared with that at the endogenous hprt locus.

There is considerable interest in developing methods that will allow investigators to determine whether people have been exposed to harmful levels of chemical carcinogens. Of the laboratories that are developing such methods, the goal of one is to develop a quantitative in vitro assay for measuring increases in the frequency of thioguanine-resistant T-lymphocytes in humans exposed to environmental mutagens. Because practical methods to measure frequency of mutations induced in vivo have not been available, indirect measurements, such as determining the frequency of chromosome damage, sister chromatid exchanges or the induction of tumors are often used. These methods have limitations in applicability due to the relatively low number of cells examined in chromosome-chromatid studies and the long latent period required for cancer. The recent isolation of the T-cell growth factor, interleukin 2, now makes it possible to grow human T-cells isolated from peripheral blood. This has allowed the cloning of T-cells under both nonselection and selection conditions, but has necessitated a study of the quantitative nature of the assay. Requirements for T-cell colony growth in vitro have been investigated to define the optimal conditions for cloning. The parameters studied have included medium, serum, mitogen treatment, amount of T-cell growth factor and

density of irradiated feeder or accessory cells. From these studies, a methodology was established that yields quantitative measurements of thioguanine-resistant mutant frequencies arising in vivo in human T-lymphocytes. In a subsequent study, a large-scale molecular analysis of human in vivo-derived hprt mutant T-cell clones, using a variety of techniques, was undertaken due to the importance of defining mutational spectra and of quantifying in vivo somatic gene mutations. Southern blot analyses were used to determine frequency and extent of gross structural alterations in the hprt gene in 50 wild-type and 164 in vivo-derived hprt mutant T-cell clones obtained from eight non-mutagen-exposed adult males. Sixteen (9.8%) of the mutant clones showed hprt changes with no site or type of lesion predominating. Ninety-four percent of wild-type and 89% of the hprt mutants were found to originate from independent in vivo precursors. Thus, most of the recovered hprt mutants in the study were presumed to be derived from separate in vivo mutations (2).

In addition to the development of mutagenicity test systems, projects are being supported which seek to understand how mutations and DNA or chromosome damage are generated by carcinogenic chemicals. Specifically synthesized oligonucleotides of defined base sequences are being used to examine the molecular mechanism of base-pair substitution and frameshift mutagenesis. The base sequence specificities of the interactions of mutagens with oligonucleotides are being studied and correlated to their mutagenic activity in E. coli. Newer studies in this area have focused on the use of specific genes which will either be cloned into plasmids or are present in cellular DNA as targets for the mutagenic action of various chemical carcinogens. The c-Ha-ras oncogene cloned into a plasmid, the lac gene introduced into M13 phage DNA, and the dihydrofolate reductase gene in Chinese hamster ovary cells are being used as target genes to assess the mutagenic action of chemicals such as benzo(a)pyrene diol epoxide, N-acetoxy-2-acetylaminofluorene, and other aromatic amines. DNA sequence techniques and effects of lesions on DNA synthesis will be used to determine the mechanisms of mutagenesis.

In other laboratories, methods for the analysis of mutations induced in human or other mammalian cells at the DNA sequence level are being developed. The approaches used depend on the development of recombinant DNA shuttle vectors composed of the simian virus 40 (SV40) early region, the Epstein-Barr virus (EBV) oriP element or some other sequence that allows the plasmid to be replicated in human or mammalian cells, sequences derived from the bacterial plasmid, pBR322, which permits the plasmid to also replicate in E. coli, and some selectable target genes for mutant selection. The studies seek to determine the types of DNA sequence changes induced by chemical carcinogens or mutagens and to characterize host processes that determine the frequency or types of mutations induced specifically in mammalian cells.

The shuttle vector approach was first demonstrated in simian cells, using vectors based on SV40. The sensitivity of the SV40 shuttle vector systems, however, was limited by the high frequency of mutation observed that was demonstrated to be associated with transfection into a variety of different types of eukaryotic cells. For instance, the spontaneous mutation frequency in the lacI gene after passage of the shuttle vector through human cells was found to be 3.4×10^{-7}/base pair, which is about 2 orders of magnitude higher than the spontaneous mutation frequency expected for a chromosomal gene in human cells. These types of results suggested that mutation of incoming transfected DNA may be a general property of all eukaryotic cells. In one such study, a shuttle vector p3AC, containing a 200-base pair gene for a tyrosine suppressor tRNA (supF) that was inserted at the

EcoRI site, was used as a target for detecting mutations resulting from covalently bound residues of benzo(a)pyrene diol epoxide (BPDE). When introduced by transformation, a functioning supF gene in progeny plasmids recovered from the monkey kidney cell line, COS7, allows suppression of a lacZ amber mutation in the indicator E. coli host. Untreated plasmids and plasmids containing 6.6 BPDE residues were transfected into COS7 cells and the progeny were assayed for mutations in the supF gene. Gel electrophoresis analysis of the size alterations of 77 mutant plasmids obtained with untreated DNA and 45 obtained with BPDE-treated DNA showed that the majority of the mutant progeny of untreated plasmids had gross alterations, principally large deletions. In contrast, only minor alterations, mostly point mutations, were shown to be generated during replication of the BPDE-treated plasmids. The sequence analysis of progeny of untreated plasmids containing putative point mutations showed insertions and deletions of bases and a broad spectrum of base substitutions. In progeny from BPDE-treated plasmids, however, all base substitutions were shown to involve G · C pairs. The failure to recover the background level of plasmids carrying deletions suggested that the presence of BPDE adducts interferes in some way with the process that produces gross rearrangements or that the BPDE adducts increase the size of the delections so that they extend into the amp gene. The latter type plasmids would not be recovered under the currently used conditions. Future refinements in shuttle vector construction may make it possible to greatly decrease the possibility of recovering mutants containing spontaneous gross rearrangements (160).

In another study the shuttle vector approach was combined with site-specific mutagenesis methodology in order to determine the effect of a defined DNA adduct. The mutagenic specificity of 2-acetylaminofluorene (AAF) was established in mammalian cells and several strains of bacteria by using a plasmid shuttle vector (pAG75) containing a single N-(deoxyguanosin-8-yl)acetylaminofluorene (C8-dG-AAF) adduct in a gene conferring tetracycline resistance. Presence of the single DNA adduct was shown to increase the mutation frequency by eightfold in both COS cells and in E. coli. Over 80% of mutations detected in both systems were shown to be targeted and to represent G·C to C·G or G·C to T·A transversions or single nucleotide deletions. It was concluded that modification of a dG residue with AAF preferentially induces mutations targeted at this site when a plasmid containing a single C8-dG-AAF adduct is introduced into mammalian cells or bacteria (94).

Properties and Mechanisms of Tumor Promotion: Research in this subject area involves projects designed to analyze the various cellular, biochemical and molecular activities and pleiotropic effects induced in cells upon exposure to tumor promoters. The phorbol ester tumor promoters are, by far, the most widely used compounds in these studies. They have been shown to exert their effects by binding to specific receptors on cell surface membranes. A number of grants support studies on the characterization of the phorbol ester receptor protein. The results of phorbol ester binding include alterations in membrane phospholipid metabolism, membrane structure and function, alterations in the transport of small molecules, the activation of macromolecular synthesis, the induction or inhibition of terminal cell differentiation by normal or neoplastic cells, the mimicry of the transformed phenotype by normal cells and the enhancement of transformation by chemicals and oncogenic viruses. Studies in the laboratory of a new MERIT awardee are focused on the perturbation of ion fluxes by the tumor promoter, TPA. Since the action of TPA may be mediated by the phosphorylation of proteins and lipids, several studies are focused on the purification and characterization of protein kinase C, a calcium and phospholipid-dependent protein kinase whose activity is

stimulated by TPA. A characterization of the proteins phosphorylated by this enzyme is included in some of these studies. In addition, several laboratories are attempting to clone the genes for what has been shown to be a family of these enzymes. The role of free radicals in promotion, either the active oxygen species generated by TPA in cells or the hydroperoxy fatty acids generated during the induction of the arachadonic acid cascade by TPA and other first and second stage promoters, is the focus of several of the studies. The activation of expression of certain genes is thought to occur during neoplastic progression of cells. The possible activation of oncogene sequences and other viral and cellular gene sequences by TPA and other promoters is the focus of several studies. Another property of tumor promoters is their apparent ability to disrupt cell-cell communication. The mechanism of this phenomenon is the focus of at least three studies and systems exhibiting this phenomenon are being developed as indicators of potential tumor promoting agents.

Phorbol esters have been shown to increase phosphatidylcholine metabolism in a variety of cell types. This event has been closely correlated with their tumor-promoting properties, but the exact mechanism(s) involved is unclear. Phorbol esters appear to stimulate both phosphatidylcholine synthesis and degradation. Phorbol esters purportedly stimulate many cellular processes via direct activation of protein kinase C. However, phorbol esters and diacylglycerols have not produced identical results under all circumstances. With this in mind, studies were undertaken to compare the effects of phorbol esters and 1,2-diacylglycerols on phosphatidylcholine metabolism utilizing GH_3 rat pituitary cells. Different results were produced. The synthetic 1,2-diacylglycerols, 1,2-dioctanoylglycerol (diC_8) and 1-oleoyl-2-acetylglycerol (OAG), but not a series of phorbol esters, were shown to cause the degradation of phosphatidylcholine via a phospholipase A_2. Both diacylglycerols and phorbol esters were shown to stimulate synthesis of phosphatidylcholine via the CDP-choline pathway, but demonstrated additive effects at maximal concentrations. Also, diC_8 was shown to still stimulate phosphatidylcholine synthesis and degradation in cells preincubated with TPA to down-modulate protein kinase C, while TPA-induced synthesis was abolished. These studies demonstrate that phorbol esters and 1,2-diacylglycerols have different effects on phosphatidylcholine metabolism and suggest that 1,2-diacylglycerols may stimulate phosphatidylcholine metabolism via a pathway independent of the protein kinase C pathway which mediates phorbol ester action. Differences in the action of phorbol esters and 1,2-diacylglycerols were also demonstrated in a subsequent study on their effect on levels of sphingomyelin in GH_3 rat pituitary cells. The 1,2-diacylglycerols were shown to be potent stimulators of sphingomyelin hydrolysis, while phorbol esters failed to activate this pathway. This is important since it has been proposed that degradation products of sphingolipids may serve as physiologic inhibitors of protein kinase C. These results indicate that it may be necessary to consider additional pathways to those activated by phorbol esters in the evaluation of the biological effects of the 1,2-diacylglycerols (137).

Although it has been well established that phenobarbital and choline-deficient (CD) diets act as liver tumor promoting regimens, the mechanism by which these agents exert tumor promoting effects is, at present, unknown. A CD diet has been shown to induce alterations in phospholipid metabolism and to result in abnormalities of some of the cell membrane-associated functions. Since alterations of membrane receptors for growth factors are thought to play an important role in tumor promotion of other organ systems, the role of these receptors in liver tumor promotion has been examined. Both the number and affinity of insulin receptors of rat hepatocytes has been demonstrated to be altered by a CD diet. Since effects

on receptors for other peptide growth factors was not known, studies were undertaken to investigate whether a CD diet alters epidermal growth factor (EGF) receptors, whether a different liver tumor promoter, phenobarbital, produces the same alteration, and whether the degree of the alteration correlates with the biological potency of tumor promotion. The results showed that both a phenobarbital and a CD diet induced a time-dependent decrease in the number of hepatocyte EGF receptors and the combination of the two agents enhanced this effect. This decrease in surface binding of EGF was shown not to be due to the rapid internalization of the receptors. This is the first demonstration of common cellular responses by two diverse types of liver tumor promoting regimens. These changes may be linked to the underlying mechanisms of liver tumor promotion (225).

Although there is evidence supporting the fact that during the process of carcinogenesis tumor promoters may act by mediating clonal expansion of previously initiated cells, it is also possible that genetic effects involving alterations in the regulation of gene expression may play a role in tumor promotion. Phorbol ester tumor promoters have been shown to modulate the transcription of a number of cellular and viral genes in vitro. In contrast to extensive cell culture studies, very few animal experiments have been reported in which the effects of tumor promoters on the expression of specific genes have been studied. In one laboratory treatment of mouse skin by TPA has been shown to result in the induction of transin mRNA. Transin, a secreted protease, is also found in elevated levels in tumor-promoter-induced carcinomas. In addition, a number of cDNA clones, referred to as mal 1 - mal 6, which correspond to mRNAs that are overexpressed in carcinomas induced by DMBA initiation followed by TPA promotion, have been described. Two of the tumor-associated genes, mal 1 and mal 2, were found to be overexpressed already in the benign papilloma stage of mouse skin carcinogenesis. Overexpression of two other genes, mal 4 and transin, was shown to be specific for the malignant state. Treatment of the normal adult epidermis with the complete tumor promoter, TPA, and the incomplete, second-stage promoter, 12-O-retinylphorbol-13-acetate (RPA), transiently enhanced the expression of the mal sequences and transin. In fractionation experiments, enhanced expression of mal sequences by TPA was shown in both basal and differentiated cells. In contrast, transin expression, which was undetectable in cells of the normal epidermis, was shown to be enhanced in only the basal cells of the TPA-treated epidermis. The results obtained suggest that the observed stimulated expression of mal 1 and mal 2 is related to proliferative processes, whereas stimulated expression of mal 4 and transin reflects tumor-promoter-specific responses. Further information on the function of mal 1, 2, and 4 and transin and analysis of the promoter regions of these genes could result in a better understanding of the mechanism of tumor promotion at the molecular level (13).

Gap-junction-mediated intercellular communication has been proposed to have an important role in the process of tumor promotion. Many known tumor promoters have been shown to inhibit metabolic cooperation, a specific type of intercellular communication in which small molecular weight, possible growth regulatory molecules are passed between adjacent cells in physical contact via membrane structures called gap-junctions. Correlations have been reported linking the tumor promoting efficacy of tumor promoters with their ability to inhibit metabolic cooperation. Because of the postulated importance of cell-cell communication in cellular growth control, many assays have been developed to measure the ability of cells to transfer low molecular weight substances across gap junctions. Many of these assays suffer from requirements for specific instrumentation, difficult procedures, or lengthy performance times in order to obtain quantitative data. The

rapidity and ease with which one can observe cell-cell communication by using the scrape-loading/dye transfer method and the general availability of flow cytometric analysis in most research centers led one laboratory to investigate the possibility of combining scrape-loading/dye transfer with flow cytometry as an additional means of quantitating dye transfer on a population basis. Two flow cytometric assays performed on populations of cells which had been stained with various fluorescent tracer molecules by the scrape-loading technique have been described. One assay uses a simple one-color analysis on a flow cytometer by quantitating the fluorescence intensity of scrape-loaded lucifer yellow CH (LY) in individual cells. The other assay described utilizes a two-color analysis on a cell sorter whereby cells which are initially loaded (donors) are identified by their uptake of both rhodamine isothiocyanate-dextran and LY, whereas the recipients of dye transfer are identified as having LY only. Agents which have been shown to inhibit intercellular communication in other assays exhibit similar blocking activity in LY transfer and this activity is readily quantitated by flow cytometry. The two-color analysis is said to have the added advantage of being able to identify both donors and recipients in a highly quantitative manner (253).

A substantial amount of evidence has been accumulating in the last few years, suggesting that the generation of free radicals, such as superoxide anion and hydroxyl radical, may be involved in the tumor promotion stage of multistage skin carcinogenesis. The most direct evidence for free radical involvement comes from studies in which free radical generating compounds, such as benzoyl peroxide, were shown to be complete tumor promoters. Indirect evidence comes from studies in which various antioxidants were shown to be inhibitors of TPA-induced tumor promotion. A chemiluminescence assay was recently developed for measuring TPA-induced and phospholipase C-induced oxidant generation in mouse epidermal cells. The relationship of this response to tumor promotion is suggested by the findings that most classes of known inhibitors of TPA promotion also inhibit this response. In addition, a comparison between SSIN (inbred SENCAR) and C57BL/6J mice showed that the extent of the oxidant response was found to correlate with the degree of sensitivity to TPA as a tumor promoter. Since C57BL/6J mice had been previously shown to be essentially refractory to TPA as a promoter, although sensitive to benzoyl peroxide promotion, it was hypothesized that while the C57BL/6J mice are able to respond to free radical generating agents, the diminished oxidant production in these mice may be the basis for the refractoriness to TPA promotion. Also, C57BL/6J mice were shown not to respond to topical application of TPA with the hyperplasia and inflammation observed in promotion-sensitive mice such as the SSIN. To test this hypothesis, the SSIN and C57BL/6J mice were crossed to determine whether their progeny were promotable with TPA and if promotability correlated with oxidant generation, epidermal hyperplasia and edema. The F_1 progeny of the above cross were found to be sensitive to TPA as a tumor promoter. With regard to other parameters believed to be associated with TPA responsiveness, the SSIN were shown to produce a strong hyperplastic response to TPA, the C57BL/6J a negligible response and the F_1 hybrids a moderate response. In SSIN mice, TPA was shown to cause an 18% increase in the water content of the skin (edema), while no change was seen for either the C57BL/6J or F_1 hybrids. The oxidant response of the F_1 hybrids to either TPA or phospholipase C was shown to be markedly less than that observed for the SSIN and to be similar to the response previously observed for the C57BL/6J mice. These findings suggested that the oxidant response may not be an essential aspect of TPA tumor promotion. It may be related to the edema response, suggesting that at least this aspect of inflammation is not necessary for promotion. In a subsequent study, the effect of TPA treatment on arachidonic acid metabolism and ornithine decarboxylase (ODC) activity was examined. Previous

work had suggested the necessity of arachidonic acid metabolism in tumor promotion. The induction of ODC was determined to be the same both in vivo and in vitro for SSIN and C57BL/6J mice, which does not correlate with the histological observations. Since hyperplasia and inflammation can be mediated by arachidonic acid metabolites, it was thought that differences in this metabolic pathway would correlate with the histological responses. However, no significant qualitative or quantitative differences were observed in the profiles of the major cyclooxygenase products between the strains of mice. 8-Lipoxygenase activity was shown to be elevated after TPA treatment in the SSIN mice by about fourfold, while no elevation was observed in C57BL/6J mice. This data suggested that oxidant generation or possibly 8-lipoxygenase activity may be the basis for the sensitivity or resistance to TPA as a hyperplasiogen and as a tumor promoter (68, 69).

Since humans are not normally exposed to phorbol ester tumor promoters, it was deemed necessary, in 1981, to stimulate more research on agents more relevant to human exposure which might function as tumor promoters. To accomplish this a Request for Applications (RFA) was issued inviting grant applications from interested investigators for both basic and applied studies that would seek to provide insight and approaches to an understanding of the role of tumor promoters, hormones and other cofactors in human cancer causation. The studies were to be focused on one or more of five different categories: (1) the development of nonphorbol tumor promotion or cocarcinogenesis models in experimental animals using the breast, colon, lung, prostate, stomach, urinary bladder, and/or uterus organ systems; (2) the development of nonphorbol tumor promotion or cocarcinogenesis models in human and/or nonhuman cell and/or organ culture systems; (3) the study of the possible tumor promotion role of hormones and substances such as bile acids, saturated/unsaturated dietary fat, alcohol, salt or oxygen-free radicals; (4) the identification and elucidation of the mechanisms of action of nonphorbol tumor promoters and/or cocarcinogens; and (5) interdisciplinary studies involving epidemiologists and experimentalists to test hypotheses concerning tumor promotion generated by either.

In FY 82, 12 grants were funded from applications submitted in response to this RFA; ten were approved for 3 years of funding and two for 4 years. The role of dietary fat on DMBA-induced mammary carcinogenesis in rats or mice was the focus of two of the studies. The cocarcinogenic action of ethanol with nitrosamines in the oral cavity, esophagus and larynx of rats, mice and hamsters was the focus of one study. The rates of metabolic activation of nitrosamines in the target organ and cell cultures was to be measured. In a mouse lung tumor model, the mode of action of butylated hydroxytoluene (BHT) as a tumor promoter was to be examined. The metabolism of BHT, the activation of cyclic GMP- and calcium-dependent protein kinase, the effect of glucocorticoids on urethane tumorigenesis and tumor promotion and the effect of BHT on glucocorticoid receptor localization was to be studied. Using a heterotopically transplanted rat bladder system, one laboratory was to investigate the promoting effect of urine components on bladder carcinogenesis induced by N-methyl-N-nitrosourea and N-butyl-N-(3-carboxypropyl)-nitrosamine. The hypothesis that asbestos and selected nonasbestos minerals act as tumor promoters in carcinogenesis of the respiratory tract was to be studied using a hamster trachea model. The determination of whether EBV-related oncogenic mechanisms in in vitro virus-cell interaction models involves promotion was to be made in one laboratory. The hypothesis given is that a viral-mediated increase in an intracellular protein that blocks the viral lytic cycle and interferes with cell differentiation leads to uncontrolled cell proliferation and the ultimate

selection of neoplastic cells. A study of the tumor-promoting activity of a number of anthracene derivatives, such as chrysarobin and its synthetic analogs and homologs, which are related to anthralin, was to be conducted using the 7,12-dimethylbenz(a)anthracene skin tumor model system. Two in vitro model systems for testing for tumor promoters were to be developed. One model system used hepatocytes or liver cells from carcinogen-treated rats which were then promoted in culture using selected compounds. The other model system used various rodent and human cells to test the hypothesis that the induction of mutations at the hypoxanthine guanine phosphoribosyl transferase (HGPRT) locus by promoters in hypermutable cells is a common property of cancer cells. In one study on the mechanism of action of promoters, the ability of promoters to stimulate gene amplification to methotrexate resistance was to be studied. Finally, one of the studies involved a biochemical epidemiology project in which sex hormone levels in breast and prostatic cancer was studied.

In FY 85, two of the projects were successfully renewed. In FY 86 support was continued for three additional projects, mainly due to successful revised renewal applications. Two other projects were renewed and funded in FY 87. Three of the projects will not be renewed as no renewal applications have been submitted and none are anticipated. One renewed project is for continuation of studies on the mechanism of action of a nonphorbol ester tumor promoter, chrysarobin. The second project, funded in FY 85, supports studies on the potential role of oxygen-free radicals in asbestos-induced bronchogenic carcinoma.

Of the projects receiving continued support in FY 86, one involved the further investigation of mechanisms of urinary bladder carcinogenesis utilizing the heterotopically transplanted bladder system and natural bladder models in rats. In the second project, the goal was changed to investigate the possible role played by the endogenous mouse mammary tumor virus gene and cellular oncogene components in murine mammary tumorigenesis, alone and in combination with chemical carcinogens. The third project involved studies on the role of Epstein-Barr virus in neoplastic transformation. Of the two projects renewed in FY 87, one involved further studies on the mechanisms of butylated hydroxytoluene stimulation of mouse lung tumor multiplicity. The second project involved the development of mammary cell culture models for further studies on the role of dietary fat in mammary carcinogenesis. It is also evident that this RFA has stimulated more studies on nonphorbol tumor promoters of relevance to humans. Some recently funded projects seek to study the activity of compounds such as orotic acid, cyclosporine, endogenous growth factors, hormones and dietary L-tryptophan as tumor promoters. In addition, epithelial cell and organ culture systems from human endometrium are being developed to study the process of tumor promotion by a variety of agents such as hormones and TPA.

Interspecies Comparisons in Carcinogenesis: In the subject area of interspecies comparisons in carcinogenesis are studies undertaken as a result of a specific initiative from the Branch to develop scientifically sound methodology for the extrapolation of carcinogenesis data derived from studies on experimental animals to humans. The initiative was designed to encourage studies that would be supportive of the Environmental Protection Agency in the area of risk assessment. In 1980, an RFA was issued inviting grant applications from interested investigators for both basic and applied studies designed to provide insight and approaches to an understanding of similarities and differences between experimental animals and

humans in response to chemical carcinogens. The proposed studies were to emphasize the use of accessible human cells, tissues, body fluids and excreta and to focus on quantitative relationships related to the carcinogenesis process.

In FY 81, 16 grants were funded from applications submitted in response to this RFA; 15 were approved for 3 years and one for a 5 year period. Fourteen of the grants supported studies with either human cells only or with human and other rodent or monkey cells. One grant supported comparative studies in mice and rats only and another used hamsters, mice, and rats. Fourteen of the grants supported studies on the comparative metabolism of a variety of chemical carcinogens such as polycyclic aromatic hydrocarbons, aromatic amines, and nitrosamines. The development of human hepatocyte, pancreas, esophagus, and bladder in in vitro cell transformation systems was the additional goal in four of the projects. The development of direct or cell-mediated mutagenesis or genetic damage assay systems was also the focus in three of the studies. The primary goal in two of the funded studies was to develop techniques to measure mutant proteins in peripheral blood lymphocytes or in red blood cells from individuals who had been exposed to potential mutagens or carcinogens either as the result of various clinical procedures or from environmental or occupational exposure to chemicals.

By the end of FY 88, 6 of the 16 original projects will still be active. Five investigators had chosen not to submit competing renewal applications. Of the 11 applications for which competing renewal applications were received, three were renewed in FY 84 and two in FY 86. One of the projects renewed in FY 84 has been renewed a second time for a 5 year period. One project, first renewed in FY 85, has been renewed for the second time in FY 88. The main goal of this project is to provide an understanding of the mechanisms responsible for the metabolic activation, organ specificity, and species and strain specificity of the carcinogen, methylazoxymethanol. Another project involves the further development and characterization of cell culture and transplantation systems using human hepatocytes obtained from normal and cirrhotic liver. A study of the mechanism of pancreatic carcinogenesis by N-nitrosomethyl(2-oxo-propyl)amine and related compounds in rats and hamsters is the goal of the third project. The fourth project involves studies on the perfection, characterization, and application of a 6-thioguanine resistance assay in human peripheral blood cells for monitoring environmental mutagens. The fifth active project supports a continuation of studies on the characterization of cytochrome P-450 isozymes in humans in which biochemical, immunological, and molecular biological techniques are used to define the molecular basis for the genetic polymorphism of certain oxidative activities in humans. This project is now included as part of an Outstanding Investigator award. The final active project, which has been renewed for the second time, involves studies on the continued evaluation of the validity of interspecies extrapolation of carcinogenicity data using a mammary gland model, and to address the hypothesis that environmental xenobiotics are involved in the etiology of human breast cancer. Only two of the six renewal projects are still included in this subject area classification. The other projects are included in other subject areas which are now more appropriate.

While it is not yet clear what the overall impact of the funded studies will have on the ability to extrapolate animal carcinogenicity data to humans, it is clear that the initiative on interspecies comparisons in carcinogenesis has stimulated additional studies using cells from human tissues, which will increase our knowledge on the metabolism and processing of carcinogens by those tissues and on the

biological and molecular characteristics of cells transformed by chemical carcinogens. In addition, many more investigators now utilize more than one species or strain of animals in their proposed studies. Different animal species have been shown to be either sensitive or resistant to the action of various xenobiotics. Thus, use of different species can give insights into the mechanism of action of carcinogens in carcinogenesis. Two additional non-RFA-generated projects have been included in this category of research.

The ability of many polycyclic aromatic hydrocarbons (PAHs) to induce mammary tumors in rodents has been well documented. The relative tumorigenicity, however, has been shown to vary widely among these compounds. Since PAHs such as benzo(a)-pyrene [B(a)P] are ubiquitous environmental contaminants, it is of great interest to determine their potential human carcinogenicity. In the absence of clear-cut in vivo human data, the development of a rodent model which accurately reflects human risk is desirable. Methods have been recently optimized for the isolation and culture of human and rat mammary epithelial cells under identical conditions. These cells have been directly compared for their abilities to convert DMBA and B(a)P to mutagenic forms in a mammary cell-mediated assay. The metabolism of these PAHs by the mammary cells was also examined and compared as well as the quantitation of DNA binding under identical conditions. DMBA was found to be highly mutagenic in the rat mammary cell-mediated mutagenesis assay, while B(a)P exhibited little or no mutagenic activity. The opposite pattern was found in human mammary cell-mediated assays. These results could not be easily explained by the results of a comparison of Phase I PAH metabolism in these cells. When the levels of total DNA binding of DMBA and B(a)P in rat and human mammary cells were examined, higher levels of cell-mediated mutagenesis were found to be associated with higher levels of intracellular PAH-DNA binding in the mammary cells. The specific DNA adducts formed in these cells after 24 hr incubations with radio-labeled DMBA and B(a)P were analyzed to determine if there were qualitative as well as quantitative differences in the amounts of individual adducts. Similar proportions of specific DMBA-DNA adducts were found in both rat and human cells, with significantly higher total amounts of adducts in rat cells. In contrast, an essentially qualitative species-specific difference was observed in the major B(a)P-DNA adduct present in the rat and human cells. The major B(a)P adduct formed in the human mammary epithelial cells was identified as the BPDE-deoxy-guanosine adduct. However, this adduct was formed at very low levels in the rat mammary epithelial cells, about 25-fold lower. Rat cells were shown to contain a large proportion of syn-BPDE adducts and other unidentified B(a)P-DNA adducts. The high level of the (+)-anti-BPDE-dG adduct in the human, but not the rat, mammary cells is consistent with the potential role of (+)-anti-BPDE in the high mutagenic activity of B(a)P in the cell-mediated mutagenesis assays, using the human mammary cells as activators, and the low mutagenic activity of B(a)P when rat cells were used as activators. The quantitative differences in the activation of DMBA by cells from these two species are also consistent with the cell-mediated mutagenic activities of DMBA using these cells as activators. The above results suggest that the higher carcinogenic activity of DMBA, when compared to B(a)P in the rat mammary gland, may not be indicative of the relative carcinogenic potencies of these compounds for human mammary cells and raise questions about the ability to extrapolate in vitro mammary cell data to the in vivo situation (7, 85, 86).

Genetics and Regulation of Enzymes Associated with Carcinogenesis: Research projects in this subject area are focused on the use of somatic cell genetic and molecular biological approaches to study the regulation of the levels of

carcinogen metabolizing enzymes. In eight of the projects, the development and use of cloned cDNA probes to cytochrome P-450 or epoxide hydrolase genes form part of the proposed studies. These cloned cDNA probes are used to examine the levels of expression of the mRNAs for the genes in response to the modulation of enzyme activity by various chemicals. The genomic organization of the genes is also being examined in several of the studies. In two of the projects, mutants or variant cells, altered in their ability to induce the carcinogen metabolizing enzymes, are being isolated and characterized. The characterization of cytochrome P-450 genes from human cells is the focus or part of three of the projects. Using the inbred hamster model the genetic variation in aryl-hydroxamic acid acyltransferase, sulfotransferase, N-acetyltransferase, and N-deacetylase enzymes which are involved in the metabolism of aromatic amines is being studied. Also, the isolation and characterization of carcinogen-binding or receptor proteins, which may play a role in the regulation of cytochrome P-450 gene expression, is being pursued in two of the studies.

The cytochrome P-450 monooxygenase system is a large family of enzymes responsible for the oxidation of drugs and environmental pollutants, as well as many endogenous compounds. Results from enzyme purification studies and cloning of the respective mRNAs indicate that many of these enzymes are encoded by distinct structural genes and that they are under different modes of regulation. The ability of these enzymes to catalyze oxidation reactions on many physically distinct substrates lies, in part, on the multiple substrate specificities of the individual cytochrome P-450s, but also can result from the large number of physically distinct cytochrome P-450s that are part of this supergene family. DNA sequence analysis of cDNA and genomic clones that encode many of the different forms of cytochrome P-450 indicates that this superfamily of proteins is encoded by subfamilies whose individual genes are similar with regard to exonic sequence, structure, and chromosomal localization. For example, the phenobarbital inducible rat P-450b and P-450e genes have been shown to contain nine exons and eight introns, with the location of the exon/intron junctions being identical in the two genes. The exonic sequences have been demonstrated to be 95% homologous to each other and the location of the genes have been mapped to mouse chromosome 7. The polycyclic aromatic hydrocarbon-inducible rat cytochrome P-450c and P-450d are 75% homologous within the coding regions, with each gene organized into seven exons and six introns. Both genes have been localized on mouse chromosome 9. The human equivalents to rat P-450c and P-450d have been shown to share 80% homology within the coding regions to the rat counterparts and have maintained the intron/exon organization. The close homology in DNA sequence and similar gene structure exhibited between lower eukaryotes and humans indicated that the homologous human P-450 gene families can be identified and characterized by using sequence-specific probes from other species. A rabbit liver cDNA clone that encodes cytochrome P-450 1 has been identified and characterized. One of its major enzyme activities has been shown to be the 21-hydroxylation of progesterone to deoxycorticosterone, an activity that is also localized to the adrenal cortex. A comparison of the DNA sequence and its predicted translation product, however, demonstrated that P-450 1 was unrelated to the human or pig adrenal 21-hydroxylase but, instead, shared approximately 50% amino acid sequence homology to the phenobarbital-inducible rabbit P-450 2 and the equivalent in the rat, P-450b and P-450e. To determine if this gene family is conserved in humans, the rabbit P-450 1 cDNA was used as a probe to identify and characterize human liver cDNAs that are homologous to P-450 1. A highly homologous (81% within the coding region) human liver cDNA, termed Hp1-1, that encodes a 490-amino acid protein was identified. The human P-450 1 was shown to be 82% homologous to the s-mephenytoin 4-hydroxylase gene reported by

the laboratory of F.P. Guengerich. Southern blot analysis has indicated that the human P-450 1 gene is part of a larger gene family, but it can be selectively identified by using a 3'-noncoding portion of the cDNA. Using a panel of human rodent somatic cell hybrids, the location of the human P-450 1 gene was placed on human chromosome 10. The human P-450 1 gene transcript was demonstrated to be processed by an alternate RNA splicing mechanism that generates two mRNA products, one representing the functional transcript and the other a form of mRNA that is not capable of encoding a functional P-450 (254).

From mouse hepatoma Hepa-1 cell cultures treated with B(a)P, B(a)P-resistant mutants that exhibit neither control nor inducible P_1450 catalytic activity have been selected. Complementation studies have characterized several defects, each believed to represent a distinct gene encoding a product responsible for a step necessary in the P_1450 induction process. These data have led to the description of at least one structural gene and two regulatory gene classes of mutant lines (complementation groups A, B and C, respectively)--P_1^-, lacking aryl hydrocarbon hydroxylase (AHH) activity but having a normal functional Ah receptor; r^-, lacking a functional Ah receptor; and nt^-, having an impaired nuclear translocation of the TCDD-receptor complex. In one study, the P_1450 structural gene of two P_1^- mutants (clones c1 and c37) was examined in order to understand the mechanism underlying the lack of either control or inducible AHH activity. Nearly full length P_1450 cDNAs from wild type and mutant c1 and c37 cells were isolated and sequenced. The c1 cDNA was found to have a single mutation leading to premature termination of the protein after Asn-414. The c37 cDNA was found to have two point mutations, leading to a Leu-118 to Arg-118 and a Arg-245 to Pro-245 change, but to otherwise encode the normal 524-residue protein. When the various P_1450 cDNAs were inserted into the expression vector pAAH5 and expressed in yeast, the Leu-118 to Arg-118 mutation alone was found to have a negligible effect on AHH activity, while the Arg-245 to Pro-245 mutation alone led to a two- to threefold reduction in enzyme activity. The two mutations together were shown to totally eliminate AHH activity. The biologic mutant c37 provides the first evidence for the importance of Arg-245, and the complementary function of Leu-118, in normal P_1450 enzymic function. This alteration in a single amino acid from arginine to proline is thought to block electron flow directly, or to change the secondary structure of the protein, such that normal monooxygenation of B(a)P cannot occur (102).

Epoxide hydrolase is responsible for the stereospecific hydration of arene and alkene oxides to trans-dihydrodiols. At least three forms of epoxide hydrolase have been identified which are enzymatically and immunologically distinct. There are two microsomal forms and a cytosolic form. The microsomal epoxide hydrolase responsible for the hydration of arene oxides has been extensively characterized. The cloning of the cDNA for the Sprague-Dawley rat liver microsomal xenobiotic epoxide hydrolase has been reported, and its nucleotide sequence and translation of the cDNA determined. The cDNA was shown to contain an open reading frame of 1365 nucleotides coding for a 52,581 dalton protein consisting of 455 amino acids. The cDNA was used to isolate a family of clones which contained the xenobiotic epoxide hydrolase gene from a rat liver genomic DNA library. This gene was shown to be approximately 16 kilobases in length and to consist of 9 exons ranging in size from 109 to 420 base pairs with 8 intervening sequences, the largest of which was 3.2 kilobases. The 5' cap site and the 170 base pair size of the first exon was determined by S_1-nuclease mapping, primer extension studies and sequence analysis. Only a single functional epoxide hydrolase gene was identified and no evidence of hybridization to the genes for the microsomal cholesterol epoxide

hydrolase (the second microsomal form) or the cytosolic epoxide hydrolase was observed. However, during the characterization of the gene, a pseudogene of epoxide bydrolase was also isolated and characterized (174).

Occupational and environmental exposures to arylamine chemicals are known to be a major factor in the etiology of bladder cancer in humans. Arylamine carcinogens require metabolic activation by the host to highly electrophilic species in order to initiate neoplasia. Suggested mechanisms of activation involve the N-oxidation of the arylamine in the liver followed by transport to the bladder or the active role of the bladder epithelium in the metabolic activation of arylamines. Hepatic N-acetylation capacity in humans and other mammalian species has been shown to be controlled by simple autosomal Mendelian inheritance of two codominant alleles at a single genetic locus. Individuals can be identified as rapid, intermediate or slow acetylator phenotypes. Of particular interest have been human epidemiological studies suggesting a genetic predisposition to arylamine-induced bladder cancer among slow acetylators. Recent studies in inbred animal models have yielded important information regarding the acetylation polymorphism. The mode of inheritance and the biochemical basis of the hepatic N-acetylation polymorphism has been reported in the Syrian inbred hamster. The expression of N-acetyltransferase activity towards p-aminobenzoic acid, p-aminosalicylic acid and 2-aminofluorene was shown to be acetylator genotype dependent in both epithelial and nonepithelial bladder tissue. Acetylator genotype as assessed by bladder N-acetyltransferase activity was shown to be completely concordant with acetylator genotype as assessed by liver N-acetyltransferase activity. The results obtained suggested that genetic control of arylamine N-acetyltransferase in bladder urothelium may be a factor in hereditary predisposition to arylamine-induced bladder cancer. The data also suggest that different isozymes are present in the two genotypes (104).

Role of DNA Repair in Carcinogenesis: The types of projects in this subject area include studies on the characterization of DNA damage produced by bulky chemical carcinogens, alkylating agents, ultraviolet (UV) light and ionizing radiation; the isolation and characterization of proteins responsible for DNA nucleotide excision repair and base excision repair; the cloning and characterization of the DNA nucleotide and base excision repair genes; and the determination of the role of chromosome structure, location and site of DNA lesions and poly(ADP-ribosylation) in the repair of DNA damage. A variety of rodent, frog, yeast, bacterial and normal and repair-deficient human cells are being used in these studies. Since DNA modification by chemical carcinogens has been shown to lead to mutations or other alterations in gene expression, DNA repair may have evolved as a defense mechanism to eliminate such damage and thus restore the correct genetic information and DNA structure. The significance of this process to human well being was emphasized by the demonstration that, in contrast to normal cells, cells from individuals with xeroderma pigmentosum, a hereditary genetic disease, did not remove UV radiation-induced pyrimidine dimers from their DNA. These individuals develop multiple skin cancers at an early age only on exposed surfaces, thus establishing a link between genetic damage, defective repair of the damage, and cancer in the exposed tissue.

Very little is known about the molecular mechanism of nucleotide excision repair in eukaryotes. Studies on human cells have been stimulated by the availability of excision repair-defective cell lines from patients suffering from the autosomal recessive disease xeroderma pigmentosum (XP). Such studies have contributed significantly to an understanding of the genetic complexity of excision repair in

human cells. For unknown reasons, the isolation of human excision repair genes or gene products known to complement the repair defect in XP cells have been difficult to achieve. The identification and isolation of human genes by complementation of mutant human cells has proven less successful than the isolation of similar genes from prokaryotes and lower eukaryotes. Standard techniques for DNA-mediated gene transfer are demonstrably less efficient in human cells. For cells with nonselectable phenotypes, complementation assays may be sufficiently laborious to preclude the routine screening of total genomic DNA. Cells from XP patients, however, have a selectable phenotype in that they exhibit a marked sensitivity to killing by UV light. In a different approach to this problem, the technique of microcell-mediated chromosome transfer was applied in one laboratory. This was shown to permit the rapid and efficient screening of human chromosomes for complementation of the mutant phenotype of XP cells. Chromosomes from an immortalized aneuploid human fibroblast cell line (VA-13) were randomly tagged with the selectable marker neo by transfection with the plasmid pSV2neo. Recipients were identified by selection for G418 resistance and colonies were secondarily screened for UV resistance. Somatic cell fusions between transfected human cells and mouse A9 cells generated pools of G418-resistant human-mouse hybrid clones containing various numbers of human chromosomes. Screening of subclones from these selected pools facilitated the identification of hybrids containing a single neo-tagged human chromosome. The transfer of this chromosome to XP complementation group A (XP-A) cells by microcell-mediated chromosome transfer was shown to result in cells with enhanced resistance to UV light and enhanced excision repair capacity. The complementing chromosome was shown by cytogenetic analysis to be rearranged and has not yet been definitively identified. These results constitute a critical first step toward chromosomal assignment, subchromosomal mapping, and molecular cloning of a gene required for excision repair of DNA in human cells (78).

Excision repair of DNA damage is one mechanism that protects cells from damaging agents. This process has been shown to be complex and to require the participation of a number of different enzymes. The first step involves the recognition of the damage and incision of the damaged strand near the lesion. Incision is followed by the removal of a short stretch of nucleotides containing the damage, synthesis of a repair patch presumably using the complementary strand as a template, and finally, ligation of the repair patch to the adjacent parental DNA strand. Many of the details of excision repair have yet to be characterized in mammalian cells. The laboratory of an Outstanding Investigator Awardee has reported that UV-induced pyrimidine dimers are preferentially removed from transcriptionally active genes. In one example, dimers were shown to be efficiently removed from the transcriptionally active dihydrofolate reductase (DHFR) gene, but poorly removed from a sequence near the gene and from the bulk of the DNA in Chinese hamster cells. Transcription has been shown to be blocked by pyrimidine dimers in template DNA and thus the preferential removal of a transcription-blocking lesion from an active gene would be expected to be of biological significance. In human cells, most dimers are eventually removed from the entire genome, but preferential repair has been shown to be manifested as a more rapid rate of repair in active DNA sequences. One plausible model considered to explain selective repair in mammalian cells is one in which the chromatin structure of active sequences is in a more open conformation, which would allow the DNA to be more accessible to repair enzymes. An alternative possibility that was considered is that an active mechanism has evolved that directly couples repair with transcription. In an attempt to improve the understanding of preferential repair of active gene sequences, repair in the transcribed and nontranscribed strands of the

DHFR gene in both hamsters and humans was measured. A dramatic difference in the efficiency of removal of UV-induced pyrimidine dimers in the respective strands was found. In both cell types repair in the transcribed strand of the DHFR gene was shown to be very rapid with most of the dimers having been removed within 4 hr after UV irradiation. In contrast, little repair was detected in the nontranscribed strand of the hamster DHFR gene during 24 hr after irradiation. In human cells, repair was shown to be significantly faster in the transcribed strand than in the other strand. In addition, it was shown that in the 5' flanking region of the human DHFR gene, selective rapid repair occurs in the opposite DNA strand relative to the transcribed strand of the DHFR gene. This strand is thought to serve as a template for a divergent transcript. The above results have important implications for excision repair pathways and mutagenesis in mammalian cells (101).

Many methylating and ethylating agents have been shown to be potent carcinogens when administered to animals. The initial amount of alkylpurines in DNA and their persistence or half-life in vivo has been measured repeatedly. For O^6-methylguanine (O^6-meG) or O^6-ethylguanine (O^6-etG), the half-life has been found to be a function of dose, species, and tissue and cell type. The repair of these lesions has been correlated with the protein, O^6-alkylguanine DNA alkyltransferase (AGT), which catalyzes the transfer of the alkyl group from the DNA to a cysteine residue in its amino acid sequence. This process thus restores the DNA in a single step, but the active site of the AGT is not regenerated. Therefore, cells can only remove rapidly as many O^6-alkylguanine residues as there are molecules of the AGT. In common with E. coli, mammalian cells also contain a second activity, 3-methyladenine DNA glycosylase, which can remove N-3 and N-7 alkylpurines from DNA by catalyzing cleavage of the sugar-base bond. In attempts to investigate the mechanism of alkylpyrimidine loss from mammalian cell systems, several research groups have focused on corresponding repair activities in mammalian cells. The enzymatic repair of the O-alkylpyrimidines and alkyl phosphotriesters has been studied in E. coli and the two proteins involved, 3-methyladenine DNA glycosylase and AGT, have been well characterized. In mammalian cells treated with carcinogenic alkylating agents, loss of these derivatives has been repeatedly demonstrated. However, mammalian repair proteins that are analogous to those from E. coli do not detectably act on these alkyl derivatives. A variety of techniques have been used by many investigators in the United States and Europe, and they have concluded that the mode of O-alkylpyrimidine and alkyl phosphotriester repair in mammalian cells differs from that in E. coli. New approaches and methods are said to be needed to characterize these processes at the biochemical and molecular level (193, 230).

Thymine is the most susceptible of the DNA bases to modification by chemical oxidants, oxygen-containing free radicals, and ionizing radiation. The oxidative derivative that is most readily formed has been shown to be the cis-5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) moiety. Thymine glycol has also been identified in DNA exposed to a variety of oxidative stresses, where it has been shown to be a block to DNA replication in vitro. Thymine glycol was shown to be removed from DNA by an N-glycosylase activity of E. coli endonuclease III. Surprisingly, thymine glycol N-glycosylase activity has been difficult to characterize with certainty in mammalian cells. To definitively characterize this important enzyme activity, a facile microderivatization scheme was devised which quantitatively converts cis-thymine glycol to 5-hydroxy-5-methylhydantoin (HMH). The reaction products were analyzed by high pressure liquid chromatography before and after

derivatization by using independently synthesized *cis*-^{14}C-thymine glycol and ^{14}C-HMH as reference compounds. The results obtained were considered to unambiguously prove the existence of both human (HeLa cell) and bacterial N-glycosylase activities that affect removal of thymine glycol from DNA (248).

MOLECULAR CARCINOGENSIS

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. ADAIR, Gerald M. University of Texas System Cancer Center 5 R01 CA28711-06	Expression of Genetic Variation in Cultured Cells
2. ALBERTINI, Richard J. University of Vermont & St Agric College 5 R01 CA30688-05	Direct Mutagenicity Testing in Man
3. AMES, Bruce N. University of California Berkeley 5 R35 CA39910-03	Mutagenesis and Carcinogenesis
4. ASHENDEL, Curtis L. Purdue University West Lafayette 2 R01 CA36262-04	Interactions of Tumor Promoters with Receptors
5. AUTOR, Anne P. Gordon Research Conferences 1 R13 CA44275-01	Gordon Conference--Oxyradicals in Biology and Medicine
6. AVADHANI, Navayan G. University of Pennsylvania 5 R01 CA22762-10	Mitochondrial DNA Damage during Chemical Carcinogenesis
7. BAIRD, William M. Purdue University West Lafayette 5 R01 CA40228-03	Molecular Mechanisms of Hydrocarbon DNA Interactions
8. BAXTER, C. Stuart University of Cincinnati 2 R01 CA34446-04	In Vivo Immunotoxicology of Tumor-Promoting Agents
9. BENFIELD, John R. City of Hope National Medical Center 5 R01 CA29373-06	Model of Bronchogenic Lung Cancer
10. BIGGART, Neal W. San Diego State University 1 R29 CA46818-01	Reactive Oxygen-Mediated Mutagenesis by CDC1-2 and TPA
11. BILLINGS, Paul C. Harvard University 1 R01 CA45734-01	Target Proteases of Anticarcinogenic Protease Inhibitors
12. BOUCK, Noel P. Northwestern University 5 R01 CA27306-09	Genetic Analysis of Malignant Transformation

13.	BOWDEN, George T. University of Arizona 5 R01 CA40584-03	Oncogene Activation During Skin Tumor Progression
14.	BOWDEN, George T. Federation of Amer Soc for Exper Biol 1 R13 CA45276-01	FASEB Summer Research Conference
15.	BOX, Harold C. Roswell Park Memorial Institute 5 R01 CA29425-06	Molecular Studies of Carcinogenesis and Mutagenesis
16.	BOX, Harold C. Roswell Park Memorial Institute 1 R01 CA44808-01A1	DNA Damage, Promotion and the Prooxidant State
17.	BOYNTON, Alton L. University of Hawaii at Manoa 5 R01 CA39745-03	Assays for and Mechanisms of Action of Tumor Promoters
18.	BOYNTON, Alton L. University of Hawaii at Manoa 5 R01 CA42942-02	Tumor Promoters, Second Messengers, and Carcinogenesis
19.	BRASITUS, Thomas A. Michael Reese Hosp & Med Ctr (Chicago) 5 R37 CA36745-05	Colonic Epithelial Cell Plasma Membranes
20.	BRESNICK, Edward University of Nebraska Medical Center 5 R01 CA35994-03	Epoxide Hydrolase in Hyperplastic and Neoplastic Livers
21.	BRESNICK, Edward University of Nebraska Medical Center 2 R01 CA36106-05	Polycyclic Hydrocarbon Metabolism and Carcinogenesis
22.	BRESNICK, Edward University of Nebraska Medical Center 5 R01 CA36679-05	DNA Repair After Polycyclic Hydrocarbon Administration
23.	BRIGGS, Robert C. Vanderbilt University 5 R01 CA26412-09	Experimental Hepatocarcinogenesis
24.	BRIGGS, Robert C. Vanderbilt University 5 R01 CA36479-03	Nuclear Toxicity of Heavy Metals
25.	BROIDO, Michelle S. Hunter College 1 R29 CA46713-01	NMR Studies of Phosphate-Alkylated DNA Oligomers

26. BROYDE, Suse B.
New York University
2 R01 CA28038-07
Carcinogen - DNA Adducts: Linkage Site and Conformation

27. BUTEL, Janet S.
Baylor College of Medicine
5 R01 CA33369-05
Mammary Cancer Stage: Viral and Chemical Interactions

28. BUTLER, Andrew P.
University of Texas System Cancer Center
1 R01 CA46629-01
Regulation of Ornithine Decarboxylase by Phobol Esters

29. BYUS, Craig V.
University of California Riverside
5 R01 CA35807-03
Mechanism of Tumor-Promoter Action

30. CALOS, Michele P.
Stanford University
5 R01 CA33056-06
Mutation in Human Cells at the DNA Sequence Level

31. CARR, Brian I.
City of Hope National Medical Center
1 R29 CA44602-01A1
TGF-Beta Receptors in Hepato-carcinogenesis

32. CARTER, Timothy H.
St. John's University
2 R01 CA37761-04
Regulation of Transcription by a Tumor Promoter

33. CHEN, Fu-Ming
Tennessee State University
5 R01 CA42682-02
Conformational Lability of POLY(DG-M5DC):POLY(DG-M5-DC)

34. CHRISTMAN, Judith K.
Michigan Cancer Foundation
7 R01 CA25985-09
Response of Phagocytic Leukocytes to Tumor Promoters

35. CHRISTMAN, Judith K.
Michigan Cancer Foundation
1 R01 CA45028-01A1
Mechanism of 5-AzaCR-Mediated Alteration in Gene Action

36. CHU, Gilbert
Stanford University
1 R01 CA44949-01
Finding the Molecular Defect in Xeroderma Pigmentosum

37. CHUNG, Fung-Lung
American Health Foundation
5 R01 CA43159-02
Enals in Tumorigenesis

38. CLAWSON, Gary A.
George Washington University
7 R01 CA21141-12
Pathology of Chemical Carcino-genesis

39.	CLAWSON, Gary A. George Washington University 7 R01 CA40145-03	Specificity of RNA Transport In Vitro
40.	COHEN, Samuel M. University of Nebraska Medical Center 1 R01 CA44886-01	Acrolein and Urinary Bladder Carcinogenesis
41.	CONNER, Mary K. University of Pittsburgh at Pittsburgh 5 R01 CA39401-03	Murine Lymphocyte SCE Model for Predicting Genotoxic Risk
42.	CONTI, Claudio J. University of Texas System 5 R01 CA42157-02	Chromosome Alteration During Chemical Carcinogenesis
43.	CORDEIRO-STONE, Marila University of North Carolina Chapel Hill 1 R01 CA40504-01	Timing of Oncogene Replication and Carcinogenesis
44.	COSTA, Max New York University 5 R01 CA43070-03	Mechanism of Metal Carcinogenesis
45.	CRAIGHEAD, John E. University of Vermont & St Agric College 5 R01 CA36993-03	Experimental Asbestos-Induced Mesothelioma
46.	CURPHEY, Thomas J. Dartmouth College 5 R01 CA30650-06	Pancreas and Liver Carcinogen Metabolism in Two Species
47.	CZECH, Michael P. University of Massachusetts Medical Sch 5 P01 CA39240-03	Growth Factor Receptor Regulation by C Kinase
48.	DANIELSON, Keith G. Baylor College of Medicine 5 R23 CA38650-03	Transformation of Mammary Epithelial Cells In Vitro
49.	DAVIDSON, Richard L. University of Illinois at Chicago 5 R01 CA31781-07	Mechanisms of Chemical Mutagenesis in Mammalian Cells
50.	DETRISAC, Carol J. IIT Research Institute 5 R29 CA40501-03	Transitional Cell Carcinoma: In Vitro/In Vivo Correlates
51.	DIAMOND, Leila Wistar Institute of Anatomy and Biology 5 R01 CA23413-10	Tumor Promoters and Cell Differentiation

52. DIAMOND, Leila
Wistar Institute of Anatomy and Biology
5 R01 CA37168-03
Chemical Transformation, Neoplastic Progession and Oncogene

53. DIGIOVANNI, John
University of Texas System Cancer Center
2 R01 CA36979-04
The Role of DNA Binding in Mouse Skin Tumor Initiation

54. DIGIOVANNI, John
University of Texas System Cancer Center
5 R01 CA37111-05
Mechanism of Skin Tumor Promotion by Chrysarobin

55. DIGIOVANNI, John
University of Texas System Cancer Center
5 R01 CA38871-03
Genetics of Susceptibility to Skin Tumor Promotion

56. DRESLER, Steven L.
Washington University
5 R01 CA37261-03
Molecular Analysis of Carcinogen-Induced DNA Repair

57. DRINKWATER, Norman R.
University of Wisconsin Madison
5 R01 CA37166-04
Molecular Analysis of Carcinogen-Induced Mutations

58. DUKER, Nahum J.
Temple University
2 R01 CA24103-07A1
Molecular Pathology of Carcinogenic DNA Damage

59. ECHOLS, G. Harrison, Jr.
University of California Berkeley
5 R01 CA41655-03
Mutagenesis and its Control in *E. coli*

60. ELLIOTT, Mark S.
Old Dominion University
1 R29 CA45213-01
Modulation of Queuine Levels with Tumor Promoters

61. ESSIGMANN, John M.
Massachusetts Institute of Technology
1 R01 CA43066-01
Extrachromosomal Probes for Mutagenesis

62. ETHIER, Stephen P.
Michigan Cancer Foundation
5 R01 CA40064-03
Proliferative Life Span of Mammary Epithelial Cells

63. FAGAN, John B.
Maharishi International University
5 R01 CA38655-02
Cytochrome P-450 Gene Structure and Regulation

64. FAHL, William E.
University of Wisconsin Madison
2 R37 CA42024-03
Carcinogen-Transformed Human Cells: Genetic Traits

65.	FAHL, William E. University of Wisconsin Madison 5 R01 CA42066-03	Hydrocarbon Carcinogenesis in Mouse and Human Cells
66.	FAUSTO, Nelson Brown University 2 R01 CA35249-04A1	Proto-Oncogenes and Cell Lineages in Liver Carcinogenesis
67.	FINK, Gerald R. Whitehead Institute for Biomedical Res 5 R01 CA39961-04	Chemical Carcinogens and Frameshift Mutation in Yeast
68.	FISCHER, Susan M. University of Texas System Cancer Center 2 R01 CA34443-04	Role of Arachidonate Metabolites in Tumor Promotion
69.	FISCHER, Susan M. University of Texas System Cancer Center 5 R01 CA42211-02	Tumor Promoter-Induced Oxidants from Epidermal Cells
70.	FISHER, Paul B. Columbia University New York 2 R01 CA35675-04	Analysis of Progression of the Transformed Phenotype
71.	FISHER, Paul B. Columbia University New York 1 R01 CA43208-01A1	Multifactor Interactions in Carcinogenesis
72.	FLOYD, Robert A. Oklahoma Medical Research Foundation 1 R01 CA42854-01	Oxygen-Free Radicals in Carcinogenesis
73.	FOILES, Peter American Health Foundation 5 R01 CA42235-02	Monoclonal Antibodies Specific for Cyclic DNA Adducts
74.	FOSTER, Patricia L. Boston University 5 R01 CA37880-03	Mechanisms of Mutagenesis by Aflatoxin and other Agents
75.	FOX, C. Fred University of California Los Angeles 1 R13 CA47041-01	Conference on Mechanisms and Consequences of DNA Damage Process
76.	FRENKEL, Krystyna New York University 5 R01 CA37858-03	Tumor Promoters Effecting Base Modification in DNA
77.	FRIEDBERG, Errol C. Stanford University 5 R01 CA12428-17	DNA Repair and its Relationship to Carcinogenesis

78.	FRIEDBERG, Errol C. Stanford University 1 R01 CA44247-01	DNA Repair and Cancer-Prone Hereditary Human Disease
79.	GALLAGHER, Patricia E. West Virginia University 1 R29 CA47457-01	Enzymatic Repair of Carcinogenic Damage to Human DNA
80.	GARTE, Seymour J. New York University 2 R01 CA36342-04	Transforming Genes in Inhalation Carcinogenesis
81.	GEACINTOV, Nicholas E. New York University 2 R01 CA20851-10	Characterization of Carcinogen Nucleic Acid Complexes
82.	GERSON, Stanton L. Case Western Reserve University 1 R01 CA45609-01	Prevention of Leukemogenesis in Hematopoietic Precursors
83.	GOLD, Barry I. University of Nebraska Medical Center 5 R01 CA29088-04	Activation and Transportation of Nitrosamines
84.	GORDON, Nancy R. American University 1 R15 CA43552-01	Cr-VI Induced DNA-Protein Cross-links
85.	GOULD, Michael N. University of Wisconsin Madison 5 R01 CA28954-06	Carcinogen Activation by Cultured Mammary Cells
86.	GOULD, Michael N. University of Wisconsin Madison 2 R01 CA30295-07	Mammary Carcinogenesis-Interspecies Comparisons
87.	GOULD, Michael N. University of Wisconsin Madison 1 R01 CA44387-01	Characterizing Early Events in Mammary Carcinogenesis
88.	GREENBERGER, Joel S. University of Massachusetts Medical Sch 5 R01 CA39851-04	Stem Cell Age and X-Ray Chemotherapy Leukemogenesis
89.	GRIFFITH, O. Hayes University of Oregon 5 R01 CA11695-18	Photoelectron Imaging of Eucaryotic Cells
90.	GRISHAM, Joe W. University of North Carolina Chapel Hill 5 R01 CA24144-09	Mechanisms of DNA-Dependent Cytotoxicity by Chemicals

91. GRISHAM, Joe W.
University of North Carolina Chapel Hill
2 R01 CA29323-07A1
Clonal Analysis of Carcinogenesis In Vitro

92. GRISHAM, Joe W.
University of North Carolina Chapel Hill
5 R01 CA32036-05
DNA Adducts, Replication, Mutation and Transformation

93. GRODZICKER, Terri I.
Cold Spring Harbor Laboratory
1 R13 CA46087-01
Eukaryotic Transposable Element as Mutagenic Agents

94. GROLLMAN, Arthur P.
State University New York Stony Brook
5 R01 CA17395-13
Molecular Pharmacology of Tumor and Virus Inhibitors

95. GRUNBERGER, Dezider
Columbia University New York
5 R01 CA39547-03
Mechanism of Mutation Induced in Mammalian Genes

96. GUENGERICH, F. Peter
Vanderbilt University
5 R01 CA30907-07
Purified Human Enzymes and Carcinogen Metabolism

97. GUENGERICH, F. Peter
Vanderbilt University
1 R35 CA44353-01
Enzymic Activation of Chemical Carcinogens

98. GUPTA, Pawan K.
Michigan Cancer Foundation
5 R23 CA38844-03
Molecular Basis of Carcinogen-Induced Mutagenesis

99. GUPTA, Ramesh C.
Baylor College of Medicine
5 R01 CA30606-07
Reaction of Carcinogenic Aromatic Amines with DNA

100. HAM, Richard G.
University of Colorado at Boulder
5 R01 CA30028-06
Human Mammary Cell Growth and Function in Defined Media

101. HANAWALT, Philip C.
Stanford University
1 R35 CA44349-01
Cellular Processing of Damaged DNA: Role in Oncogenesis

102. HANKINSON, Oliver
University of California Los Angeles
5 R01 CA28868-08
Carcinogen Activation and Screening in Variant Cells

103. HECHT, Stephen S.
American Health Foundation
5 R01 CA23901-09
Environmental Nitrosamines-Metabolism and Carcinogenesis

104.	HEIN, David W. Morehouse School of Medicine 2 R01 CA34627-04A1	Pharmacogenetics of Drug and Carcinogen Metabolism
105.	HENDERSON, Earl E. Temple University 5 R01 CA35471-03	Carcinogen Enhancement of EBV Induced Transformation
106.	HENDRICKS, Jerry D. Oregon State University 5 R01 CA39398-03	Mechanism of Promotion of Cancer in Trout
107.	HERSCHMAN, Harvey R. University of California Los Angeles 5 R01 CA42887-02	Molecular Mechanisms--Single and Two-Stage Transformation
108.	HITTELMAN, Walter N. University of Texas Hlth Sci Ctr Houston 2 R01 CA27931-07A1	Chromosome Aberrations with Therapeutic Agents
109.	HITTELMAN, Walter N. University of Texas System Cancer Center 5 R01 CA39534-03	Visualization of Chromosome Events in DNA Repair
110.	HIXSON, Douglas C. Rhode Island Hospital (Providence, RI) 5 R01 CA42715-02	Cellular Origins of Liver Cancer
111.	HIXSON, Douglas C. Rhode Island Hospital (Providence, RI) 5 R01 CA42716-03	Membrane Glycoproteins during Hepatocinogenesis
112.	HOGAN, Michael E. Princeton University 5 R01 CA39527-03	Mapping Carcinogen Binding Sites on Genes
113.	HUMAYUN, M. Zafri University of Medicine & Dentistry of NJ 2 R01 CA27735-07A1	Mutagenesis by Carcinogens: A Molecular Approach
114.	HUNT, John M., Jr. University of Texas Hlth Sci Ctr Houston 2 R01 CA37150-04A1	Alloantigens as Probes for Hepatocarcinogenesis
115.	HUTCHINSON, Franklin Yale University 5 R01 CA40195-03	DNA Sequence Changes in Mutated Mammalian Cells
116.	HUTCHINSON, Franklin Gordon Research Conferences 1 R13 CA48592-01	Gordon Research Conference on Mutagenesis, 1988

117. IVARIE, Robert D.
University of Georgia
2 R01 CA34066-04A1
Inactivation of Gene Expression by DNA Agents

118. JACOBSON, Myron K.
Texas College of Osteopathic Medicine
5 R01 CA43894-02
Alteration of NAD Metabolism by Chemical Carcinogens

119. JACOBSON, Myron K.
Texas College of Osteopathic Medicine
1 R13 CA44757-01
Niacin Nutrition, ADP-Ribosylation and Cancer Symposium

120. JENSEN, David E.
Temple University
2 R01 CA38077-03
The Biochemistry of DNA Alkyl Phosphotriesters

121. JIRTLE, Randy L.
Duke University
2 R01 CA25951-07
Survival and Carcinogenesis in Transplanted Hepatocytes

122. JONES, Peter A.
University of Southern California
5 R01 CA39913-04
5-Azacytidine-Induced Differentiation

123. KALLENBACH, Neville R.
New York University
2 R01 CA24101-09
Ligand Interactions of DNA Junctions

124. KAUFMAN, David G.
University of North Carolina Chapel Hill
5 R01 CA20658-10
Chemical Carcinogenesis and Cell Proliferation

125. KAUFMAN, David G.
University of North Carolina Chapel Hill
5 R01 CA31733-06
Promotion of Chemical Carcinogenesis in Uterine Tissue

126. KAUFMAN, David G.
University of North Carolina Chapel Hill
5 R01 CA32238-05
Factors Influencing Initiation of Hepatocarcinogenesis

127. KAUFMAN, David G.
University of North Carolina Chapel Hill
1 P01 CA42765-01A1
Cycle-Dependent Mechanisms of Chemical Carcinogenesis

128. KAUFMANN, William K.
University of North Carolina Chapel Hill
5 R01 CA36906-03
Operation of a DNA Excision Repair Pathway In Vitro

129. KENNEDY, Ann R.
Harvard University
2 R37 CA22704-10
Radiation and Chemical In Vitro Malignant Transformation

130.	KING, Charles M. Michigan Cancer Foundation 1 R01 CA45639-01	Site-Directed Mutagenicity
131.	KLEIN-SZANTO, Andres J. Fox Chase Cancer Center 5 R01 CA44980-02	Markers of Skin Tumor Progression
132.	KLEIN-SZANTO, Andres J. Fox Chase Cancer Center 7 R01 CA44981-01	Carcinogenesis of Xeno-transplanted Human Epithelia
133.	KNOLL, Brian J. University of Texas Hlth Sci Ctr Houston 5 R01 CA39792-03	Gene Expression in Liver Carcinogenesis
134.	KNUDSON, Alfred G., Jr. Institute for Cancer Research 5 R01 CA43211-02	Hereditary and Induced Cancer in Model
135.	KOESTNER, Adalbert Michigan State University 5 R01 CA32594-05	Neuro-oncogenesis by Resorptive Carcinogens
136.	KOHWI-SHIGEMATSU, Terumi La Jolla Cancer Research Foundation 5 R01 CA39681-03	Studies of Non-B DNA Structure with Chemical Carcinogens
137.	KOLESNICK, Richard N. Sloan-Kettering Institute for Cancer Res 5 R01 CA42385-02	Mechanism of Phorbol Ester-Induced Lipid Metabolism
138.	KRAFT, Andrew S. University of Alabama at Birmingham 5 R01 CA42533-02	Control of Differentiation of Human Promyelocytes
139.	KRAUTER, Kenneth S. Yeshiva University 5 R01 CA39553-03	Induction of Gene Expression by Chemical Carcinogens
140.	KRUGH, Thomas R. University of Rochester 2 R01 CA35251-04A2	Structure of Carcinogen-Nucleic Acid Complexes
141.	KULESZ-MARTIN, Molly F. Roswell Park Memorial Institute 5 R01 CA31101-07	Quantitative Carcinogenesis in Cultured Epithelial Cells
142.	LAPEYRE, Jean-Numa University of Texas System Cancer Center 5 R01 CA31487-06	Regulation and Enzymology of DNA Methylase in Cancer

143.	LEADON, Steven A. University of California Lawrence Berkeley Lab 5 R01 CA40453-03	DNA Repair in Specific Sequences of Mammalian Cells
144.	LEBOWITZ, Jacob University of Alabama at Birmingham 2 R01 CA17077-10A4	Chemical Inhibition of Transcription and DNA Supercoiling
145.	LEBRETON, Pierre R. University of Illinois at Chicago 1 R01 CA41432-01A1	UV Photoelectron Studies of Electronic Influence
146.	LEGERSKI, Randy J. University of Texas System Cancer Center 5 R01 CA36486-04	Isolation and Cloning of Human DNA Repair Genes
147.	LEWIS, James G. Duke University 1 R29 CA44734-01	Xenobiotics, Inflammation and Carcinogenesis
148.	LIEBERMAN, Michael W. Fox Chase Cancer Center 5 R01 CA39392-04	Carcinogen Activation of Unexpressed Mammalian Genes
149.	LIEBERMAN, Michael W. Fox Chase Cancer Center 5 R01 CA40263-03	Gene Expression in Carcinogen-Induced Liver Cancer
150.	LIEHR, Joachim G. University of Texas Med Br Galveston 5 R37 CA43233-03	Mechanism of Estrogen-Induced Renal Carcinogenesis
151.	LILLY, Frank Yeshiva University 5 P01 CA31855-06	Mechanisms of Chemical Lymphomagenesis
152.	LINDAHL, Ronald G. University of Alabama in Tuscaloosa 5 R01 CA21103-08	Gene-Enzyme Correlates of Liver Aldehyde Dehydrogenases
153.	LOEB, Lawrence A. University of Washington 5 R35 CA39903-03	Fidelity of DNA Replication
154.	LOMBARDI, Benito University of Pittsburgh at Pittsburgh 2 R01 CA23449-09A1	Choline Deficiency and Hepatocarcinogenesis
155.	LU, Lee-Jane W. University of Texas Med Br Galveston 5 R01 CA44163-03	DNA Damage During Pregnancy

156. LUDLUM, David B. — The Role of DNA Adducts in Ethylene-Oxide Exposure
University of Massachusetts Medical Sch
1 R01 CA47103-01

157. MACLEOD, Michael C. — Specificity of Diol Epoxide: Chromatin Interactions
University of Texas System Cancer Center
5 R01 CA35581-05

158. MACMANUS, John P. — Incidence, Quantitation of a Tumor Protein (Oncomodulin)
National Research Council of Canada
5 R01 CA31898-06

159. MAGUN, Bruce E. — Mechanisms of Tumor Promotion In Vitro
University of Arizona
2 R01 CA39360-04

160. MAHER, Veronica M. — Interaction of Carcinogens with DNA: Repair of Lesions
Michigan State University
5 R01 CA21253-11

161. MAHER, Veronica M. — Environmental Factors in Inherited Malignant Melanoma
Michigan State University
5 R01 CA36520-04

162. MAHER, Veronica M. — In Vivo/In Vitro Human T-Cell Environmental Mutagenesis
Michigan State University
5 R01 CA37838-03

163. MALKINSON, Alvin M. — Promotion of Lung Tumors by BHT
University of Colorado at Boulder
2 R01 CA33497-05A1

164. MARCHOK, Ann C. — Preneoplastic Markers in Specific Lesion Cell Population
Oak Ridge National Laboratory
5 R01 CA42798-02

165. MARLETTA, Michael A. — Properties of a Carcinogen Binding Receptor-Like Protein
University of Michigan at Ann Arbor
7 R01 CA37770-03

166. MARNETT, Lawrence J. — Studies on Malondialdehyde and Related Compounds
Wayne State University
5 R01 CA22206-09

167. MARNETT, Lawrence J. — Peroxyl Radicals, Hydroperoxides and Carcinogenesis
Wayne State University
5 R01 CA43209-02

168. MATRISIAN, Lynn M. — Role of Transin in Tumor Promotion and Progression
Vanderbilt University
1 R01 CA46843-01

169. MC CORMICK, J. Justin
Michigan State University
2 R01 CA21289-10
In Vitro Transformation of Human Cells

170. MEDINA, Daniel
Baylor College of Medicine
1 R01 CA47112-01
Early Events in Chemical Carcinogen-Induced Mammary Tumors

171. MEEHAN, Thomas D.
University of California San Francisco
5 R01 CA40598-04
Physical and Chemical Interactions of BPDE and DNA

172. MICHALOPOULOS, George K.
Duke University
5 R01 CA30241-07
Cell Culture and Transplantation of Human Hepatocytes

173. MICHALOPOULOS, George K.
Duke University
1 R01 CA43632-01
A1 Adrenoreceptor, Liver Carcinogenesis and Regeneration

174. MILLER, Elizabeth C.
University of Wisconsin Madison
5 P01 CA22484-10
Biochemical Studies in Chemical Carcinogenesis

175. MILO, George E.
Ohio State University
2 R01 CA25907-07A1
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176. MILO, George E.
Ohio State University
1 R01 CA42313-01
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177. MITCHELL, Ann D.
Genesys Research, Inc
1 R43 CA45903-01
L5178Y Lymphoma TK Locus Host-Mediated Assay

178. MITRA, Sankar
Oak Ridge National Laboratory
5 R01 CA31721-06
DNA Repair and Nitrosamine-Induced Carcinogenesis

179. MOORE, Peter D.
University of Illinois at Chicago
5 R01 CA37145-03
Replication of Damaged DNA in Mammalian Cell Extracts

180. MORDAN, Lawrence J.
University of Hawaii at Manoa
5 R01 CA38806-03
Tumor Promoter Effects Upon Initiated Cell Communication

181. MORIZOT, Donald C.
University of Texas System Cancer Center
5 R01 CA39729-02
Multistep Chemical Initiation in Genetic Melanoma Models

182. MORRIS, Rebecca J. — University of Texas System Cancer Center — 1 R29 CA45293-01 — Epidermal Stem Cells in Two-Stage Carcinogenesis

183. MORRISON, Harry A. — Purdue University West Lafayette — 5 R01 CA18267-09 — Cutaneous Photobiology

184. MOSES, Robb E. — Baylor College of Medicine — 5 R01 CA37860-03 — Transfer of DNA Repair Genes

185. MOSSMAN, Brooke T. — University of Vermont & St Agric College — 5 R01 CA33501-06 — Oxygen Radicals in Mineral Damage/Tumor Promotion

186. NIEDEL, James E — Duke University — 1 R01 CA43816-01 — Oncogene Regulation of Diacylglycerol Metabolism

187. O'BRIEN, Thomas G — Wistar Institute of Anatomy and Biology — 2 R37 CA36353-04 — Ionic Regulation and Tumor Promotion

188. OBERLEY, Larry W. — University of Iowa — 5 R01 CA41267-02 — Superoxide Dismutase Levels in Tumor Cells

189. OYASU, Ryoichi — Northwestern University — 5 R01 CA33511-05 — Experimental Urinary Bladder Carcinogenesis

190. OYASU, Ryoichi — Northwestern University — 1 R01 CA43574-01 — Role of Stroma in Urinary Bladder Carcinogenesis

191. PARSA, Ismail — Health Science Center at Brooklyn — 5 R01 CA38955-03 — Quantitative Model of Human Pancreas Carcinogenesis

192. PATEL, Dinshaw J. — Columbia University New York — 1 R01 CA46533-01 — DNA Damage Sites--Mutagenic and Carcinogenic Lesions

193. PEGG, Anthony E. — Pennsylvania State Univ Hershey Med Ctr — 5 R01 CA18137-12 — Persistence of Alkylated DNA in Carcinogenesis

194. PELLING, Jill C. — University of Nebraska Medical Center — 5 R01 CA40847-03 — Two-Stage Skin Carcinogenesis and Altered Gene Expression

No.	Investigator / Institution / Grant	Title
195.	PENMAN, Sheldon Massachusetts Institute of Technology 5 R01 CA37330-03	Cytoarchitecture in Tumor Promotion and Transformation
196.	PERCHELLET, Jean-Pierre H. Kansas State University 5 R01 CA40083-03	Glutathione Metabolism during Skin Tumor Promotion
197.	PEREIRA, Michael A. Environmental Health Research & Testing 1 R43 CA45110-01	Initiation-Promotion Bioassay in Liver
198.	PITOT, Henry C. University of Wisconsin Madison 1 R01 CA45700-01	Instability of Tumor Promotion in Hepatocarcinogenesis
199.	PIZER, Lewis I. University of Colorado Hlth Sciences Ctr 5 R01 CA42444-02	Mutagenic Activity of Anti-Herpes Drugs
200.	RAJALAKSHMI, S. R. University of Toronto 1 R01 CA45361-01	Glycosylation in Experimental Liver Carcinogenesis
201.	RANDERATH, Kurt Baylor College of Medicine 2 R37 CA32157-06	32P-Labeling Test for Nucleic Acid Damage by Carcinogens
202.	RAO, M. Sambasiva Northwestern University 5 R01 CA36043-03	Carcinogen-Induced Acinar Cell Lesions in Pancreas
203.	RAO, M. Sambasiva Northwestern University 5 R01 CA36130-03	Y-Glutamyltranspeptidase Negative Hepatocarcinogenesis
204.	REDDY, Arram L. University of Washington 2 R01 CA32716-04A1	Skin Tumorigenesis Studied with Cell Markers
205.	RICE, Robert H. Harvard University 1 R01 CA46928-01	Carcinogen Suppression of Keratinocyte Differentiation
206.	RICH, Alexander Massachusetts Institute of Technology 5 R01 CA29753-07	Chemical Carcinogenesis and DNA Structure
207.	RICHIE, Ellen R. University of Texas System Cancer Center 5 R01 CA37912-03	Mechanisms of MNU-Induced Lymphoma

208.	ROGAN, Eleanor G. University of Nebraska Medical Center 5 R01 CA25176-06	Binding of Aromatic Hydrocarbons to Nucleic Acids
209.	ROKITA, Steven E. State University New York Stony Brook 1 R01 CA43593-01	Oligonucleotide Photochemistry
210.	ROMANO, Louis J. Wayne State University 2 R01 CA35451-04	In Vitro Function of DNA Containing Carcinogen Adducts
211.	ROMANO, Louis J. Wayne State University 5 R01 CA40605-02	Biological Consequences of Site-Specific Damage to DNA
212.	RONEN, Amiram Hebrew University of Jerusalem 5 R01 CA40922-02	A Direct Test for Somatic Mutation In Vivo
213.	ROSENSTEIN, Barry S. Brown University 7 R01 CA45078-01	Repair of 290-320 NM-Induced Non-Dimer DNA Damage
214.	ROSNER, Marsha R. University of Chicago 7 R01 CA35541-05	Modulation of Cellular Phosphorylation by Tumor Promoter
215.	ROSNER, Marsha R. University of Chicago 7 R01 CA40407-03	Regulation of Protein Kinase C
216.	ROSSMAN, Toby G. New York University 5 R01 CA29258-06	Mutagenesis by Metals of Environmental Significance
217.	SAFFRAN, Wilma A. Queens College 7 R01 CA42377-02	Psoralen-Induced Mutation and Recombination
218.	SARMA, D. S. University of Toronto 2 R01 CA37077-04	Promotion of Liver Carcinogenesis by Orotic Acid
219.	SARMA, D. S. University of Toronto 1 R01 CA46261-01	Cell Proliferation and Liver Carcinogenesis
220.	SCARPELLI, Dante G. Northwestern University 5 R01 CA34051-05	Metabolism of Pancreatic Carcinogens: Species Differences

221. SEVILLA, Cynthia L.
Proteins International
1 R43 CA45878-01

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222. SHARMA, Surendra
Roger Williams General Hospital
1 R01 CA46959-01

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223. SHAW, Barbara R.
Duke University
1 R01 CA44709-01

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224. SHAY, Jerry W.
University of Texas Hlth Sci Ctr Dallas
5 R01 CA40065-02

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225. SHINOZUKA, Hisashi
University of Pittsburgh at Pittsburgh
2 R01 CA26556-08

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226. SHINOZUKA, Hisashi
University of Pittsburgh at Pittsburgh
2 R01 CA36175-04

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227. SHINOZUKA, Hisashi
University of Pittsburgh at Pittsburgh
5 R01 CA40062-03

Liver Cancers by Hypolipidemic Peroxisome Proliferators

228. SIDRANSKY, Herschel
George Washington University
5 R01 CA41832-02

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229 SIEGAL, Gene P.
University of North Carolina Chapel Hill
1 R29 CA45727-01

Chemical Progression and Inhibition of Neoplasia

230. SINGER, Bea A.
University of California
Lawrence Berkeley Lab
5 R01 CA42736-03

Alkylation of Polynucleotides In Vitro and In Vivo

231. SINGER, Bea A.
University of California
Lawrence Berkeley Lab
1 R01 CA47723-01

Biochemical Mechanisms of Vinyl Chloride Carcinogenesis

232. SIRICA, Alphonse E.
Virginia Commonwealth University
5 R01 CA39225-03

Hepatic Oval Cells in Culture and In Vivo

233. SIROVER, Michael A.
Temple University
2 R01 CA29414-07A1

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234.	SLAGA, Thomas J. University of Texas System Cancer Center 5 R01 CA43278-02	Critical Changes and Factors in Skin Tumor Progression
235.	SMULSON, Mark E. Georgetown University 5 R01 CA25344-09	Carcinogens and Chromatin Structure and Function
236.	SNOW, Elizabeth T. New York University 1 R29 CA45664-01	Mechanisms of Metal Mutagenesis: Cr, Ni, and Be
237.	SOLT, Dennis B. Northwestern University 2 R01 CA34160-05	Sequential Analysis of Oral Carcinogenesis
238.	SOROF, Sam Institute for Cancer Research 5 R01 CA05945-23	Target Polypeptide of Carcinogen Mitosis and Oncogenesis
239.	STAMBROOK, Peter J. University of Cincinnati 2 R01 CA36897-04	Mammalian Cell Assay for Mutagenesis and Carcinogenesis
240.	STONER, Gary D. Medical College of Ohio at Toledo 5 R01 CA28950-06	Carcinogenesis Studies in Cultured Esophagus
241.	STRAUSS, Bernard S. University of Chicago 5 R01 CA32436-06	Error Prone DNA Synthesis and Oncogene Mutagenesis
242.	STRAUSS, Bernard S. University of Chicago 5 P01 CA40046-03	Etiology of Treatment-Induced Secondary Leukemia
243.	SUDILOVSKY, Oscar Case Western Reserve University 5 R01 CA35362-03	DNA Content of Dysplastic Lesions in Human and Rat Liver
244.	SUDILOVSKY, Oscar Case Western Reserve University 1 R01 CA45716-01	Pathobiology of High Dose Sucrose
245.	SUMMERHAYES, Ian C. New England Deaconess Hospital 5 R23 CA42944-02	Oncogenesis of Bladder Epithelial Cells
246.	SUMMERS, William C. Yale University 5 P01 CA39238-02	DNA Repair, Recombination and Mutagenesis

247.	TANG, Eric M. University of Texas System Cancer Center 5 R01 CA42897-02	Repair of Antitumor Antibiotic-Induced DNA Damage
248.	TEEBOR, George W. New York University 5 R01 CA16669-12	Repair of Radiation-Induced Carcinogenic Damage to DNA
249.	TELANG, Nitin T. Memorial Hospital for Cancer & Allied Dis 1 R29 CA44741-01A1	In Vitro Induction and Modulation of Mammary Preneoplasia
250.	TERZAGHI-HOWE, Margaret Oak Ridge National Laboratory 5 R01 CA34695-05	Cell Interactions: Expression of Preneoplastic Markers
251.	TOPAL, Michael D. University of North Carolina Chapel Hill 5 R01 CA28632-06	Effects of Carcinogen Modification of DNA Precursors
252.	TOPAL, Michael D. University of North Carolina Chapel Hill 9 R01 CA46527-09	Molecular Basis of Environmentally-Induced Mutations
253.	TROSKO, James E. Michigan State University 2 R01 CA21104-10	Mutation and Derepression of Genes in Carcinogenesis
254.	TUKEY, Robert H. University of California San Diego 2 R01 CA37139-04	Cytochrome P-450 Genes and Chemical Carcinogenesis
255.	UPTON, Arthur C. New York University 1 R13 CA47472-01	Symposium on Advances in Environmental Health
256.	VERMA, Ajit K. University of Wisconsin Madison 2 R01 CA35368-04A1	Ca2+-Dependent Processes Involved in Phorbol Ester Tumors
257.	VOLSKY, David J. St. Luke's-Roosevelt Inst for Hlth Sci 5 R01 CA33386-06	Epstein-Barr Virus and Tumor Promotion
258.	WALKER, Graham C. Massachusetts Institute of Technology 5 R01 CA21615-11	Mutagenesis and Repair of DNA
259.	WANI, Altaf A. Ohio State University 1 R01 CA39397-01A3	DNA Damage in Oncogene Activation

260. WEBB, Thomas E.
Ohio State University
1 R01 CA42886-01
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261. WEBER, Wendell W.
University of Michigan at Ann Arbor
5 R01 CA39018-03
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262. WEINSTEIN, I. Bernard
Columbia University New York
5 P01 CA21111-11
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263. WEINSTEIN, I. Bernard
Columbia University New York
5 R01 CA26056-08
Cellular and Biochemical Effects of Tumor Promoters

264. WEISSMAN, Bernard E.
Children's Hospital of Los Angeles
5 R01 CA39602-02
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265. WENDER, Paul A.
Stanford University
5 R01 CA31841-07
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266. WETTERHAHN, Karen E.
Dartmouth College
5 R01 CA34869-05
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267. WETTERHAHN, Karen E.
Dartmouth College
1 R01 CA45735-01
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268. WHALEN, Dale L.
University of Maryland Balt Co Campus
5 R01 CA17278-09
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269. WHITLOCK, James P., Jr.
Stanford University
5 R37 CA32786-05
Carcinogen-Metabolizing Enzymes: Action in Variant Cell

270. WILLIAMS, Gary M.
American Health Foundation
5 R01 CA39545-02
Biochemical Toxicity of Agents Increasing Reactive O_2

271. WINKLER, Jeffrey D.
University of Chicago
1 R01 CA45686-01
Synthesis of Tumor Promotors and Inhibitors

272. YAGER, James D., Jr.
Dartmouth College
2 R01 CA36701-04A1
Role of Gonadal Steroids in Hepatocarcinogenesis

273. YAGER, James D., Jr.
Dartmouth College
5 R01 CA36713-05

DNA Sequence Changes during Hepatocarcinogenesis

274. YAMASAKI, Hiroshi
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Role of Intercellular Communication in Carcinogenesis

275. YU, Fu-Li
College of Medicine at Rockford
5 R01 CA30093-06

Aflatoxin B1 and Nucleolar RNA Synthesis

276. ZURLO, Joanne
Dartmouth College
1 R01 CA42419-01A1

Mechanisms of Mutagenesis by Environmental Agents

SUMMARY REPORT

SMOKING AND HEALTH

The Smoking and Health component within the Chemical and Physical Carcinogenesis Branch includes 11 grants with FY 87 funding of $2.30 million and one Interagency Agreement with FY 88 funding of $0.3 million. Support is continuing for research directed toward understanding and mitigating the deleterious effects of tobacco products on health. Significant past efforts have included identifying smoking-related diseases and the chemical analyses of major whole smoke components and their subsequent metabolic products. These efforts have been expanded to include smokeless tobacco products, since these items have shown increased usage in recent years, especially among younger people. Current activities are focused on the toxicological and pharmacological aspects of the problem, with emphasis on nicotine and nicotine metabolites, and their affect on tobacco dosimetry in humans.

A program project grant (6) at the American Health Foundation is directed towards the elucidation of major questions in tobacco carcinogenesis which remain unresolved. During the past year, chemical analytical studies have been concerned with the identification of specific carcinogens such as benzene and butadiene in the vapor phase of cigarette smoke. They were detected at levels of <1,500 micrograms and ≤80 micrograms per cigarette, respectively, with a highly specific and sensitive method based on cryogenic capillary gas chromatography. Aliquots of the vapor phase of cigarette smoke are injected into the cryogenic capillary gas chromatograph, which is interfaced with a mass selective detector. The system is connected with a computer that affords the quantitative assessment of several hundred compounds in the vapor phase (detection limit: 0.1 ng/injection). This high sensitivity also enables the detection of vapor phase carcinogens in indoor air, polluted with tobacco smoke, which is termed "environmental tobacco smoke" (ETS). Furthermore, it is possible to utilize this method for analyzing the exhaled breath of smokers for certain vapor phase compounds which can be suitable biomarkers of exposure to the combustion products of tobacco. The methodology is now being extended to the determinations of formaldehyde and other volatile carbonyl compounds in mainstream and sidestream smoke of tobacco products in ETS, and the exhaled breath of smokers.

The chemical analysis, the biochemistry, and the bioassays of the carcinogenic, tobacco-specific N-nitrosamines (TSNA), and especially those derived from nicotine, continue to be major aspects of the investigations in tobacco carcinogenesis at this laboratory. The nicotine-derived 4-(methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the strongest carcinogen in chewing tobacco (≤0.4 micrograms/g), in snuff (≤13 micrograms/g), and tobacco smoke (≤1 microgram/-cigarette). It has been shown that NNK is a potent carcinogen in mice, rats, and hamsters in which it induces cancer of the lung, esophagus, nasal cavity, and liver. Recently, experimental evidence has documented that NNK and its enzymatic reduction product 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) induce cancer of the pancreas, in addition to cancer of the lung, upon administration of these agents in drinking water. This represents the first identification of a pancreas carcinogen in tobacco and tobacco smoke and supports the epidemiological observation of the tobacco user's increased risk for cancer of the pancreas.

Chemical-analytical studies have led to the identification of two additional nicotine-derived N-nitrosamines in smokeless tobacco products. These were 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL; ≤2.5 micrograms/g snuff) and 4-(methylnitrosamino)-4-(3-pyridyl)-1-butyric acid (iso-NN-acid; ≤0.7 micrograms/g snuff). Bioassays of iso-NNAL are indicative of carcinogenic activity in the lungs of rats. The iso-NN-acid is now being synthesized in sufficient quantities for later bioassays.

Methods for a biochemical assessment of the uptake of TSNA by chewers, snuff-dippers, and smokers are being developed at the present time. Upon metabolic activation, NNN and NNK yield highly reactive species which are capable of forming hemoglobin adducts as well as DNA adducts. A micro-method permits the quantification of the liberated TSNA moiety of the globin adduct from the blood of smokers and snuff-dippers for the dosimetry of the uptake of the tobacco-specific carcinogens by the tobacco user.

Tobacco-containing betel quid is a known oral carcinogen in both men and women. Studies supported at this laboratory have shown that the major alkaloid in betel nut, arecoline, gives rise to four N-nitrosamines during the storage of the product and during chewing. Among these nitrosamines is 3-(methylnitrosamino) propionitrile (MNPN), a powerful carcinogen in rats, which, upon metabolic activation, methylates and cyanoethylates guanine in the DNA of the cells in the nasal cavity, esophagus, and liver. It is noteworthy that these are the target organs for tumor induction by MNPN in the rat. Through its determination in serum, arecoline, the primary precursor for MNPN in betel quid, is targeted for use as an indicator for absorption of betel quid carcinogens.

It has long been the concern that flavor additives to tobacco may have an impact on the biological activity of tobacco extracts and the cigarette smoke. Therefore, analytical investigations have been aimed at the identification of semi-volatile compounds that are not germane to tobacco, and the assesment of known flavorants which appear to be significantly enhanced in tobacco products. Approximately 100 semivolatile agents have been found in snuff and about 40 such compounds have been identified in cigarette tobaccos. Further analytical refinement of capillary gas chromatography techniques with mass detection is underway, and all potentially bioactive semivolatile compounds are undergoing in vitro tests for genotoxicity and will later be tested for their ability to modify the biological activity of other tobacco and smoke constituents.

Large-scale radioimmunoassays of nicotine and cotinine in the serum and urine of smokers, and of nonsmokers exposed to environmental tobacco smoke (involuntary smokers), have documented that the exposure of the latter is equivalent to the smoker's uptake of nicotine from one cigarette per day. However, these studies have also shown that the elimination of cotinine, the major nicotine metabolite, by the involuntary smoker is 50 to 100% slower than that by the smoker. This finding underscores that concentration of nicotine and cotinine determined in the serum and urine of smokers and invouluntary smokers cannot simply be compared as an exposure index.

Another study (10) is designed to determine if NNK, a potent respiratory tract carcinogen in adult rodents, is metabolized by fetal respiratory tract tissue of Syrian golden hamsters, nonhuman primates (Cynomolgus monkeys), and humans, and if such metabolism leads to cancer in the offspring of pregnant females injected with the compound. Specific DNA-lesions such as 7-methylguanine induced by NNK also

will be assayed. Short-term acute toxic and long-term carcinogenicity bioassays will complement such biochemical studies. In preliminary studies, Syrian golden hamsters were time-mated, and fetal tracheas and lungs were cultured with NNK for 24 hours. Metabolites released into the medium were assayed by HPLC. Eight explants metabolized 83% of NNK (5.25 nmoles) added to the culture medium. Carbon hydroxylation (40%) and pyridine N-oxidation (32%) were the two main metabolic pathways in tracheal explants from 15-day-old fetuses. NNK was metabolized extensively (80-90%) by lung explants of 14- and 15-day-old fetuses. Pyridine N-oxidation was more extensive at 15 days than at 14 days of gestation. Ratios of a carbonyl reduction metabolite to NNK were 0.75 (15 days) and 4.3 (14 days). The same ratio was 0.75 for fetal trachea. Covalent binding of NNK metabolites to protein in trachea (15 days) and lung (15 and 14 days) was similar. These results indicate that if NNK crosses the placenta, the fetal respiratory tract tissue can activate the nitrosamine by alkylating intermediates. The effects of NNK on human fetal trachea and lungs also were studied. Tissues were excised from 21-week-old fetuses after therapeutic abortion. Tracheal and lung explants were cultured with (5 micro M) and without NNK for 4 or 24 hours. Light and electron microscopic examination showed that the human respiratory tract explants were comprised of undifferentiated precursor cells (respiratory epithelium) as well as primitive alveolar cells (no differentiation into alveolar type I and alveolar type II cells). All epithelial cells contained cytoplasmic organelles commonly linked with zenobiotic metabolism (e.g., endoplasmic reticulum). The explants cultured with NNK demonstrated acute toxicity (swelling of cytoplasmic organelles), while the control explants did not.

A short-term (4 weeks) study was conducted in pregnant hamsters to assess the acute toxicity of different dose levels of NNK on the newborns. High dose levels (200 mg/kg) caused a 50% mortality and significant reduction in birth weight in the newborns. One hundred mg/kg NNK caused reduction of birthweight but no mortality in the newborns, while 50mg/kg NNK had no detectable adverse effects in this assay. Based on these results, a long-term bioassay now has been started to test the transplacental carcinogenicity of NNK in hamsters. All of these experiments are conducted with a single dose of NNK given on the 15th day of gestation. Short-term (4 weeks), acute toxicity assays also are underway to test the effects of multiple NNK doses (days 13 to 15 of gestation) on the offspring. The results of these studies will be the basis of a long-term bioassay (lifetime) on the transplacental carcinogenicity of multiple NNK doses.

In another laboratory (4) five different smokeless tobaccos were analyzed for mutagenic components. Three of these were popular brands of moist tobaccos and two were dry snuffs. Neutral aqueous extracts of all of these were mutagenic in Salmonella TA100, but there was considerable variation in activity. Mutagenesis was only observed in the presence of a rodent liver fraction, and it was found that a mixture of rat and hamster 9000xg supernatants for Aroclor-1254 pretreated animals was most effective in the general activation of the extracts. In addition the use of a slightly acidic preincubation increased mutagenesis. This behavior is typical of mutagenesis by nitrosamines. Formerly, there was about a tenfold range in mutagenic activities of the extracts when taken from the most linear portions of the dose-response curves. However, the curves had only limited regions of apparent linearity, and this comparison may not be striclty valid. Typically, there was an apparent threshold, above which mutagenesis increased rapidly, and then a region where mutagenesis leveled off and declined. The linear mutagenic portion of the curve actually represented a very small fraction of the curve.

In order to facilitate comparison of mutagenic activities between different tobaccos or different fractions of the same tobacco, an attempt was made to linearize the dose-response curves. As N-nitroso compounds are known to be present in smokeless tobaccos, and N-nitroso-N-methyl or ethyl compounds are known to exhibit thresholds in their dose responses, the possiblity was considered that these compounds might be responsible for much of the mutagenic activity in smokeless tobacco. Previous work in this laboratory had shown that the reason for the threshold was that the O^6-methylguanine repair activity was satuarable and that mutagenesis rapidly increased after this activity was saturated. In order to test the hypothesis that mutagenesis induced by smokeless tobaccos resulted at least partially from an N-nitroso-N-methyl compound, bacteria were pretreated with a subthreshold dose of N-nitroso-N-methylurea (NMU), to saturate the repair activity, and then used in the mutagenesis assay. This treatment greatly reduced the threshold and greatly facilitated the comparison of mutagenic activity between different samples. This experiment also provided strong evidence that a substantial portion of the mutagenic activity in the smokeless tobacco results from N-nitroso-N-methyl or ethyl compounds.

Two of the tobacco extracts exhibiting strong activity were further analyzed. These were extracted with either methylene chloride or ethyl ether. Surprisingly, after extraction with ethyl ether, mutagenic activity in the aqueous layer increased, and the activity remained the same in the chloroform-extracted aqueous layer. This suggested that inhibitors of mutagenesis were present which were extracted (at least partially) into the organic solvent. This possibility was subsequently investigated. The aqueous layers were then made acidic and extracted again with organic solvent. Most of the mutagenic activity remained in the aqueous layer. The aqueous layer was then made alkaline and again extracted with organic solvent. Again, most of the activity remained in the aqueous layer. Mutagenic activity was detected in both TA100 and TA104. Potencies of 5-20,000 revertants/g tobacco were obtained from the aqueous layers. Since some of the extractions with organic solvents led to increased mutagenesis in the residual aqueous layers, the possibility was considered that inhibitors of mutagenesis were present in the original aqueous extracts and some of these inhibitors extracted into the organic layers. In order to test this possibility mutagenesis, induced by a relatively high dose of dimethylnitrosamine, was assayed in the presence of a weakly mutagenic dose of tobacco extracts. Although additional mutagenesis would have been expected in the presence of the two mutagens, the opposite effect was observed and mutagenesis actually declined. The decline in mutagenesis was dose-dependent and confirmed the hypothesis that the extracts contained inhibitors of mutagenesis. All of the aqueous and organic layers contained some inhibitory activity, but the alkaline aqueous layer contained the least.

Studies (11) are being conducted to determine how smoking behavior and biological exposure levels are influenced when smokers switch to cigarettes with differing yield characteristics. Human subjects smoked five different commercially available cigarette brands in random order for one week each. These brands delivered 1.1 mg nicotine (usual brand), 1.1 mg nicotine (altered brand), 0.7, 0.4, and 0.1 mg nicotine. Both biological exposure and behavioral measures of smoking were examined concurrently. The study showed that cotinine levels achieved during chronic smoking are related in an orderly manner to package nicotine yields, although they cannot be predicted directly from these package yields. Carbon monoxide exposure, in contrast, was similar when subjects smoked all but the lowest (0.1 mg nicotine) yield cigarettes. The study also provided evidence that smokers compensate for lowered yield by smoking more cigarettes and

taking larger puffs. Health risks related to nicotine, but not to carbon monoxide exposure, may be lowered by smoking lower yield (0.7-0.4 mg nicotine) cigarettes; risks are lower for both constituents when smoking 0.1 mg cigarettes. A second series of studies is designed to identify the factors that influence smoke exposure from 0.1, 0.4, and 1.0 mg nicotine yield cigarettes. Variables being manipulated in systematic fashion include number of puffs, puff volume, and puffing intensity (interpuff interval). These studies will determine the conditions under which 0.1 and 0.4 mg cigarettes can produce exposure levels similar to those obtained by smoking high yield (1.0 mg nicotine) cigarettes.

One study (3) has as its specific aim the development of monoclonal antibodies specific for DNA adducts formed by the tobacco-specific nitrosamine, NNK. There are two types of adducts formed by NNK--methylation adducts and adducts which contain the pyridyl group of NNK. In the past year these workers succeeded in developing a monoclonal antibody specific for the methylation adduct O^6-methyldeoxyguanosine (O^6-MedGuo). This antibody has been used in the high performance liquid chromatography, enzyme-linked immunosorbent assay (HPLC-ELISA) assay, which was previously developed using polyclonal antibodies, resulting in a fivefold increase in sensitivity. This modified assay was then used in a collaborative study to screen DNA from placentas of smoking and nonsmoking women for the presence of O^6-MedGuo. Detectable levels of O^6-MedGuo were found in 2/10 nonsmoking women and 3/10 smoking women. The level of O^6-MedGuo in the two groups was not significantly different. This would seem to indicate that the presence of O^6-MedGuo is not a reliable indicator of exposure to tobacco smoke.

In a related project, radiolabeled NNK, with the label in the pyridine ring, was used to demonstrate binding to both protein and DNA. The exact structure of this adduct has not yet been determined, but work is now in progress. This compound has been coupled to various protein carriers that have been used to immunize mice for the development of monoclonal antibodies. To date, numerous cell lines have been developed which produce antibodies which bind these immunogens, but in no case has the binding been inhibited by this adduct. This indicates that these antibodies are reacting with some other modification that was introduced into the protein carrier during the coupling process. Currently we are coupling the adduct ficoll, a synthetic sucrose polymer. Hopefully this will give a more chemically well-defined antigen and will yield specific antibodies. Different immunization protocols are being tried. The development of antibodies specific for this class of NNK adduct is the highest priority for the coming year. As noted above, O^6-MedGuo has not proven to be a specific marker for tobacco exposure. Because of the nature of the pyridyl adduct of NNK, it will serve as a specific marker for exposure to tobacco products, and will allow development of assays for this adduct in DNA and or protein. This could then serve as an accurate marker for exposure to tobacco products.

With increasing evidence that enviromental tobacco smoke is hazardous to the health of nonsmokers, a need has been perceived for reliable dosimetry. At present, measurement of concentrations of cotinine in plasma, saliva, or urine is felt to be most sensitive and specific. As a prelude to studies of human exposure to environmental tobacco smoke, one laboratory (1) modified an analytical method for nicotine and continues to increase sensitivity for determining low levels in nonsmokers' plasma, which range from 0-5 ng/ml. This has been achieved by concentrating the final extract to a volume of about 20 microliters. The determination is carried out by capillary column gas chromatography using either nitrogen-phosphorus detection or selected ion monitoring mass spectrometry. The

limit of sensitivity is about 0.2 ng/ml. Both nicotine and cotinine can be determined, although determination of nicotine concentrations in nonsmokers is confounded by ubiquitous presence of nicotine in the environment which may interfere with analysis. These workers have just completed analysis of several hundred plasma samples from nonsmokers from the NHANES II study. The data are currently being analyzed.

Additional work has documented that trans-3'-hydroxycotinine is a major metabolite of nicotine, with concentrations in urine generally exceeding those of cotinine. Consequently, 3'-hydroxycotinine is likely to be a good marker for human tobacco exposure. A gas chromatograph/mass spectrometry (GC/MS)-selected ion monitoring assay has been developed and data obtained on urinary concentrations in smokers. In six subjects, concentrations of 3'-hydroxycotinine ranged from 1.2 to 6.5 micrograms/microliter (mean 3.8 micrograms/microliter). In these same subjects, cotinine concentrations ranged from 0.8 to 2.7 micrograms/microliter (mean 1.8 micrograms/microliter). Currently, work is continuing on improving the sensitivity of the assay for determination of plasma levels in pharmacokinetic studies.

There is strong association between cigarette smoking and alcohol consumption, as well as synergy between the two in causation of upper gastrointestinal tract cancer. To examine possible mechanisms of the alcohol-tobacco interaction, this laboratory initiated studies of the effect of alcohol on the metabolism of nicotine and on nicotine and tar intake during cigarette smoking. Nicotine metabolism is being studied using deuterium-labeled nicotine infusions. Four subjects have participated in this protocol to date; 12 will be studied altogether.

The other ETS research (12) addresses analytical chemical technology and characterization of cigarette smoke, again, with particular emphasis on environmental tobacco smoke exposure. Ongoing research seeks to characterize exposures in terms which support toxicological and epidemiological studies by others.

Methodology for the quantitative determination of gas phase organic constituents of environmental tobacco smoke in this laboratory has been refined and has been applied to the analysis of natural environments. The method involves the quantitative collection of volatile organics on a triple sorbent resin followed by thermal desorption gas chromatographic analysis using flame ionization and nitrogen-selective detection. Eleven hydrocarbons, ranging in volatility from isoprene to limonene, and ten organonitrogen compounds, ranging in volatility from acetonitrile to vinyl pyridines, can now be confidently quantitated in indoor air containing otherwise undectable levels of ETS. Many other constituents are observed in the chromatograms, but have not been identified. Nicotine is observed in the chromatograms but analysis is semiquantitative. Evidence is also observed for the presence of naphthalene and higher molecular weight constituents.

Hydrocarbon and organonitrogen compounds have been determined in a restaurant, bowling alley, laboratory test office, and two locations in a residence. Residence samples include the room in which smoking occurred and an upstairs bedroom remote from smoking. Two important observations were made. First, the relative concentrations of the gas phase constituents found in all locations was very similar except for several chemicals that are known to be common indoor air contaminants from sources other than ETS. The ratios of concentrations are also found to be very similar to that of fresh sidestream smoke. Second, most ETS

organonitrogen compounds were undetected in non-ETS contaminated environments. These observations suggest that appropriate nitriles, pyridenes, or pyrroles can serve as high sensitivity markers of gas phase ETS exposure and can allow calculation of the quantities of other gas phase contaminants due to ETS.

Fourier transform mass spectrometry (FTMS) continues to show great promise for the identification of ETS particulate matter constituents. Model compounds suggested to be present in earlier work have been acquired and are being examined by FTMS. Techniques are being developed to incorporate an internal standard in ETS particulate matter to allow quantitative mass spectrometric profiling at trace concentrations. Results continue to indicate that ETS particulate matter is compositionally unique from that of mainstream particulate matter.

SMOKING AND HEALTH

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. BENOWITZ, Neal L. University of California San Francisco 5 R01 CA32389-06	Nicotine and Tar Intake During Cigarette Smoking
2. BRADLOW, H. Leon Rockefeller University 1 R01 CA44458-01	Smoking and Estrogen Metabolism Related to Cancer Risk
3. FOILES, Peter American Health Foundation 5 R01 CA32391-05	Tobacco-Specific Nitrosamine: RIA for DNA-Adducts
4. GUTTENPLAN, Joseph B. New York University 1 R01 CA44986-01	Mutagenic Components in Smokeless Tobacco
5. HECHT, Stephen S. American Health Foundation 5 R01 CA21393-11	Metabolism of the Carcinogen Nitrosonornicotine
6. HOFFMANN, Dietrich American Health Foundation 2 P01 CA29580-07	Experimental Tobacco Carcino-genesis
7. HOFFMANN, Dietrich American Health Foundation 1 R01 CA44161-01A1	Bioassay of Snuff for Carcino-genic Activity in Rats
8. MARSHALL, Milton V. Univ of Texas Hlth Sci Ctr San Antonio 7 R01 CA33069-05	Xenobiotic Metabolism in the Cigarette Smoking Baboon
9. POMERLEAU, Ovide F. University of Michigan at Ann Arbor 2 R01 CA42730-03	The Role of Anxiety in Cigarette Smoking
10. SCHULLER, Hildegard M. University of Tennessee Knoxville 5 R01 CA42829-02	Transplacental Carcinogenicity of NNK
11. STITZER, Maxine L. Johns Hopkins University 5 R01 CA37736-04	Tobacco Yield Changes: Behavioral and Biological Effect

CONTRACTS ACTIVE DURING FY 88

Investigator/Institute/Contact Number	Title
12. GUERIN, Michael Department of Energy Y01-CP-60513	Collection, Separation and Elucidation of the Components of Cigarette Smoke and Smoke Condensates

SUMMARY REPORT

CHEMICAL RESEARCH RESOURCES

The Chemical Research Resources component of the Branch endeavors to make available to the scientific research community those critical resources which are difficult or impossible for most investigators to obtain on their own, but which are necessary for pursuit of studies on the chemical and physical aspects of carcinogenesis. Six resource contracts totaling $1.47 million in FY 88 dollars presently comprise this program. There are no grants included in this effort. The major thrust of these contracts has involved the synthesis and distribution of chemical carcinogens and their derivatives and metabolites for use as authentic research standards. Some of these compounds of major interest are available with ^{14}C- or ^{3}H-labeling.

The Branch supports one contractor, Midwest Research Institute (1), who has as its prime responsibility the maintenance and operation of the Chemical Carcinogen Reference Standard Repository (CCRSR). The repository receives unlabeled chemical compounds, stores them, assures quality control, and upon receipt of orders which have been cleared by the National Cancer Institute (NCI), ships these compounds and bills the user. The funds received for these compounds are used to partially offset the expense for the other contractors which supply the CCRSR with stocks of the chemical compounds.

The current CCRSR catalog lists 638 items; 616 are in stock, 22 are backordered. During the year, 153 requests for 639 aliquots were received; 49 came from industry, 79 from academia, and 23 from Government facilities. One hundred thirty-two requests came from organizations within the United States; 19 were from foreign organizations. The various polynuclear aromatic hydrocarbons (PAH) represented over 75% of the aliquots requested. Of this 75%, the PAH metabolite class was, by far, the most often ordered. Benzo(a)pyrene metabolites were eight of the ten most frequently requested chemicals.

Midwest Research Institute, under the direction of NCI, has revised the chemical compound catalog. The cover has the original design from the previous folder, but the listings of the compounds have been arranged so the user will find them easier to use. This catalog is in routine use at the present time.

Another project, currently underway, is the assembly of pertinent data sheets on the compounds in the CCRSR in the form of a handbook. When this project is completed, plans are to make it available to the scientific community for a fee.

There are four contractors who collaborate closely and who are involved in the synthesis of carcinogenic compounds and/or their metabolites for supply to the CCRSR. These four synthesis contractors develop suitable routes for the unequivocal organic synthesis of compounds designated by the NCI Project Officer, and then develop methods for production of adequate quantities of well-characterized compounds of high purity (generally greater than 98%). Compounds are analyzed by a meaningful combination of techniques to assess purity and confirm structure. These may include ultraviolet, fluorescence, and/or infrared spectrometry, nuclear magnetic resonance, mass spectrometry,

high pressure liquid chromatography, thin-layer chromatography, and elemental analysis. Over 80 chemicals were analyzed during this report period to assure that the purity was acceptable.

One of the support contractors, Eagle-Picher Industries, Inc. (5), is charged with the resynthesis, purification, and characterization of selected metabolites (e.g., quinones, dihydrodiols, epoxides, diolepoxides, and phenols) of benzo(a)pyrene and cyclopenta[c,d]pyrene; and the synthesis of selected aromatic amines, steroid derivatives, physiologically active natural products, and other PAHs. The products include both unlabeled and labeled (^3H and ^{14}C) compounds at a purity level of $\geq$ 98%.

Most of the PAH derivatives have been previously prepared by other NCI contractors who have developed the synthesis routes, characterized the products, determined their relative stabilities, and maximized product yields. The resyntheses of these compounds on this contract utilize these proven procedures to produce well-characterized compounds of high purity to resupply the depleted stores of the NCI CCRSR.

During this report period, 11 compounds were synthesized and sent to the repository. Of the 11 compounds, eight were benzo(a)pyrene metabolites, two were metabolites of 7,12-dimethylbenz(a)anthracene, and one was a 2-aminofluorene derivative. The benzo(a)pyrene metabolites included phenols, epoxides, and tetrols. The present effort is directed to other benzo(a)pyrene metabolites including the 3,6- and 1,6-diones, and the potassium salt of benzo(a)pyrene-9-sulfate. In addition to the effort directed toward resynthesis of carcinogens for CCRSR, a portion of the effort has been directed toward the synthesis of methidiumpropyl ethylinediamine tetraacetic acid (MPE). This compound complexed to iron, and in the presence of oxygen, has been shown to cleave DNA with low sequence specificity. When the synthesis is completed, MPE will be a useful addition to the CCRSR.

A second support contractor, American Health Foundation (2), has placed emphasis on the synthesis of dimethylchrysenes. Five dimethylchrysenes, in quantities of about 20 mg each, were prepared and sent to the CCRSR; also, 14 mg of 5-methylchrysene tetraol was supplied. Substantial progress has been made on the synthesis of the deoxyguanosine 3'-monophosphate adducts of anti-5-methylchrysene-1,2-diol-3,4-epoxide and benzo(a)pyrene-7,8-diol-9,10-epoxide. These adducts should be ready within 60 days and will be shipped to the repository.

Another support contractor, SRI International (4), expends a major portion of effort on the synthesis of fecapentaenes (Fec) and their radiolabeled isotopes. The route used to synthesize them remains the same as the one originally used. This sequence has proved to be satisfactory to prepare the needed amounts of Fec-12 and can be readily adapted for use in the synthesis of 5-^3H-Fec-12, one of the desired radiolabeled analogs.

There have been requests to prepare Fec-12 labeled with tritium at the terminal position of the alkyl chain--i.e., 12-^3H-Fec-12. A synthetic route has been devised for this compound that is derived from tritiated propionaldehyde and trimethyl 3-phosphonyl crotonate. This synthesis should be developed during the coming months. In addition to the work on the fecapentaenes, this

laboratory has recently supplied the CCRSR with 7-methyl-3,4-dihydro-3,4-dihydroxybenz(a)anthracene, trans-8,9-dihydroxy-syn-10,11-epoxy-8,9,10,11-tetrahydrobenz(a)anthracene, and trans-1,2-dihydroxy-syn and anti-3,4-epoxy-1,2,3,4-tetrahydrochrysene (B-26 and B-27).

The contract at Chemsyn Science Laboratories (CSL) (6) differs from the other three described above in that it operates and maintains a repository for radiolabeled PAH derivatives. At the direction of NCI, labeled compounds are subdivided and shipped, with the appropriate documentation, to designated recipients as instructed by the Project Officer. A small prorated fee (payback) is charged for each sample. This fee includes a minor portion of the sample synthesis cost plus handling and shipping charges.

Since the last report, a total of 145 samples of radiolabeled compounds and 31 samples of unlabeled compounds were shipped to 51 different investigators after authorization and after receipt by CSL of documentation demonstrating that the user (or his institution) possesses a license from the Nuclear Regulatory Commission for handling the isotope and quantity requested. The total billed cost was $70,677.

Many times recipients of the radiolabeled compounds also require the unlabeled analog, so it was felt that there would be a considerable savings in shipping and handling costs if the compounds were combined in one shipment; therefore unlabeled compounds, on occasion, are shipped by CSL instead of referring the order to CCRSR. New synthesis work in progress at CSL includes benzo(a)pyrene-1,6 and a 6,12-tritiated quinone derivative for 1-electron oxidation studies and ideno[1,2,3-c,d]pyrene D-ring diolepoxides.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY 88

Investigator/Institute/Contract Number	Title
1. GRAVES, Steven Midwest Research Institute N01-CP-51012	Chemical Carcinogen Reference Standard Repository
2. HECHT, Stephen S. American Health Foundation N01-CP-61041	Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons
3. PETRAZZUOLO, Gary Technical Resources, Inc N01-CM-57658	Working Group on Neoplasms and Related Disorders in Fishes
4. REIST, Elmer J. SRI International N01-CP-71108	Synthesis of Selected Chemical Carcinogens
5. RUEHLE, Paul H. Eagle-Picher Industries, Inc N01-CP-71007	Synthesis of Selected Chemical Carcinogens
6. WILEY, James C. Eagle-Picher Industries, Inc N01-CP-61037	Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons

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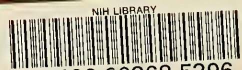
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