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Division of

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# CANCER ETIOLOGY

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Volume II

October 1, 1983-September 30, 1984

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OF HEALTH  
AND HUMAN SERVICES

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ANNUAL REPORT  
DIVISION OF CANCER ETIOLOGY

NATIONAL CANCER INSTITUTE  
October 1, 1983 through September 30, 1984

TABLE OF CONTENTS  
VOLUME II

CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM (CPCP)		Page No.
<u>Laboratory of Biology</u>		
Summary Report		1
<u>Project Reports:</u>		
CE 04629	Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents	6
CE 04673	The Immunobiology of Carcinogenesis	22
<u>Laboratory of Cellular Carcinogenesis and Tumor Promotion</u>		
Summary Report		29
<u>Project Reports:</u>		
CE 04504	Model Systems for the Study of Chemical Carcinogenesis at the Cellular Level	37
CE 04798	Metabolism and Mode of Action of Vitamin A	56
CE 05177	Use of Immunological Techniques to Study the Interaction of Carcinogens with DNA	66
CE 05178	Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis	73
CE 05270	Molecular Mechanism of Action of Phorbol Ester Tumor Promoters	79
CE 05356	Phorbol Ester Receptor Occupancy-Response Coupling in Hormone Responsive Cells	88

Laboratory of Chemoprevention

Summary Report	97
----------------	----

Project Reports:

CE 05051	Isolation of Polypeptide Transforming Factors from Cells	101
CE 05267	Identification of Platelet-derived Transforming Growth Factor-beta	106
CE 05396	Development of Analogs for Study of Oncogenes and Development of the Rat	109
CE 05397	Role of Ectopic Peptides in Phenotypic Transformation	112
CE 05398	Functional Characterization of Transforming Growth Factors and Their Receptors	117

Laboratory of Comparative Carcinogenesis

Summary Report	121
----------------	-----

Project Reports:

CE 04542	Chemistry of N-Nitroso Compounds	133
CE 04580	The Role of Lipotropes in Carcinogenesis	137
CE 04582	Metal Interactions in Carcinogenesis	141
CE 04680	Development and Application of In Vitro Systems Involving Epithelial Cells	145
CE 04812	Cell Interactions During Transformation of Epithelial Cells	148
CE 04967	Role of Genetic and Physiological Factors in Ontogeny and Carcinogenesis	152
CE 05092	Transplacental Carcinogenesis and Tumor Promotion in Nonhuman Primates	157
CE 05093	In Vitro Studies on Organ Specificity in Transplacental Carcinogenesis	164
CE 05157	Biochemical Mechanisms of Organ Specificity in Chemical Carcinogenesis	170
CE 05299	Interspecies Differences in Transplacental Carcinogenesis and Tumor Promotion	175

		Page No.
CE 05301	Biology and Pathology of Natural and Experimentally Induced Tumors	179
CE 05303	Pathogenesis of Natural and Induced Tumors	184
CE 05350	The Thymic Microenvironment During T-Cell Lymphoma Development	188
CE 05351	Ultrastructural Characterization of Normal and Neoplastic Cells	191
CE 05352	Metabolic and Pharmacological Determinants in Perinatal Carcinogenesis	193
CE 05353	Sensitivity Factors in Special Carcinogenesis Models	197
CE 05399	Oncogene Expression in Chemically Induced Tumors	203

#### Laboratory of Experimental Carcinogenesis

	Summary Report	209
	<u>Project Reports:</u>	
CE 04986	Steroid Hormone Action; MMTV Oncogenesis; Viral Oncogenes and Transformation	220
CE 05118	Analysis of Transcriptional Regulation of Murine Retroviruses	226
CE 05260	Regulation of Gene Expression and Differentiation in Neoplasia	230
CE 05261	Metabolism and Mutagenicity of Chemical Carcinogens	237
CE 05262	Mechanism of Chemically Induced Murine Hepatomas	245
CE 05263	Computer Analysis of Carcinogenesis by Two-Dimensional Gel Electrophoresis	248
CE 05313	Early Events in Chemically Induced Hepatocarcinogenesis	253
CE 05315	Regulation of Cell Surface Protein Expression in Normal and Neoplastic Cells	258

CE 05316	Hepatic Asialoglycoprotein Receptor Mediated Gene Transfer	261
CE 05369	Chemical Leukemogenesis: A New Approach	265
CE 05370	Normal Stem Cell Biology and Hemopoietic Regulation	268
CE 05371	Effect of Transforming Proteins of Oncogenic Viruses on Hemopoietic Cells	272
CE 05372	Hormone Sensitivity and Growth Control of Tumor Cells	275
CE 05373	Hepatic Proliferation Inhibitors	278
CE 05374	Structural and Physicochemical Studies of Proteins Relevant to Tumorigenesis	282
CE 05375	Effect of Chemical Leukemogens on Hemopoietic Target Cells	287
CE 05376	The Role of Fatty Acid Acylated Polypeptides in Cellular Transformation	290
CE 05377	Receptor and Growth Factor Genes: Cloning and Characterization	293
CE 05378	Hormone Regulatory Elements: Structure and Function	296
CE 05379	Analysis of Polypeptide Changes During Cellular Differentiation	299
CE 05380	Analysis of Gene Expression During Transformation	302

#### Laboratory of Experimental Pathology

	Summary Report	307
	<u>Project Reports:</u>	
CE 04491	Quantitative Studies on Concurrent Factors in Neoplastic Transformation	320
CE 04493	Bioenergetic Pathways in Chemically-Transformed Epithelial Cells	324
CE 05265	Effects of Chemical Carcinogens on Transforming DNA Sequences and Expression	330

CE 05273	Molecular Mechanisms in Multistage Carcinogenesis	336
CE 05274	Respiratory Carcinogenesis by Chemical and Physical Factors in the Hamster Model	341
CE 05275	Arsenic Metabolism and Carcinogenesis Studies	350
CE 05276	Growth Control in Epithelial Cells and Its Alteration in Carcinogenesis	354
CE 05277	Hamster Respiratory Epithelial Culture Carcinogenesis Model	361
CE 05381	Cellular/Molecular Stages of Carcinogenesis in Respiratory Epithelia	365

#### Laboratory of Human Carcinogenesis

Summary Report		369
Contract Narratives		386

#### Project Reports:

CE 04513	Metabolism of Chemical Carcinogens by Cultured Human Tissues and Cells	396
CE 04846	Biochemical Analysis of Viral Infection and Its Control at the Cellular Level	402
CE 05130	Carcinogenesis Studies Using Cultures of Human Epithelial Cells	405
CE 05133	Epidemiological Studies Using Monoclonal Antibodies to Aflatoxin B <sub>1</sub> -DNA Adducts	410
CE 05192	Repair of Carcinogen-Induced Damage in Human Cells	413
CE 05193	Alterations in Growth and Differentiated Properties in Human Carcinoma Cells	417
CE 05291	DNA Adducts in People Exposed to Benzo[a]pyrene	424
CE 05293	Oncogene Transfection of Human Cells	428
CE 05321	Carcinogen-DNA Adducts by Synchronous Fluorescence Spectrophotometry	431
CE 05322	Role of Hepatitis B Virus in Hepato-cellular Carcinoma	434

CE 05324	Human Lung Carcinoma/Bronchial Epithelial Cell Hybrid Genetics	438
CE 05325	Chemical Carcinogenesis and 5-Methylcytosine Patterns in DNA	440
CE 05326	HLA Antigens: Structure, Function and Disease Association	444
CE 05327	Development and Use of Antibodies to Detect Carcinogen-DNA Adducts	449
CE 05328	Immunologic Studies of Human T-Cell Lymphoma Virus	453
CE 05338	Characterization, Mode of Action, and Evolution of the Oncogene <u>raf</u>	457
CE 05341	Model Systems for the Study of Physical Carcinogens at the Cellular Level	462
CE 05402	Lymphokine Production from Human T-Cell Leukemia Virus-Infected Cell Lines	467
CE 05403	Molecular Analysis of Gene Regulation and Proliferative Control in Human Cells	470
CE 05405	Transfection of TBE-1 with v-myc, Burkitt's myc, raf, and raf/myc Oncogenes	473
CE 05406	Construction of a Genomic Library from the Lung Carcinoma, A1146 DNA	476
CE 05407	Effects of Tumor Promoters and Cocarcinogens on Human Esophageal Cells	478
CE 05408	Drug Metabolism Phenotyping of Nonhuman Primates	481
CE 05409	Control of Growth and Differentiation of Human Bronchial Epithelial Cells	484
CE 05410	Hepatitis B Virus Carcinogenesis	488
CE 05411	Studies of Proliferative Response in Normal Human Bronchial Epithelial Cells	492
CE 05412	Model System for the Investigation of Putative Tumor Promoters in NHBE Cells	497
CE 05413	Enzyme Immunoassay for Endogenous N-Nitrosation Products	500

Laboratory of Molecular Carcinogenesis

Summary		503
<u>Project Reports:</u>		
CE 04496	The Role of Chromosomal Proteins in the Structure and Function of Chromatin	512
CE 04516	Cellular and Molecular Effects of Psoralen Plus Ultraviolet Light	517
CE 04517	DNA Repair in Human Cancer-Prone Genetic Diseases	520
CE 04525	Studies on Electrophoretic Techniques for Protein, RNA, and DNA	525
CE 04555	Multiple Steps Involved in Neoplastic Transformation by Chemical Carcinogens	528
CE 04578	Role of Molecular and Functional Alterations of Actin in Neoplastic Transformation	532
CE 04782	Hormones and Breast Tissue Interactions	537
CE 04785	DNA Repair Studies on Human and Mouse Normal, Tumor and Transformed Cells	541
CE 05086	Monoclonal Antibodies to Human Carcinogen Metabolizing Enzymes	548
CE 05109	Changes in Chromatin Structure During Carcinogenesis	551
CE 05125	Preparation of Monoclonal Antibodies to Rat Liver Cytochromes P-450	554
CE 05196	Cloning, Structure and Regulation of the Genes for the Cytochromes P-450	558
CE 05204	DNA Sequence Alterations In Vivo Following DNA Modification by Carcinogens	564
CE 05208	Phenotyping of Cytochrome P-450 in Human Tissues Using Monoclonal Antibodies	567
CE 05240	Rearrangement of a Plasmid Sequence in Mammalian Cells	570
CE 05241	Role of Left-Handed Helical DNA in Carcinogenesis	573

CE 05242	Monoclonal Antibody Mapping of Cytochromes P-450 in Different Species and Tissues	577
CE 05317	Opal Suppressor tRNA Genes in Human, Chicken, Rabbit and Xenopus Genomes	580
CE 05318	Immunopurification and Characterization of Cytochrome P-450	584
CE 05320	Regulation of Oncogene Expression	587
CE 05339	Radioimmunoassay of Cytochromes P-450 Using Monoclonal Antibodies	591
CE 05415	Structure of Human Actin Genes: Characteristics and Evolutionary Considerations	594

### Chemical and Physical Carcinogenesis Branch

Summary Report	599
----------------	-----

### Biological and Chemical Prevention

Summary Report	604
Grants Active During FY 84	611
Contracts Active During FY 84	615

### Carcinogenesis Mechanisms

Summary Report	617
Grants Active During FY 84	628

### Diet and Nutrition

Summary Report	637
Grants Active During FY 84	640

### Molecular Carcinogenesis

Summary Report	644
Grants Active During FY 84	672

### Smoking and Health

Summary Report	690
Grants Active During FY 84	695
Contracts Active During FY 84	696

### Chemical Research Resources

Summary Report	697
Contracts Active During FY 84	701



CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM

ANNUAL REPORT OF

THE LABORATORY OF BIOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

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

The major emphasis of the Laboratory of Biology concerns the identification of relevant alterations in target cells that result in malignancy. For this purpose several different mammalian cell model systems have been used; the models, for the most part, have originated in the laboratory. Because of our ability to obtain neoplastic conversion of diploid cells with our transformation models, we are directing our efforts toward understanding the basic molecular events occurring during transition to malignancy. The application of gene cloning and flow cytometry has added new dimensions to the study of stages of carcinogenesis. For example, we are currently analyzing guinea pig and hamster chemically transformed lines for cellular proto-oncogenes. In addition, human cells exposed to known carcinogen insult are being cataloged in terms of cell surface changes shared with spontaneous human malignant cells.

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. By superimposing molecular events on biological observations, it should be possible to make conclusions concerning gene expression relevant to control of differentiation and cancer. The Tumor Biology Section emphasizes host interactions particularly from an immunological point of view. The objective of these studies is to examine phenotypic changes at the cell surface to identify the effectors biochemically and to study their mode of action.

The process of transformation leading to neoplasia can be readily demonstrated in a number of systems, particularly rodents. For example, hamster cells are transformed by a variety of carcinogens that are also mutagens as well as nonmutagens. On the other hand, human cells obtained from normal individuals or those with genetic disorders appear refractory by transformation by known carcinogens. When protocols that have been successful in demonstrating transformation of hamster cells have been used with human cells and with carcinogens known to cause cancers in people, fully neoplastic lines have not been obtained. Furthermore, cocarcinogenic experiments utilizing combination of carcinogens from different categories such as viruses, chemicals, or radiation have also

failed. Because carcinogen treatment of human cells has not resulted in either permanent or tumorigenic lines, the term "transitory" transformation is suggested to describe the transformation that results with human cells. This statement is made even though cells subjected to chemicals such as alkylating agents, polycyclic hydrocarbons, lactones, or ultraviolet irradiation will result in cells that produce progeny that grow in suspension and form small nodules when injected into nude mice. It is apparent, however, that control mechanisms differ between hamster and human cells. Although hamster and human cells have a similar survival with similar doses of the chemical or physical agent, they rely on different repair mechanisms; human cells have been shown to excise DNA damage rapidly relative to their replication rate while hamster cells continue to divide with unrepaired DNA lesions in the template strand. To demonstrate neoplastic transformation of human cells in vitro, we conclude that it will be necessary to alter cells to have at least two properties in common with tumor cells: cell immortality and loss of growth control. A cell that has become immortal but exhibits growth control will cease dividing when a signal or contact inhibition is activated. On the other hand, a cell that has lost its growth control will only divide for a finite number of generations if it is not immortal.

One can identify the property of loss of growth control in human cells treated with chemical, physical or biological agents. These "transitorily" transformed cells have extended life-spans but are not immortal. Therefore, a new approach for obtaining permanently malignant human cell lines in vitro is to first obtain immortal but not transformed human cells. The activated myc proto-oncogene from chicken or mouse has been reported to confer immortality to primary rat cells. Therefore, we have transfected a chicken-derived activated myc oncogene into otherwise not treated human foreskin fibroblasts. Isolated cell lines containing the myc DNA have been obtained which are now being evaluated for myc gene expression, immortality and susceptibility to further transformation. Another putative oncogene that may partially transform human cells is human papilloma virus, HPV-16. In nonhuman experimental systems papilloma viruses are cocarcinogens. There is a high association of HPV-16 with cervical carcinoma compared to its infrequent appearance in cervical warts. Therefore, a recombinant DNA construct, antibiotic selectable, with HPV-16 in a form that will allow full expression of biological activity has been made. This DNA is currently being used for transfection into human epithelial cells.

A number of "transitory" transformations of human cells have been obtained by first blocking cell multiplication using amino acid deficient medium. As a result of treating cells during the S phase of the cycle with aflatoxin B1 or ultraviolet light and then immediately culturing in agarose, cell lines were obtained from single colonies, propagated in culture for several passages, and analyzed for chromosomal alterations. With both carcinogens, cells exhibiting an abnormal chromosome constitution were obtained. Subsequent high resolution banding analysis showed that in the ultraviolet light line there was an abnormal chromosome originating from chromosome No. 22 with an extra band present in the long arm; the latter was a tandem duplication at the locus of the c-sis oncogene. The aflatoxin line which also produced nodules in nude mice had chromosomal abnormalities involving the short arms of chromosomes 1, 11, and X. The c-Ha-ras 1 oncogene resides on the short arm of chromosome 11. These observations are important for two reasons: (1) they reflect an attribute of neoplasia, namely aneuploidy; and (2) the rearrangements observed are often associated with oncogenes that become active as a result of new chromosomal configurations. Therefore, it is imperative to determine the relationship between chromosome

rearrangements and oncogene activation in human cells because it will probably be pertinent to the mechanism responsible for the conversion of normal cells into neoplastic cells. For this reason we have undertaken the mapping of a series of oncogenes utilizing a new method developed in this laboratory for obtaining good resolution G bands on chromosome preparations processed for the hybridization and autoradiography required for grain localization on chromosomes. The method is the first successful use of trypsin to produce G bands on chromosome preparations covered with emulsion. Two proto-oncogenes molecularly cloned from a normal DNA library using viral oncogene probes have been used to identify the N-ras and the c-Ki-ras 1 sequences. The N-ras was assigned to the short arm of the chromosome 1 (1p13) and c-Ki-ras 1 to the short arm of chromosome 6 (6p21). The ability to identify oncogene sites will make it possible to determine when and where oncogenes become active as a result of treating human cells with carcinogens. The question remains whether "transitory" human cell transformations have any translocations or duplications of proto-oncogenes.

It is known that spontaneous tumor-derived cells have an increased sensitivity to natural killer (NK) cell destruction, reflected by the release of <sup>51</sup>chromium as a result of cell lysis. Furthermore, this effect by NK can be enhanced by treating the cells with leukoregulin (LR). "Transitory" transformed human cells are sensitive to LR enhanced NK cytotoxicity induced by carcinogens or transfection with HeLa or viral DNA, whereas their normal counterparts are not. Increased sensitivity to LR induced membrane permeability as measured cytofluorometrically using a fluorescein dye occurs without loss of cell viability in tumor-derived cells as well as those exhibiting "transitory" transformation. The similarity of these results with "transitory" transformants and with tumor-derived cells originating from either human or other mammals suggests that these markers are not specific for malignancy. Nevertheless, the ability to select cells by sorting specific subpopulations will enable characterization of their properties relative to carcinogenesis. It will be possible to compare the effects of insulting subsets of cells that do or do not exhibit increased membrane permeability.

The characteristics of Syrian hamster cells in vitro makes it a prototype model for studying transformation and its relevance to cancer. Transformed cells developed in vitro mimic tumor-derived cells resulting from in vivo carcinogen treatment. With hamster cells it has been possible to prove two important primary factors relevant to carcinogenesis. First, the dose response relationships coupled with a Poisson distribution of transformation indicate that an inductive rather than a selective mechanism is responsible for transformation. Secondly, the inability to demonstrate viral activation in this system provides the best evidence to date that the effects of chemical and physical agents that induce transformation must be considered directly responsible for transformation. Thus, this specific system is a valid in vitro approach for investigating carcinogenesis. It is now possible to study the steps and/or stages involved in a transition of normal cells to malignant ones in the absence of host modification. Because the model lends itself to quantitation, the modulation of transformation by a variety of carcinogens and noncarcinogens can be investigated. Furthermore, the expression of the initiated and promoted stages of transformation can be separated for study.

Bisulfite, a non-mutagen at neutral pH is being studied because this compound was reported to enhance UV mutations. Therefore, its effect on UV transformation was determined. Bisulfite alone was able to induce a dose-dependent increase in transformation and a combination of bisulfite and UV resulted in an additive effect. Because it is considered an ubiquitous pollutant in the form of SO<sub>2</sub> and induces a

dose-dependent transformation frequency up to 3% without cytotoxicity, the underlying biochemical mechanism responsible for transformation is important. The acute effects of this compound are limited. It does not cause chromosome aberrations but it does minimally increase sister chromatid exchanges at concentrations higher than are necessary for transformation. There was also no indication of induced excision or post-replication repair. However, bisulfite slowed down the rate of DNA synthesis as a result of a reduction in the number of replicons contributing to DNA synthesis. An examination of the neoplastic lines capable of producing progressively growing tumors show that all lines have both structural and numerical chromosome abnormalities. An unusual characteristic of the chromosome picture produced by transformation with bisulfite was an increase of polyploidization. Because bisulfite is a non-mutagen having a minimal effect on DNA metabolism, an analysis of protein alterations using 2D gel electrophoresis has begun. Controls and tumor lines have over 800 proteins that are identical. However, all the transformed lines examined have specific protein changes; there are three additional new proteins and two protein shifts; one protein is missing. These identical protein changes raise the question of whether they reflect genetic changes critical for transformation. The isolation of these proteins and identification of their genes will make it possible to elucidate their role in carcinogenesis. This is now under investigation.

The ability to demonstrate transformation of Syrian hamster cells with chemicals of diverse classes including polycyclic hydrocarbons, alkylating agents, lactones, trace metals, and hormones as well as the ability to modulate the transformation frequency with other carcinogens and noncarcinogens raises a very important question. If viruses do not have a direct effect, are there other elements that make it possible to transform hamster cells with what appears to be relatively easy compared to human cells. Interstitial A-particles (IAP) are retrovirus-like entities expressed in many mouse tumor cells. However, their function is unknown and they are not considered to be oncogenic. Recently, such sequences have been shown to exist in hamster cells. It is estimated there are between 1000-2000 copies per genome. Because IAP-DNA sequences have been detected in a rearranged mouse cellular oncogene it is of interest to determine if their transposition or enhanced expression of neighboring genes might be associated with transformation. No increased expression of A-particles was observed in hamster cells transformed by physical or chemical carcinogens, or by combinations of chemical and physical carcinogens that gave enhanced transformations or in tumors that resulted from the direct transformation by carcinogens. Evidence for the distribution of these sequences in hamster cells was obtained by in situ hybridization on fixed chromosome preparations. Approximately half of the IAP-DNA sequences were found concentrated in the constitutive heterochromatin of the chromosomes; the remaining exist randomly throughout the genome with a few exceptions. Their random distribution is useful as a probe for detecting the presence of hamster DNA in recipient cells of other species which have been transfected with hamster DNA.

In vitro carcinogenesis models can be used to study the mechanism of activation as well as the presence of oncogenes in transformed cells. The hamster model is useful because a large number of independent transformed lines induced by different carcinogens are available for assessment. The sensitivity of DNA of five transformed cell lines induced by 3-methylcholanthrene to inactivation by several restriction enzymes differs. This suggests that the carcinogen can activate different oncogenes. The guinea pig model is important because it demonstrates distinct extended stages. A number of tumorigenic cell lines as well as their preneoplastic progenitors are available for study. Five guinea pig lines induced

by four different carcinogens contain activated oncogenes related to ras. Because none of their preneoplastic progenitor cells do, therefore, the activation of this gene occurs at a late stage of carcinogenesis and is closely associated with, if not responsible for, malignancy. The activated oncogene from one of these lines has been cloned and is under further investigation.

Lymphokine preparations from antigen or mitogen-stimulated lymphocytes from normal hamster or guinea pig cells contain a potent anticarcinogenic activity. They prevent as well as inhibit the subsequent development of carcinogenesis. The new physiologic state, like many hormone responses, is induced rapidly and persists for a short time. When the lymphokine-induced anticarcinogenic state is present at the time of carcinogen exposure, carcinogenesis is prevented and when present shortly after carcinogen exposure, the further development of initiated or complete carcinogenesis is irreversibly inhibited. In fully tumorigenic cells, cellular proliferation is inhibited; cellular destruction may also occur with high lymphokine concentrations. Lymphokine also has the ability to increase the sensitivity of cells to destruction by natural killer cell cytotoxicity.

With molecular size fractionation techniques the lymphokine co-purifies with lymphotoxin. With a combination of isoelectric focusing and molecular size fractionation by column chromatography the anticarcinogenic activity of hamster lymphokine was separated into two fractions with mean isoelectric pHs of 4.6 and 5.0 and 45,000 molecular weight proteins. Lymphotoxin activity, identified by the ability to lyse L929 murine target cells, was present only in the isoelectric 5 fraction suggesting that the anticarcinogenic activities were either a new form of lymphotoxin or a distinct new lymphokine. The anticarcinogenic activities, moreover, were free of interleukin 1 and 2, macrophage activating factor, macrophage migration inhibitory factor, and interferon activities. Human lymphokine preparations prepared from phytohemagglutinin-stimulated freshly isolated peripheral blood lymphocytes also has a new lymphokine with anticancer activities. Using a combination of isoelectric focusing and high performance liquid chromatography within a silica based molecular sizing column, a lymphokine distinct from previously recognized lymphokines with a mean molecular weight of 50,000 by silica column HPLC and isoelectric pHs of 5 and 7.5 has been isolated. The lymphokine possesses the cell proliferation inhibitory and natural killer cell sensitizing activity for neoplastic cells and is free of lymphotoxin activity. Further support for the existence of the new lymphokine is that the ratio of the tumor cell proliferation inhibitory activity to lymphotoxin activity varies from one lymphokine preparation to another and that the tumor cell growth inhibitory activity is decreased after protease but not neuraminidase digestion, whereas lymphotoxin activity is decreased after either treatment. The new lymphokine has been named leukoregulin to indicate its origin from leukocytes and its regulatory action.

It is apparent that the research program of the Laboratory of Biology has evolved from a strictly biological one to one that applies its resources of material and experience to molecular biological and immunological approaches toward understanding the mechanisms responsible for the critical steps leading to the neoplastic state. The study of genetic and extracellular biological factors important to the carcinogenesis process will enable the answers of critical questions such as: Does a relationship exist between chromosome rearrangements of transformed cells and enhanced expression of oncogenes?; How many changes are required to make a cell immortal?; What is the significance of an oncogene to the induction of the neoplastic state and cancer?

PERIOD COVERED

October 1, 1983 to September 30, 1984

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)

P.I.: Joseph A. DiPaolo Chief, LB, NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biology

SECTION

Somatic Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

3.0

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.)

Transformation leading to neoplasia of "target" cells from different species, including human, is being studied with the ultimate aim of understanding critical steps leading to transformation of human cells. There are differences in control mechanisms between rodent species that are readily and reproducibly transformable and human cells that are relatively refractory to transformation. Carcinogen treatment of cells from normal or individuals with genetic disorders only results in "transitory" transformation. Therefore, to induce immortality in human cells proto-oncogenes are being introduced into cells to facilitate the expression of malignancy when combined with pre- or post-carcinogen exposure. The quantitative Syrian hamster embryo cell (HEC) transformation model is being used to study some of the underlying biology of transformation. A lymphokine, leukoregulin (LR), inhibits promoted transformation. Results of pulse experiments prove that it causes a transient physiological alteration of normal cells making them refractory to carcinogen-induced transformation. Dose-dependent transformation of HEC occurs with the non-mutagen, bisulfite, a chemical ineffective in inducing DNA damage or chromosome aberrations. Permanent, tumorigenic lines with a variety of chromosome changes, including increased ploidy and chromosome markers, were obtained. Regardless of the chromosome changes, all neoplastic cell lines have identical protein changes (new, missing, and shifted proteins). Comparison of O-6 methylguanine lesions and methylating carcinogen-induced HEC transformation suggests that the target for initiation is 50-500 genes, a number inconsistent with the concept of a single base mutation. Tumorigenic HEC contain activated oncogenes not related to ras. In the guinea pig transformation model a ras-related proto-oncogene becomes activated concurrently with tumorigenicity many months after carcinogen treatment. An 11KB EcoR1 DNA fragment related to ras has been cloned from 3T3 cells transformed by DNA from a guinea pig cloned line.

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

J.A. DiPaolo	Chief	LB NCI
S. Amsbaugh	Microbiologist	LB NCI
J. Doniger	Senior Staff Fellow	LB NCI
N.C. Popescu	Senior Staff Fellow	LB NCI

Objectives:

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

Methods Employed:

The "competence" of the target cells for transformation differs from species to species. Therefore, the susceptibility to transformation is studied with cells from a variety of species: hamster, guinea pig, rat, and human. Each has its own advantage which ranges from quantitative transformation to defined stages of carcinogenesis and in the case of human cells, to the refractiveness to transformation. All procedures performed are with the view of quantitating the progression of the transformation phenomenon. Such an approach is required to determine whether or not the observed transformation is due to the direct or indirect effect of the carcinogen and in order to study the early events associated with in vitro transformation. Cultures are made with freshly isolated cells from animals and humans and human cell strains obtained from patients with metabolic disorders that as controls have many of the attributes of "normal" cells, and from cell lines which are known to exhibit some of the properties associated with nontransformed cells. Cells derived from whole embryos or specific organs are grown in complete medium in the presence or absence of irradiated cells (feeder cells) and exposed to carcinogen transplacentally or prior to or subsequent to seeding the cells in plastic dishes. The transformation frequency takes into consideration the observed rate of transformation on a per cell basis or on the number of colonies obtained.

Procedures utilized for detection of acute carcinogen-induced damage include sister chromatid exchange (SCE), chromosome aberrations and micronuclei identification. Structural chromosome alterations are determined by high resolution analysis of G band of prophase or metaphase chromosomes in conjunction with procedures for localization of constitutive heterochromatin (C band) and nucleolar activity (Ag-AsNOR). In situ hybridization on fixed chromosome preparations is used for

gene mapping. Autoradiography and liquid scintillation procedures are used for DNA, RNA and protein metabolism.

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

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

#### Major Findings:

Emphasis has continued to focus on the problems associated with obtaining transformed human cells utilizing Syrian hamster embryo cells (HEC). The use of cells from the two species is important because they permit comparison of a cell model that is readily transformable with one that is refractory to experimental transformation. Both the normal hamster and normal human have a stable karyotype and senesce. We are concerned with why it is possible to obtain dose-dependent transformation of HEC that results in permanent neoplastic cell lines. No permanent malignant lines have resulted from treatment of normal human cells *in vitro* with a physical or chemical carcinogen. Experimental transformation of normal human fetal or foreskin cells as a result of carcinogenic insult (chemical, physical or viral) should be called "transitory" or intermediate transformation. Of the many properties of tumor cells, two of the most important are cell immortality and loss of growth control. In fact, both at least are necessary for tumorigenicity. A cell that has lost only its growth control will not form a tumor because it will only divide for a finite number of generations before it senesces and dies. A cell that has become immortal, indefinite life span, but exhibits growth control will cease dividing when a signal of contact inhibition is activated. Therefore, we are using new approaches to study and to attain critical characteristics of tumor cells.

The study of the relationship between chromosome rearrangements and oncogene activation in human cells is essential for the understanding of the mechanisms responsible for the conversion of cells into neoplastic cells. A primary requirement is the precise localization (mapping) of proto-oncogenes in the human genome. Although cloned DNA is utilized as a probe for human genes in cell hybrids preserving particular human chromosomes, the location of the gene in the chromosome is not precise. By contrast, in situ hybridization allows gene localization to a specific chromosome location. In situ hybridization involves radiolabeling the cloned DNA probe with very high specific activity and hybridizing the DNA to fixed chromosome preparations in which each chromosome is identifiable under the microscope. A series of treatments prior to chromosome preparation are necessary to produce cell synchrony and to obtain elongated prophase or prometaphase chromosomes suitable for high resolution banding analysis. We developed a new method for obtaining good resolution of G bands on chromosome preparations processed for in situ hybridization. Optimal G banding resolution is crucial for accurate grain localization. The presence of emulsion has an adverse effect on chromosome banding; thus, known methods for inducing G banding have been relatively unsuccessful. Our method consists of two steps to produce high quality G banding. Shortly after autoradiographs of hybridized chromosome are developed, the slides are



stained for 3 min with Wright stain. Labeled metaphases are photographed with a plan 60X objective and these slides are destained through a series of absolute methanol and 95% ethanol, air dried, immersed in 0.03% Gibco trypsin 0.12% EDTA in HBSS for 2.5 min and immediately restained with Wright stain. The metaphases are relocated and a second photograph is obtained to identify grains on specific chromosome regions. This method is the first successful use of trypsin to produce G bands on chromosome preparations covered with emulsion and is highly reproducible. It has a major advantage over the existing techniques because G banding can be obtained even in contracted chromosomes. Therefore, this method is used for banding chromosomes of tumor cells in culture of *in situ* which ordinarily exhibit more contraction than chromosomes obtained from normal lymphocyte cultures. Three proto-oncogenes (N-ras, c-Ki-ras-1 and c-K-ras-2) have been molecularly cloned (by the Laboratory of Cellular and Molecular Biology) from a normal DNA library using viral oncogene probes to identify these sequences. N-ras was isolated from DNA of a human neuroblastoma cell line, SK-N-SH, and is similar to other members of the ras oncogene family. Under stringent hybridization conditions, N-ras detects only its homologue and none of the other ras gene family members. Radiolabeled probes were used on normal human chromosomes derived from peripheral blood lymphocyte cultures. N-ras was assigned to the short arm of the chromosome 1 (1p-13) and c-K-ras-1 to the short arm of the chromosome 6 (6p21), and c-Ki-ras-2 is on the long arm of chromosome 12, but is yet to be precisely mapped.

Several leukemias, lymphomas and solid tumors are associated with specific chromosomal translocations. These translocations can transpose inactive oncogenes to new chromosomes near active promoters. This results in high expression of the gene playing a crucial role in carcinogenesis. Although human cells exposed *in vitro* to chemical or physical carcinogens exhibit fewer chromosome rearrangements than human tumors derived from the same type of target cells, aneuploidy occurs with both structural and numerical chromosomal aberrations. Normal fibroblasts "transitorily" transformed *in vitro* by chemical or physical carcinogens have chromosome translocations that can involve loci in the vicinity of human protooncogenes. Freshly obtained foreskin cultures were exposed to ultraviolet light, and cultivated in agarose. A cell line derived from a single colony (UV-1) isolated from agarose was obtained and propagated in culture for several passages prior to the chromosome analysis. An abnormal chromosome originating from chromosome 22 was found in 80% of these cells. High resolution banding analysis of this chromosome showed that an extra band was present in the long arm as a tandem duplication near the locus for the c-sis proto-oncogene. Another human cell line derived from foreskin fibroblasts treated with aflatoxin B<sub>1</sub> was examined cytogenetically at passage 40 after carcinogen insult. These cells produced nodules after subcutaneous inoculation into nude mice. The cells had abnormalities involving the short arms of chromosome 1; 11 and X chromosome. Alterations of the short arm of chromosome 11 is important because it involves the segment for the c-Ha-ras-1 oncogene. Very few reports of physical or chemical carcinogen induced stable chromosome rearrangements in normal human cells exist because of the remarkable *in vitro* stability of the human chromosome constitution. Chromosome changes observed in these "transitory" transformants are meaningful because they show that human cells treated with physical or chemical carcinogens exhibit aneuploidy, an attribute for neoplasia. More importantly, these changes involve chromosome rearrangements of segments with proto-oncogenes which may become activated as a result of new chromosome configurations.

A new approach for obtaining human cells susceptible to malignant transformation by chemical or of physical carcinogenesis to use a cancer promoting virus. Papilloma viruses are known to work in concert with chemical carcinogens in rabbits and to be responsible for laryngeal and cervical papillomas in humans. The laryngeal papillomas were converted to carcinomas by X-irradiation. The high incidence of human papilloma viral (HPV) related sequences (HPV-16 or HPV-18) associated with cervical carcinoma but not condyloma acuminatum (cervical warts) suggests the HPV-16 (or 18) are involved in the establishment or maintenance of the malignant state. To determine the role of papilloma viral DNA in transformation, HPV sequences are being transfected into human fibroblasts and epithelial cells. HPV-16 was originally cloned from circular DNA using a unique Bam HI site. Because this Bam HI cut could inactivate HPV-16 biologically, a new construct was made with a head to tail duplication of HPV-16 cloned into the Bam HI site of pSV2 neo. After DNA transfection, G-418 (neo) selection is being used to isolate human cell clones containing HPV-16 genome. Following demonstration of expression of the papilloma virus, the cells will be exposed to chemical carcinogens and will be studied for further development of neoplastic properties.

Activated myc genes are reported to immortalize mammalian cells. Myc is a normal proto-oncogene of fowl that has homologues in mice and humans. Amplification of gene expression has been found in translocations that exist in Burkitt's lymphoma; it is also involved in non-hematopoietic diseases such as kidney, pancreatic, and liver carcinomas, mesothelioma, and neuroblastoma. Therefore, it is not tissue specific. Furthermore, myc was effective in transforming primary rat fibroblasts after co-transfection with the EJ transforming gene of human bladder. In order to develop immortal human cell lines for further carcinogenesis studies, activated myc genes are being transferred into normal human fibroblasts by protoplast fusion. A 9.1 Kb Eco RI DNA fragment, cloned from MC29 transformed quail cells (provided by Dr. Takis Papas) containing the MC29 long terminal repeat (LTR) and the myc gene, was cloned into pSV2 neo. After protoplast fusion into human neonatal foreskin, fibroblasts cells containing pSV2 neo were selected for using the antibiotic G418. Three G418 resistant cell lines contain the 9.1 Kb sequence as determined by Southern blot hybridization. These lines are being evaluated for myc gene expression and extended life span.

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

analyzed by the Southern blot protocol with nick translated mouse v-bas sequence as a probe. V-bas specifically hybridizes to the Harvey ras sequence of Ha-MuSV.

DNA from both GM3498B-HV1 and GM3498B-HV2 transformed foci as well as Ha-8 contain a 4.5 Kb fragment after digestion with Sst 1 that is homologous to v-bas as well as p-14 (a fragment of the Ha-MuSV genome containing the LTR but not Harvey ras). This demonstrates that the transformed phenotype was the result of incorporation of the Ha-MuSV sequence into the human recipient cell DNA. These experiments demonstrate that transformation of NIH 3T3 cells by transfection with DNA from human tumor cells is accomplished in a manner analogous to transfection of non-malignant human cells by Ha-MuSV DNA. However, because these eventually senesce they are classified as "transitory" transformations. Parental GM3498B cells were also transfected with HeLa DNA. Three of nine foci have an extended life span; all had high SCE. Because human DNA was transfected into human cells, it is difficult to determine if the transformants contain HeLa DNA. However, HeLa cells contain HPV-18 DNA in their genomes which is suspected of contributing to their malignant phenotype. Therefore, we are examining the GM3498B-HeLa transformants for the presence of HPV-18 DNA sequences.

Even though transformation of normal human cells by a variety of chemical and physical agents or by transfection procedures results in transitory or intermediate transformation, no laboratory can reproducibly obtain malignant cells. This raises the question: is it logical to ask whether what is being observed is a preneoplastic stage. Therefore, it is important to determine whether human cells that have been subjected to known carcinogens differ from normal cells and share unique properties of tumor cells. One property of cancer cells compared to normal cells is their high sensitivity to natural killer cell (NK) cytolytic destruction which is measured by the release of  $^{51}\text{Cr}$  from labeled target cells. The NK anti-cancer activity can be enhanced by leukoregulin (LR), a natural lymphocytic hormone that inhibits the growth of tumor cells and irreversibly inhibits carcinogenesis (see below). After a 2 hr treatment plus NK,  $^{51}\text{Cr}$  released from human leukemia (K562), carcinoma (RPM12650), or sarcoma (HS1) cells was double that with NK alone, whereas the  $^{51}\text{Cr}$  release from non-transformed Bloom Syndrome cells by NK was similar with or without LR. As in the case of spontaneous tumor cells, the release of  $^{51}\text{Cr}$  by NK was also enhanced twofold when "transitorily" transformed (by transfection) GM3498B-HV2 and GM3498B-HeLa cells were the targets. LR also alters increased membrane permeability, as measured by decreased fluorescein retention using flow cytometry. LR-treated HS1 or K562 tumor cells or GM3498B-HV2, or GM3498B-HeLa but not parental GM3498B cells exhibited increased membrane permeability after LR for 1 hr. UV-1 fibroblasts derived from a progressively growing colony in agarose after UV-radiation of foreskin fibroblasts also showed LR stimulated membrane permeability as did an anchorage independent, nontumorigenic HeLa X normal human cell hybrid, ESH5A-4-4-4. The development of NK sensitivity during preneoplastic stages of carcinogenesis in other species suggests that this is an important stage of carcinogenesis because human cells "transitorily" transformed not only by radiation but also by DNA transfection with a known oncogene are LR sensitive; susceptibility of transformed cells to cellular recognition and destruction by NK has a genetic basis. Finally, the ability to select specific subpopulations by cell sorting will enable characterization of their properties relative to normal and malignant cells.

In human cancer a correspondence exists between chromosomal localization of cellular proto-oncogenes and break points associated with several specific chromosome

translocations. Fragile sites have been identified in the human genome; some specific translocations occur as a result of preferential breakage at these sites. Direct evidence for the involvement of fragile sites in chromosome translocations in carcinogenesis is obtainable by examining the location of carcinogen-induced damage and breakage points of stable chromosome translocation. A suitable model for such an analysis is the rat cell because the rat genome has several highly vulnerable sites to carcinogen-induced damage. Furthermore, both fibroblast and epithelial cells can be transformed in vitro by chemical carcinogens. The first two chromosomes of rat embryo cells have an increased vulnerability to 7-12-dimethylbenzo(a)anthracene (DMBA) damage within one day of treatment. Four regions on chromosome 2 (q-22, 24, 26 and 34) were the most susceptible sites. Chromosome 2 has a special significance to rat neoplasia because alterations of this chromosome frequently occur as a result of treatment with DMBA regardless of whether a carcinoma, sarcoma or leukemia develops. The chromosomes of in vitro transformed lines of rat embryo fibroblasts, mammary epithelial cells, and fibrosarcomas induced in vivo by DMBA were analyzed by banding methods. The first three chromosomes of the complement were nonrandomly involved both in vitro and in vivo transformed cells. All structural abnormalities involving chromosome 2 were examined to locate the break points and to determine whether a correlation exists between their location and vulnerable site to acute damage. From a total of 9 chromosome markers derived from chromosome 2 only one site vulnerable to breakage (2q34) was coincidental to the translocation points. This analysis indicates that stable chromosome changes may occur either as a direct result of carcinogen interaction with the DNA or may be the result of a secondary event independent of carcinogenic insult.

The HEC transformation model is a favorably rapid quantitative bioassay that uses diploid cells and compares with both experimental long-term animal studies and epidemiologic data. Similar to the human, normal HEC senescence and spontaneous transformation is a rare event. Thus, the HEC model for transformation is relevant to the study of biology of carcinogenesis. The induction of the quantitative carcinogen-induced morphologic transformation is observed in a 7-day colony assay. The morphologic transformation frequency can be modulated so that the mechanism of factors that are responsible for enhancing or inhibiting transformation can be elucidated. Furthermore, in vitro morphologic transformation occurs in a dose-dependent manner and is characterized by random criss-crossing and piling up of cells not seen in controls; transformation correlates with tumorigenicity because individually transformed cell colonies can be isolated, cell lines developed, and the formation of tumors demonstrated by injecting the transformed cells into either Syrian hamsters or athymic nude mice. Furthermore, in vitro morphologic transformation is similar to that observed when primary tumors from in vivo hamster experiments are cultured.

Bisulfite is a food and pharmaceutical additive and is an ubiquitous pollutant in the form of SO<sub>2</sub>. Some epidemiological evidence indicates an association between SO<sub>2</sub> and cardiac and respiratory illness including lung cancer. Sodium bisulfite, a non-mutagen at neutral pH, induces neoplastic transformation of HEC. Treatment of HEC with 5-20 mM bisulfite in phosphate buffer for 15 min results in dose-dependent transformation (up to 3%) without cytotoxicity. Colonies exhibiting altered morphology characteristic of transformation were isolated and cell lines were developed for evidence of growth in agarose, tumorigenicity, and study of chromosome changes. Colony formation in agarose was observed within 10

days from all bisulfite cell lines; inoculation of  $5 \times 10^6$  cells into nude mice resulted in progressively growing fibrosarcomas. Five bisulfite cell lines were analyzed cytogenetically 10-15 passages after isolation of the transformed colonies; chromosomes of cells derived from agarose colonies and cultured tumor cells removed from the animals were also analyzed. Chromosome analysis included prometaphase and metaphase G and C banding, and determination of the ploidy. All five neoplastic cell lines were chromosomally abnormal with structural and numerical alterations. An unusual characteristic of transformation by bisulfite was an increased polyploidism. Moreover, the karyotype of tumor cells was in general similar to the parental transformed line. One line, BS-A, exhibited a near diploid chromosome number with a stemline of 43-46 chromosomes and a low number of tetraploid cells (8%). This line was the most heterogeneous in structural alterations; 6 abnormal chromosome (markers) have been identified and their origin determined. Marker chromosomes derived from chromosomes 1, 2, 6, 9 and 17 resulted from translocations, or interstitial, or terminal deletions. In addition, a homogeneously stained chromosome was observed after G banding in 100% of the cells; it also stained positive for constitutive heterochromatin. This type of abnormality is considered indicative of gene amplification in human or animal tumors. The X chromosome was trisomic in all cells, while chromosomes 3, 19, 20 and 22 were frequently monosomic. Another line, BS-D, had two populations: 68% of the cells were near diploid (42-47 chromosomes) and 32% were near tetraploid (86-90 chromosomes). Three abnormal chromosomes derived from deletions of the heterochromatic long arm of the X-chromosome and chromosomes 1 and 6 were identified. Chromosomes 1, 6, 17 and 19 were trisomic and chromosome 13 was monosomic. Line MBS-B<sub>2</sub>-E was the only line with the majority (87%) of the cells having a near tetraploid chromosome number. No detectable structural alterations were observed even by high resolution prometaphase banding analysis. Chromosome 6 and 13 were underrepresented in most of the cells.

Line BS-F had 32% and 68% of the cells with near diploid and near tetraploid populations, respectively. Both populations had the same chromosome alterations involving a translocation on chromosome 17 and an additional chromosome 9. The tumor-derived cell line also had two cell populations. To assess the tumorigenic potential of the near diploid and near tetraploid cells, two populations were separated by fluorometric cell sorting based on DNA content. Two near diploid and two near tetraploid lines were isolated from agarose. Each was inoculated into nude mice. Both near diploid and near tetraploid cell lines both produced tumors with a similar latency period suggesting that multiploidy is not a prerequisite for tumorigenicity.

The relationship between the occurrence of chromosome abnormalities and the acquisition of the tumorigenic potential is not clear. A sequential chromosome analysis of line BS-I was carried out. The transformed cells were chromosomally abnormal at passage 5 (after recognition and isolation of the transformed colonies); there was a mixture of a number of normal and abnormal male cells. The abnormalities were a deletion of the long arm of the X chromosome, a deletion of the short arm of chromosome 1 with a possible translocation of the deleted segment to the long arm of chromosome 6 and trisomy of 17. At passage 13, no additional alterations were observed. At this time the cells were tumorigenic as indicated by growth in agarose and tumor formation in nude mice. The karyotype of the tumor-derived cell line at the second culture passage after tumor excision showed that cells with abnormal chromosome constitution from the parental line produced the tumor in vivo. Although the features of the parental cell line were

maintained, a new large marker chromosome derived from chromosome 13 was detected in all the tumor cells. The bisulfite concentrations effective in producing transformation did not cause detectable DNA damage as indicated by excision or post replication analysis and induced a minimal increase in sister chromatid exchange. At lethal concentrations bisulfite caused a low frequency of chromosome aberrations. Bisulfite, however, did cause a dose-dependent inhibition of DNA synthesis as a result of a decrease in the number of functioning replicons. Because of the minimal acute effects of bisulfite on DNA metabolism and the profound chromosomal changes observed in neoplastic cells, an analysis of protein alterations has been initiated. Preliminary data indicates all bisulfite lines share the same protein changes; there are 3 new proteins, one missing protein, and two shifted proteins.

The morphologic transformation of HEC is irreversibly inhibited by LR obtained from mitogen-stimulated peritoneal lymphocytes. Sensitivity of HEC to the anticarcinogenic action of hamster LR depends on the stage of transformation, initiation, and promotion. Twice as much LR was required to obtain a 50% reduction in 12-Otetradecanoylphorbol 13-acetate (TPA) promoted transformation as in non-promoted transformation, suggesting a difference in the mechanism of initiated and promoted cell sensitivity to LR. In a study of promoted transformation, 48 hr LR treatment, either before or immediately after X-irradiation or during TPA exposure, caused a persistent inhibition of transformation independent of when LR was added. The degree of sensitivity of different steps in carcinogenesis as the cells underwent the physiological changes associated with transformation was examined more precisely with 6 hr LR treatments. LR treatment before irradiation and TPA caused a transient cellular change. When the cells were initiated within 2 days but not 3 after lymphotoxin exposure, the induction of promoted or non-promoted transformation was inhibited. LR became a more effective anticarcinogen as the interval between the LR pulse and carcinogen insult or TPA addition was reduced. When added during the last 6 hr of the experiment, LR was equally inhibitory whether or not TPA was present. Thus, LR induces an anticarcinogenic physiological state in noncarcinogen-treated HEC that is transient.

Increased in vitro transformation frequencies resulting from TPA promotion can be inhibited free radical scavengers, a phenomenon also observed in vivo with mouse skin painting experiments. The effects of superoxide dismutase (SOD) and Copper (II) 3,5-disopropylsalicylate (CuDIPS); SOD-mimetic on UV radiation-induced transformation of HEC in vitro was studied. UV was used because it is not suspected to intake carcinogenesis by free radical. Neither SOD or CuDIPS effected transformation by UV alone. However, both compounds caused a dose-dependent inhibition of TPA-promoted UV transformation when given concurrently with TPA. No toxicity is attributable to either reducing agent. High molecular weight SOD acts at the cell surface, whereas CuDIPS penetrates throughout the cell. These results suggest that the superoxide anion radical ( $O_2^-$ ) has a role in the promotion of transformation. Further experiments are required to precisely determine the role of free radicals in the biologic effects of TPA.

Comparison of sister chromatid exchange (SCE) induction and morphologic transformation of HEC suggest that SCE may be essential for the initiation of transformation. Furthermore, molecular and cytologic evidence support the conclusion that for certain carcinogens common DNA lesions may be involved in the induction of SCE and transformation; because the SCE frequency exceeds the transformation frequency, only a fraction of the total DNA lesions reading to SCE represents damage

relevant to carcinogenesis. Lesions which persist several cell generations after carcinogenic insult may be most critical. Thus, the persistence of SCE and its relevance to the transformation process has been assessed. After a 1 hr exposure of HEC to a carcinogen (at a dose that induces 30-40 SCE/cell) 5-bromodeoxyuridine (BUDR) required for SCE visualization, was added either at 0, 24 or 48 hr later. N-acetoxy-2-fluorenylacetaimide (AcaAF) (0.5  $\mu\text{g/ml}$ ), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (0.2  $\mu\text{g/ml}$ ) mitomycin-C (MMC) (50 ng/ml) and ultraviolet-light (UV) (3 J/m<sup>2</sup>) were used; cultures were further incubated for 24 hr until chromosome fixation. Thus, a direct comparison of SCE persistence with carcinogen was possible. Sequential SCE analysis over a 72 hr interval showed that DNA damage leading to SCE can be either partially or completely removed. Lesions caused by AcaAF were only partially removed as reflected in the decrease in the number of SCE from 39 to 28 to 21 after 0, 24, 48 hr post AcaAF, respectively. At the last interval approximately twice the number of SCE compared to the control value (10  $\pm$  2) still persisted. The lesions caused by MNNG, MMC and UV light were completely removed within 72 hrs. UV-induced lesions, however, were more rapidly removed than those caused by MNNG or MMS as indicated by the SCE values obtained when BUDR was added 24 hr after carcinogen. Comparison of SCE values to the transformation frequencies determined with the HEC colony model did not show a correlation between the persistence of SCE and the induction of transformation for the majority of the carcinogens. The lesions caused by AcaAF, for example, were only partially removed, whereas those induced by MNNG or UV were completely removed as indicated by SCE assay. MNNG and UV produced 2.76 and 1.58% transformation frequencies, respectively, and AcaAF resulted in a lower transformation frequency, 1.1%. Thus, the persistence of SCE is not directly related to transformation.

Sister chromatid exchanges (SCE) are induced by a variety of chemical and physical carcinogens but the mechanism of their formation is unknown. SCE formation is intimately dependent on DNA replication. DNA precursor imbalances can have severe genetic consequences as DNA replication fidelity is dependent on proper balances of deoxyribonucleic triphosphates during DNA synthesis. Therefore, any factor influencing replication, either by causing DNA damage or inhibiting fork progression, may lead to SCE. The importance of deoxycytidine (dC) metabolism in SCE formation has been demonstrated in our studies with normal human and rodent cells subjected to amino acid deprivation and a mouse cell line deficient for deoxycytidine deaminase. Another approach to the study of the role of DNA precursor imbalance to SCE induction is to examine the effect of increasing BUDR concentrations and to assess the influence of specific nucleotides in this process. A dose dependent increase in SCE occurs with BUDR concentrations up to 100  $\mu\text{M}$ . For instance, 3  $\mu\text{M}$  BUDR caused 8 SCE per HEC and 100  $\mu\text{M}$  BUDR medium induced 24 SCE per cell. The BUDR dependent increase in SCE can be suppressed by the presence of 200  $\mu\text{M}$  dC during BUDR incubation. dC also suppressed SCE induction by BUDR in the presence of 5-fluorodeoxyuridine, which prevents the conversion of exogenously supplied dC to thymidine nucleotides. dC is the only nucleotide that suppresses SCE induced by BUDR. Deoxyadenine (dA) and deoxyguanosine (dG) have an enhancing effect on BUDR induction of SCE even with relatively low concentrations of BUDR (3-30  $\mu\text{M}$ ). Two-hundred  $\mu\text{M}$  dA doubled the number of 17 SCE per cell induced by 60  $\mu\text{M}$  BUDR and 50  $\mu\text{M}$  dG, a concentration which did not adversely effect cell progression. This data suggests that BUDR inhibits ribonucleotide reductase which converts CDP to dCDP. Because dC reverses BUDR enhancement of SCE, the enhancement may be a reflection of reduced cellular levels of dCDP. Future experiments are planned to determine the molecular basis for this phenomenon.

HEC are free of viruses commonly associated with neoplastic transformation. Consequently, transformation is considered to be due to the direct action of the carcinogenic insult. However, HEC do have moderately reiterated sequences in their genome related to mouse genes. The sequences are retrovirus-like entities and are expressed in embryonic Syrian hamster and mouse tumor cells. HEC contain between 1000-2000 copies of the sequences homologous to intracisternal "A" particle IAP-DNA. Because these DNAs contain LTR sequences which could be transposed and/or enhance expression of neighboring genes, the role of IAP-DNA in HEC transformation is being investigated. Overall, IAP expression in carcinogen-treated HEC remains the same. IAP expression is also unaffected by X-ray or MMS pretreatments that greatly enhance transformation by other carcinogens. The chromosomal location of IAP-DNA in the HEC genome has been determined by the *in situ* hybridization. The Syrian hamster genome has a relatively large amount of sex chromosome and autosomal heterochromatin; the long arm of the X chromosome, the entire Y, the short arms of 8 autosomal pairs, and the long arm of the smallest metacentric are heterochromatic. Constitutive heterochromatin is usually formed by highly repetitive DNA sequences. About half of the IAP-DNA sequences are concentrated in regions with constitutive heterochromatin, whereas the remaining sequences, with the exception of the centromeric region of the chromosome 5 and the terminal segment of the short arm of chromosome 15, are randomly spread throughout the rest of the genome. Because of their random distribution, IAP sequences are being used for detecting the presence of HEC-DNA in recipient cells of other species which have been transfected with HEC-DNA.

Although DNA repair and metabolism are considered relevant to carcinogenesis, the underlying processes are still obscure. Slow removal of  $O^6$ -methylguanine has been the basis for the hypothesis that this lesion is critical for carcinogenesis induced by methylating agents. For example, a positive correlation exists between the persistency of  $O^6$ -methylation in various tissues of the rat and their susceptibility to tumor induction by N-methylnitrosourea (MNU) or dimethylnitrosamine, the brain being the most susceptible organ followed by the kidney and then the liver. The HEC system allows for a quantitative comparison of the induction and repair of methylated DNA lesions with the induction of transformation and lethality by a variety of methylating carcinogens. We determined that MNNG, MNU, or MMS concentrations that induce equivalent transformation frequencies in HEC also induced similar levels of  $O^6$ -methylguanine but not of  $N^7$ -methylguanine. Therefore, it is highly probable that  $O^6$ -methylguanine is the lesion responsible for the initiation of carcinogenesis induced by methylating agents. To determine how these lesions might effect DNA metabolism and thus contribute to the initiation process of carcinogenesis, the effects of MNNG and MMS at concentrations that induce equivalent levels of  $O^6$ -methylguanine on the size of both parental and nascent daughter DNA were examined by alkaline sucrose sedimentation analysis. The number of single strand breaks in parental DNA is greater in cells treated with MMS than in those treated with MNNG. Because the  $O^6$ -methylguanine level is the same with the conditions used for the two chemicals but the level of  $N^7$ -methylguanine is 30-fold high after MMS treatment, we concluded that the  $N^7$ -methylguanine lesion is responsible for the breakdown of parental DNA. We calculate, however, that there are approximately 250  $N^7$ -methylguanine lesions for each single strand break in parental DNA. These breaks probably represent incomplete repair of these lesions at the time of sampling. The  $N^7$ -methylguanine lesions do not appear to have any biological consequences in terms of carcinogenesis because transformation is induced in MNNG-treated cells without the appearance of these breaks. At a concentration of MNNG that induces approximately one  $O^6$ -methylguanine



per four replicons and one N<sup>7</sup>-methylguanine per third of a replicon, there is no effect on size distribution of nascent daughter DNA. Therefore, this level of lesions has no effect on either replicon initiation or chain elongation. The rate of DNA replication, however, is 20% lower than that of untreated cells. Therefore, a group of replicons exists that do not function. When four times as many lesions are present, a slight effect on chain elongation occurs as well as a further reduction in the rate of DNA synthesis. Because both the number of lesions remaining as well as the DNA replication rate are lower 24 hrs after MNNG treatment, it is unlikely that the inhibition of the rate of DNA synthesis is directly related to the number of lesions in DNA. The rate of DNA synthesis is much lower in cells treated with MMS having the same level of O<sup>6</sup>-methylguanine as those treated with MNNG. This further reduction in rate could be due to N<sup>7</sup>-methylguanine because there are approximately 100 per replicon. However, 24 hours after carcinogen treatment, the rate of DNA synthesis recovers to about 50% of untreated controls while there are still at least 25 N<sup>7</sup>-methylguanines remaining per replicon. Therefore, it is also possible that the reduction in the rate of DNA synthesis is due to methylation of RNA and/or protein in the cell as opposed to direct inhibition due to the presence of DNA lesions in the DNA. These results indicate that the initiation of carcinogenesis by O<sup>6</sup>-methylguanine is probably not mediated directly by effects on the rate of DNA replication but is more likely due to the miscoding properties of this lesion. Finally, quantitative analysis of O<sup>6</sup>-methylguanine and transformation induction indicates that the target for initiation of carcinogenesis is one O<sup>6</sup>-methylguanine per 50 to 500 genes. This large target size is inconsistent with the concept of cancer induction by mutation.

An understanding of the nature of the DNA sequence of the daughter strand DNA replicated from carcinogen-damaged parental DNA is critical in the study of carcinogenesis. Therefore, DNA sequences putatively responsible for induction and maintenance of the transformed state are being isolated and characterized. DNA isolated from six different HEC tumorigenic cell lines, five originally treated with 3-methylcholanthrene and one with benzo[a]pyrene, contain DNA sequences that can transform 3T3 cells by DNA transfection. Five of these DNAs have been analyzed for sensitivity to inactivation of their transforming sequences by a battery of restriction endonucleases. Since DNA from different tumorigenic cell lines appears to be inactivated by a different set of restriction enzymes, the DNA transforming sequences in these different cell lines are different. Transformed cell lines have been isolated from the foci derived from 3T3 cells transfected with DNA from the hamster tumorigenic cell lines. The DNA from these foci has been analyzed for the presence of hamster "middle repeat" DNA sequences using Southern blot analysis. Because of some homology between the "middle repeat" sequences from hamster DNA and those from mouse DNA, the presence of these "middle repeat" hamster sequences in the DNA of the transformed foci cannot be confirmed. However, several of the transformed foci do contain hamster IAP-DNA sequences, confirming that these foci were transformed by incorporation of HEC oncogenes. DNA from these foci were examined for the presence of hamster ras sequences by hybridization to Kirsten ras (ras<sup>K</sup>) under relaxed conditions. Because no HEC ras K-related sequences were detected, it appears that activated DNA transforming sequences are not in the ras family.

Cells at distinct preneoplastic stages of carcinogenesis can be identified and isolated in an in vitro guinea pig transformation system. These cells have been

utilized for studying various aspects of transformation leading to malignancy and are considered particularly appropriate material for analysis by DNA transfection. In collaboration with Dr. Mariano Barbacid, Z01CP04948-08-LCMB, we have found that five independent tumorigenic clones derived from carcinogen-treated (using four different carcinogens either in vitro or in utero) guinea pig cells contain DNA sequences capable of transforming 3T3 cells, whereas DNA from untreated normal guinea pig cells cannot. In addition, anchorage independent cells isolated from the agar but negative in all tumorigenicity assays are also negative as a donor in 3T3 transfection assays. By restriction analysis we have determined that all five positive lines contain a similar 11Kb EcoRI-activated oncogene derived from a guinea pig ras-related proto-oncogene. Because activated oncogenes can be detected only in the guinea pig neoplastic cells but not in their neoplastic progenitors, we concluded that activation of this oncogene is a late step in carcinogenesis closely associated if not responsible for tumorigenicity. Both laboratories are now cloning activated oncogenes from different cell lines. We have isolated and restriction mapped an 11.3 Kb EcoRI fragment from transformed 3T3 cells containing a ras related sequence. We are assessing the biological activity of this DNA as well as confirming its guinea pig origin. Stable chromosome changes may occur either as a direct result of carcinogen interaction with the DNA or may be the result of a secondary event independent of carcinogenic insult. It is now important to determine if any oncogenes are located on chromosome 2 of the rat complement.

#### Significance to Biomedical Research and the Program of the Institute:

The prevention of cancer in humans depends to a large extent on understanding the process that is responsible for the development of transformation and on removing potentially harmful environmental agents. The evidence indicates that DNA is the target for the initiation of carcinogenesis. Furthermore, certain genes which become activated appear responsible for the expression of the neoplastic phenotype. The exact process by which the relevant genes become activated is not clear, nor are the pivotal stages known. This research addresses these problems by attempting to identify the critical stages of carcinogenesis and to understand the underlying DNA and chromosome changes in order to find methods for intervening with the process or for preventing it.

#### Proposed Course:

In this project we will establish conditions and methods for in vitro quantitative study of transformation by chemical and physical carcinogens to determine the underlying biochemical and molecular processes responsible for the somatic changes which result in malignancy. The project will continue to define the conditions necessary for the quantitative transformation of mammalian cells with specific emphasis on human cells. A relevant question concerns the difficulty of transforming human cells relative to the ease of transforming Syrian hamster cells. Human and hamster cells senesce and have stable karyotypes; but only hamster cells, after carcinogen treatment, exhibit dose-dependent transformation that results in permanent tumorigenic lines. Because the hamster transformation model is responsive to promoters and anticarcinogens, it will be used to study free radical formation and surface alterations during transformation. The hamster cell contains approximately 100 copies of sequences related to mouse intracisternal A-particle genes. Mouse intracisternal A-particles are known to be transposable and with in situ hybridization techniques we have shown that in the

hamster about 80% of these are localized in the heterochromatin regions of the chromosomes. Mouse intracisternal A-particles have been shown to activate mos genes in that species. The possibility that hamster intracisternal A-particles can act in a similar way or that the long terminal repeat sequences can be involved in the transformation of hamsters will also be considered. Because the mechanism(s) involved in transformation are probably independent of cell type, various sources of human fibroblasts will be used, including those obtainable from patients with genetic disorders and from fresh normal foreskin. The use of normal cells (from individuals not cancer prone) results in a transitory transformed state. Carcinogen-treated human cells eventually lose their transformed attributes and fail to evolve into permanent lines. Therefore, we plan to embark on a series of experiments to determine the changes required to make a cell immortal. The approaches will include transfection using plasmids which contain sequences conferring extended growth potential compared with the nontransformed counterparts. Carcinogen insult would be either prior to or after transfection. Other experiments will be concerned with decreasing chromosome stability. In view of the high degree of aneuploidy associated with solid human tumors, it is logical to consider decreasing chromosome stability and/or increasing aneuploidy to increase the probability of transformation. The results utilizing cells from xeroderma pigmentation have not been any more encouraging than those with normal cells. Therefore, we plan to use cells from individuals with other syndromes such as Fanconi, Bloom, and neurofibroma. In addition, because of the success obtained with papilloma viruses and chemicals in animals, we plan to utilize type 16 human papilloma virus that we have introduced into a plasmid. The virus may serve as a promoter when used in conjunction with known carcinogens. After determining the conditions necessary for increasing the susceptibility of primary human fibroblasts, epithelial cells, such as from breast, will be used because of the possibility of relating specific markers associated with differentiation to carcinogenesis.

The role of DNA lesions caused by physical or chemical carcinogens will be investigated at the chromosomal and molecular level to determine the critical events leading to oncogene activation. In human cancer certain oncogenes have been activated at the site of specific translocations. The identification of altered DNA sequences will be correlated with chromosome rearrangements in human transformed cells. This will permit the evaluation of the role of oncogene activation in initiation and progression of neoplasia. Because of the progressive (steps/stages) nature of cancer development, preneoplastic cell populations will be identified and isolated by a fluorescence-activated cell sorter; surface changes, chromosome alterations, and DNA changes will be characterized. Human cells will also be utilized to isolate and characterize DNA sequences responsible for carcinogenesis induced by chemical or physical agents. In addition, how oncogenes bring about transformation will also be studied utilizing hamster and guinea pig cell lines transformed by chemical or physical carcinogens. The cell lines exhibit a series of progressive changes that may lead to malignancy; DNA transforming sequences will be isolated from them and compared. The transforming gene(s) will also be used to determine whether it is able to convert preneoplastic stages (carcinogen treated) to malignancy.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE04673-13 LB

PERIOD COVERED

October 1, 1983 to September 30, 1984

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)

P.I.: C. H. Evans Chief, Tumor Biology Section, LB NCI

COOPERATING UNITS (If any)

None

LAB/BRANCH

Laboratory of Biology

SECTION

Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

2.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Naturally occurring lymphocytes, macrophages, and other leukocytes and their secretory products (e.g., lymphokines, interleukins, and other immunological hormones), are being studied to define their effective anticarcinogenic and tumor cell growth inhibitory activities. Leukoregulin, a newly isolated lymphokine, can prevent carcinogenesis and inhibit tumor cell growth. Anticarcinogenic action is direct and irreversible and occurs without cytotoxicity. Inhibition of tumor cell growth is primarily reversible but can become irreversible due to increased susceptibility of preneoplastic and neoplastic cells to cytolytic destruction by natural killer cells resulting from 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, the lymphokine may be an effective homeostatic mechanism for control of carcinogenesis at its later stages of development. Leukoregulin anti-carcinogenic, tumor cell growth inhibitory, and cytoreductive sensitizing activity co-purify into two major glycoprotein classes with different isoelectric pHs. The more acidic class has an isoelectric pH of 4.6 and 5.0 and the other class an isoelectric pH of 5.0 and 7.1 for hamster and human leukoregulin, respectively. The anti-cancer actions of leukoregulin are distinct from other lymphokines including interleukin I, interleukin II, lymphotoxin, macrophage migration inhibitory factor, macrophage activating factor, and interferon. Leukoregulin alters cell surface conformation, membrane fluidity and permeability, and large molecular weight membrane glycoprotein synthesis with changes in the latter correlating directly in time and quantity to leukoregulin induced establishment of the anticarcinogenic state. Leukoregulin induces identical changes in target cells that are present during natural killer cell 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 DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. H. Evans	Chief, Tumor Biology Section	LB	NCI
J. H. Ransom	Staff Fellow	LB	NCI
J. P. Fuhrer	Expert Scientist	LB	NCI
S. A. Barnett	Visiting Fellow	LB	NCI

Objectives:

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

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

Methods Employed:

Guinea pig, human, mouse, rat, and Syrian hamster cells are utilized in this study. Freshly isolated cells are obtained from embryos, fetuses or adult animals exposed in utero or in vitro to chemical or physical carcinogens. The cells are cultured and studied for somatic cell changes such as altered morphology, morphological transformation, growth in agar, tumorigenicity and interaction with components of the immune system. Immunobiological techniques including direct and indirect immunofluorescence, flow cytometry, complement fixation, colony inhibition, radionuclide uptake and release assays, delayed hypersensitivity skin reactions, and tumor transplantation rejection are employed in analyzing cell membrane changes and in assessing host interactions to the changes.

Major Findings: During the past several years we have demonstrated that lymphokine preparations prepared from antigen-or mitogen-stimulated lymphocytes

from normal hamsters or guinea pigs contain a potent anticarcinogenic activity.

The lymphokine is able to prevent as well as inhibit the subsequent development of carcinogenesis at several stages. An anticarcinogenic state is induced in target cells for carcinogenesis that does not affect cell growth. The new physiological state, like many responses to hormones, is induced rapidly and persists for a short time. When the lymphokine-induced anticarcinogenic state is present at the time of carcinogen exposure, carcinogenesis is prevented and when present shortly after carcinogen exposure, the further development of initiated or complete carcinogenesis is irreversibly inhibited. In the later stages of carcinogenesis the anti-cancer action of the lymphokine is characterized by inhibition of cellular proliferation which in fully tumorigenic cells at high lymphokine concentrations may also be accompanied by cellular destruction. The lymphokine's ability to prevent carcinogenesis in Syrian hamster cells, moreover, is more potent than its ability to suppress proliferation of fully neoplastic cells, the latter requiring more than 20-fold as much lymphokine. Another action of the lymphokine is its ability to increase the sensitivity of cells to destruction by natural killer cell cytotoxicity. These activities--prevention of transformation, inhibition of neoplastically transformed cell growth, and the sensitization to natural killer cell cytotoxicity--co-purify with the lymphokine known as lymphotoxin using conventional molecular size fractionation techniques. Using a combination of isoelectric focusing and molecular size fractionation by column chromatography, we have separated the anticarcinogenic activity of hamster lymphokine into two fractions with mean isoelectric pHs respectively, of 4.6 and 5.0 associated with a 45,000 molecular weight glycoprotein. Lymphotoxin activity, identified by the ability to lyse  $\alpha$ 929 murine target cells, was present only in the isoelectric 5 fraction suggesting that the anticarcinogenic activities were either a new form of lymphotoxin or a distinct new lymphokine. The anti-carcinogenic activities, moreover, were free of interleukin 1 and 2, macrophage activating factor, macrophage migration inhibitory factor, and interferon activities. The examination of human lymphokine preparations prepared from phytohemagglutinin-stimulated, freshly isolated peripheral blood lymphocytes provided confirmatory evidence for the presence of a new lymphokine with anti-cancer activities. Using a combination of isoelectric focusing and high performance liquid chromatography within a silica-based molecular sizing column, a lymphokine distinct from previously recognized lymphokines with a mean molecular weight of 50,000 by silica column HPLC and isoelectric pHs of 5 and 7.5, has been isolated. The lymphokine possesses the cell proliferation inhibitory and natural killer cell sensitizing activity for neoplastic cells and is free of lymphotoxin activity. Further support for the existence of the new lymphokine is that the ratio of the tumor cell proliferation inhibitory activity to lymphotoxin activity varies from one lymphokine preparation to another and that the tumor cell growth inhibitory activity is decreased after protease but not neuraminidase digestion, whereas lymphotoxin activity is decreased after either treatment. The new lymphokine has been named leukoregulin to indicate its origin from leukocytes and regulatory action.

Leukoregulin induces specific target cell membrane changes associated with its natural killer cell sensitizing and tumor cell proliferation inhibitory activities. These can be measured rapidly by flow cytometric analysis of both light scatter and membrane permeability changes, the latter being followed by the uptake of or loss of fluorescent dyes. Measurement of these parameters has led to the development of a rapid 2 hr assay for the quantitative measurement of leukoregulin. This facilitates measurement of the lymphokine during the fractionation procedures as



well as during study of its biochemical action and biological role. For example, by simultaneously analyzing effector and target cells during a natural killer lymphocyte cytotoxicity reaction using flow cytometry we have shown that leukoregulin induces changes in target cell surface membrane conformation and permeability identical with those induced during natural killer lymphocyte cytotoxicity. This strongly suggests that leukoregulin is an intrinsic mediator or element of natural lymphoid cell cytotoxicity and may indicate its central role in the immune system.

In addition to separating and evaluating the activities of leukoregulin and lymphotoxin, specificity studies in terms of interactions with other cell surface active agents (e.g., hormones, antibodies, and lectins) are in progress. For example, insulin can render leukoregulin-responsive cells resistant to the growth inhibitory action of leukoregulin. In an opposite manner leukoregulin can reverse the desensitizing action of interferon resulting in an increased susceptibility of the target cell to the cytoreductive action mediated by naturally immune lymphocytes. Investigations are in progress to determine the molecular character of these stimulatory and opposing actions. It may be through a balance between such stimulatory and antagonistic activities that leukoregulin exerts its normal regulatory role. Leukoregulin is, therefore, an immunologic hormone with direct and indirect anticarcinogenic and tumor cell growth inhibitory activity. The hormone binds to cell surface oligosaccharide receptors and its action may be mediated through alterations in cell surface glycoproteins that directly block the carcinogenesis process at its early stages or indirectly prevent cancer by increasing the susceptibility of preneoplastic and neoplastic cells to control or to destruction by immunological or other homeostatic mechanisms.

#### Significance to Biochemical Research and the Program of the Institute:

This investigation provides the first evidence that a lymphokine distinct from interferon possesses the ability to directly prevent as well as inhibit the further development of carcinogenesis at several distinct stages during the transition from a normal to a neoplastic cell. Moreover, the evidence indicates that the lymphokine functions in concert with lymphoid cells as an intrinsic element or mediator within natural lymphoid cell immunoregulatory actions and as an extrinsic immunological anticarcinogenic hormone. The most recent observations, furthermore, suggest that the lymphokine is a unique immunological hormone distinct from lymphotoxin. The potential for the normal immunological system to prevent and to modulate carcinogenesis has been recognized for many years. Identification of the critical anticarcinogenic elements, however, remains cryptic and is essential for our understanding of normal host responses to carcinogenesis in order for their effective utilization as interventive measures in the prevention and control of carcinogenesis. This investigation provides fundamental new insights into normal anticarcinogenic immunological mechanisms increasing both our understanding of homeostasis and basic physiological mechanisms influencing the development of carcinogenesis.

#### Proposed Course:

Investigations will continue to define the mechanisms whereby neoplastically transformed rodent and human cells are preferentially susceptible to the cytotoxic activity of naturally immune host-derived cellular and humoral effectors. A major thrust will be a multidisciplinary investigative analysis of cell membranes

relevant to differentiating tumor cells from normal cells. The approaches will behavior. The ability to quantitatively follow cell surface membrane alterations flow cytometrically that are specific for a given lymphokine provides a new dimension in defining cellular and molecular mechanisms preventing and regulating carcinogenesis. Additional membrane changes due to the specific action of leukoregulin as well as those specific for other immunological and non-immunological hormones will be identified to characterize the synergistic and contravening mechanisms of these molecular mediators of host responses to carcinogenesis. Preneoplastic and neoplastically transformed cells exhibiting lymphokine-specific alterations, such as leukoregulin-induced increased membrane permeability to fluorescein, will be identified and sorted flow cytometrically. The complex cell surface oligosaccharides of the sorted cells will be biochemically defined during investigation of the receptors for individual lymphokines and the binding of cellular host defense mechanisms. A major question to be resolved is the role of leukoregulin in relationship to lymphotoxin. Lymphotoxin is present in all mammalian species in which it has been studied but except for its widely recognized ability to lyse murine L cells has an undefined physiologic role. Leukoregulin and lymphotoxin are both produced by natural killer lymphocytes and may interact in a synergistic manner in natural lymphoid cell cytotoxicity. This program will, in part, seek to explain how leukoregulin and lymphotoxin preferentially bind to and inhibit tumor cells and why cell to cell contact is necessary in most natural cellular immunocytotoxicity. New technology in cell surface topography and cell separation using computerized cytometers and cell sorting cytofluorographs enables further resolution of the relationships between the cytostatic and cytotoxic activities of macrophages, lymphocytes, and lymphokines to tumor cells. Definition of these relationships will clarify the species specificity of the effector mechanisms and our understanding of the role of in vivo natural immunity in the phenomenon of carcinogenesis.

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## ANNUAL REPORT OF

### THE LABORATORY OF CELLULAR CARCINOGENESIS AND TUMOR PROMOTION

#### NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

The Laboratory of Cellular Carcinogenesis and Tumor Promotion plans, develops and implements a comprehensive research program to determine the molecular and biological changes which occur at the cellular and tissue levels during the process of carcinogenesis. Studies are designed to (1) define normal regulatory mechanisms for cellular growth and differentiation; (2) determine the mechanism by which carcinogens alter normal regulation and the biological nature of these alterations; (3) investigate the mechanism by which tumor promoters enhance the expression of carcinogen-induced alterations; (4) identify cellular determinants for enhanced susceptibility or resistance to carcinogens and tumor promoters; (5) elucidate the mechanism by which certain pharmacologic agents inhibit carcinogenesis.

The Laboratory is composed of three sections each of which is charged with a major responsibility for portions of the Laboratory goals. Because of the integrated approach toward an understanding of mechanisms of carcinogenesis, considerable interaction occurs among the sections. Areas of interaction are defined in the individual project reports.

IN VITRO PATHOGENESIS SECTION: The In Vitro Pathogenesis (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; (5) elucidates factors which determine susceptibility to carcinogenesis.

This section has directed its efforts toward both developing in vitro model systems to study chemical carcinogenesis in epithelial cells and to use these systems to study the mechanisms of tumor initiation and promotion. Mouse epidermis, the classic model for induction of squamous cancer by chemicals, has been adapted for in vitro study. Previous investigations had demonstrated that this model is a close in vitro analogue of the mouse skin carcinogenesis system in vivo. In vitro, epidermal cells proliferate and differentiate, metabolize carcinogens, repair DNA damage, and respond to tumor promoters like epidermis in vivo.

Regulation of Epidermal Growth and Differentiation: Previous results from this Laboratory have indicated that extracellular calcium concentration regulates epidermal proliferation and differentiation. Culture medium of 0.02 - 0.09 mM calcium concentration selects for proliferating cells which have morphological, immunological and biochemical characteristics of basal cells. Culture medium of >0.1 mM induces epidermal differentiation resulting in cessation of proliferation, vertical stratification, cornification and sloughing of mature squames.

The regulation of differentiation by calcium is not associated with changes in cyclic nucleotide levels but appears dependent on a functioning  $\text{Na}^+ - \text{K}^+$  ATPase. There are strong similarities in the induction of epidermal differentiation by calcium and by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). Both alter protein synthesis as evidenced by computer analysis of polyacrylamide gel electrophoresis patterns of radiolabeled proteins. A number of protein changes are common for these inducers. TPA-induced differentiation is likely to be mediated through activation of protein kinase C, and calcium may also mediate differentiation via phospholipid metabolism and protein kinase C activation. Retinoids can inhibit the induction of differentiation by both TPA and calcium. Retinoids are potent inducers of a unique skin transglutaminase activity which is associated with a reduction in cornified envelope crosslinking. This retinoid-induced enzyme is heat labile, has a characteristic  $K_m$  for putrescine, a unique elution profile on column chromatography and is located in the soluble subcellular compartment. These characteristics are unlike those of the differentiation-associated epidermal transglutaminase and suggest that the retinoid enzyme could inhibit differentiation by crosslinking envelope precursors prior to their proper assembly at the cell periphery.

Quantitative Assay for Carcinogen-induced Altered Differentiation: The capability to selectively grow basal cells in low calcium medium and induce differentiation in high calcium has provided an assay to select for cells with altered differentiative responses. Exposures of primary cultures of mouse keratinocytes to chemical carcinogens results in foci which resist the  $\text{Ca}^{++}$  signal to differentiate and continue to proliferate under high  $\text{Ca}^{++}$  conditions, producing countable colonies which stain red with rhodamine B. Cells obtained from mouse skin initiated in vivo show the same characteristics. A series of mouse skin initiators show similar potency for the production of altered foci in vitro and initiation of tumorigenesis in vivo. For mouse skin initiated in vivo, more foci formed in vitro with higher doses of initiator or with equal doses of more potent initiators. There is an excellent correlation of focus number with the extent of DNA adduct formation for an individual initiating agent. However, certain agents are more potent focus formers at a given level of DNA adducts suggesting that qualitative factors, as well as quantitative factors, determine biological effects. Cell lines derived from foci induced by initiators in vitro are resistant to induced differentiation by phorbol ester tumor promoters and many are stimulated to proliferate by these agents. Likewise, cells derived from skin papillomas are stimulated to proliferate by promoting agents.

Infection of epidermal cells with oncogenic retroviruses containing an activated ras gene indicate that expression of ras and subsequent synthesis of p21 provides a marked proliferative stimulus to basal cells. However, such cells respond to high  $\text{Ca}^{++}$  by cessation of proliferation. These cells do not terminally differentiate but appear to be blocked in some non-terminal state of differentiation. Interestingly, blocked cells appear to remain responsive to tumor promoters and do not synthesize pemphigus antigen, a marker of suprabasal differentiation in vivo. Thus epidermal cells with an activated ras gene differentiate to a late basal cell stage but do not advance further. Analysis of RNA isolated from initiated basal cells or epidermal papillomas or carcinomas using a variety of oncogene cDNA probes has failed to show a significantly increased expression of any of these genes.

Molecular Regulation of Epidermal Specific Differentiation Products: Our studies have indicated that initiation of carcinogenesis is associated with a change in normal differentiation. In order to understand this association at the molecular level, the regulation of specific differentiation products is being explored. Keratin peptides are a family of proteins which comprise the major cytoskeletal and differentiation molecules of epidermis. We have cloned the genes which code for the major keratins of proliferating and differentiating keratinocytes. Their expression has been studied in keratinizing vaginal epithelium where a hormone-regulated, coordinated, sequential program related to proliferation and differentiation has been determined. Interestingly, the expression of these genes is markedly altered in skin treated with TPA. An initial marked decrease in expression of differentiation keratins is followed by an aberrant sequential increase in expression of these proteins. This is consistent with other evidence for a fundamental derangement of differentiation in promoter-treated epidermis. Sequence analysis of keratin cDNAs have revealed unique structural aspects of these proteins which demonstrate sites of interaction in filament formation and perhaps posttranslational modification. Isolation of a genomic fragment of one of the differentiation keratins has been completed and the gene structure has been characterized by sequence analysis in comparison to the cDNA.

Determinants for Susceptibility to Carcinogenesis: Epidemiological and medical genetic data have indicated major individual differences in cancer risk in humans. Increased risks are associated both with overall susceptibility to cancer or susceptibility in a particular target organ. In some cases specific genetic changes have been associated with increased risk, but in many examples polygenic influences appear more likely. To date biochemical epidemiological studies have focused only on genetic differences in carcinogen metabolism. In the complex and multistage evolution of cancer, it seems unlikely that carcinogen metabolism is solely responsible for enhanced risks. In fact it seems likely that factors associated with the expression of neoplastic change would play an important role in host susceptibility. The development through selective breeding of animal strains with high susceptibility at a particular organ site provides an excellent model for the study of susceptibility determinants. This Laboratory has utilized the SENCAR mouse strain for susceptibility studies since this strain is especially sensitive to chemically induced skin carcinogenesis.

SENCAR mice are markedly susceptible to two-stage skin carcinogenesis compared to BALB/c mice. Grafting studies have shown that susceptibility is a property of the skin itself and other studies indicate that sensitivity is not due to differences in metabolism of polycyclic aromatic hydrocarbons. Yet by a variety of biological and biochemical parameters SENCAR epidermal cells behave identically to epidermal cells from less sensitive strains. These include in vitro growth kinetics, DNA repair, receptor binding of growth factors and phorbol esters, density and function of Langerhans cells, production of epidermal thymocyte-activating factor, and induction of transglutaminase. Adult SENCAR epidermal cells are not more susceptible to focus induction by carcinogens in vitro but consistently show an increased rate of spontaneous foci over other strains. Thus SENCAR epidermal cells may have constitutively initiated cells or may be inherently unstable leading to spontaneous initiation in vitro. The lack of enhancement of focus number by carcinogens focuses attention on the promotion phase of carcinogenesis as the basis for susceptibility.

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 of DNA substituted with 2-acetylaminofluorene (AAF), benzo[a]pyrene (BP) or cis-dichlorodiammineplatinum (II) (cis-DDP) were subjected to immunological localization and quantitative immunoassays able to detect one adduct in one hundred million nucleotides. In hepatic DNA of rats fed a carcinogenic level of AAF for 4 weeks, adduct accumulation reached a plateau at 2-3 weeks. During 4 subsequent weeks on control diet, adduct removal was biphasic with a rapid initial phase followed by a slow second phase. A pharmacokinetic model consistent with this data proposes that adducts are formed in two DNA compartments, one from which adducts are removed rapidly and another from which adducts are removed slowly. Persistent adducts accumulate in the slow-repairing compartment, but constitute less than 7% of the total adducts formed. In contrast to the high levels of AAF adducts in liver DNA, binding of BP to deoxyguanosine in DNA of mouse epidermis and cultured epidermal cells is more than 50-fold lower. Binding levels are similar in epidermis and epidermal cells, subsequent to dosages known to induce papillomas in vivo and differentiation-altered foci in the cultured keratinocytes. The kinetics of repair for BPDg in vivo and in vitro are biphasic (as in liver) but much more rapid, with 50% removal by 1-2 days. Thus adduct accumulation and removal seem to be characteristic of interaction between a particular target tissue and an individual carcinogen, and may not be quantitatively related to efficiency of tumorigenesis or transformation. Antisera specific for cis-DDP-DNA (bidentate N<sup>7</sup> dideoxyguanosine intrastrand adduct) have been used to measure adducts in DNA of nucleated blood cells from cancer patients on platinum drug chemotherapy. Adducts appear to accumulate both as a function of total cumulative dose and increasing cycle number in individuals who have not received previous platinum drug therapy. The relationship between ability to form adducts and disease response is being analyzed in a prospective clinical study.

Mechanism of Action of Tumor Promoters and Antipromoters: Tumor promotion by phorbol esters has been an area of intense study in this Section. Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Our studies have indicated that basal cells are heterogeneous in response to phorbol esters in that some cells are induced to differentiate while others are stimulated to proliferate. This could form the cellular basis for selection. The induction of terminal differentiation by phorbol esters appears to be mediated by the phorbol ester receptor, and this action of phorbol esters is enhanced by Ca<sup>++</sup>. The molecular basis for the pharmacological heterogeneity is suggested by studies of the phorbol ester receptor in cultured keratinocytes. Multiple receptor classes are found in differentiating cultures indicating that maturation state may modify receptor affinity. Protein kinase C activation may be the major pathway which mediates phorbol ester responses in keratinocytes since exogenous diacylglycerols can mimic the effects of TPA. Furthermore generation of endogenous diacylglycerols by exposure of cells to phospholipase C reproduces the biological effects of TPA. Studies on the progression of benign to malignant tumors in vivo indicate that promoters are incapable of accelerating the conversion process while genotoxic carcinogens have a marked enhancing and accelerating effect on malignant conversion. These results suggest a mechanism of multistage carcinogenesis involving three steps. A genetic change in the program of terminal differentiation characterizes the initiation step. This is a preneoplastic change. Tumor promotion involves



cell selection and clonal expansion of initiated cells but does not alter their preneoplastic character. A second genetic change is required in the third step to convert benign to malignant lesions.

MOLECULAR MECHANISMS OF TUMOR PROMOTION SECTION: Using relevant model systems, the Molecular Mechanisms of Tumor Promotion Section 1) studies the interaction of tumor promoters with specific cellular receptors, 2) elucidates the functional importance of receptors in promoter action, 3) identifies endogenous ligands with specific affinity for receptors of exogenous promoters, 4) characterizes endogenous factors mediating receptor affinity and response, and 5) clarifies the initial biochemical steps in the cascades associated with receptor occupancy. Understanding of the early events in promoter action should permit the analysis of their control, modulation, and function in human cells under normal and pathological conditions. Determination of the ability of less specific tumor promoters to perturb indirectly the same processes will shed light on the generality of mechanisms of promotion and will assist in the development of better assays for tumor promoters.

Both our evidence and that of others strongly argue that protein kinase C is the major phorbol ester receptor. An impediment to biochemical and immunological analysis of the receptor is that the published purification protocols are time consuming, afford low yields (0.5-5%), and are difficult to scale up. Taking advantage of new advances in column chromatography, we have now developed purification protocols that permit the rapid and efficient isolation of the receptor. We have also identified stabilization procedures to preserve receptor activity, which is otherwise quite labile once the receptor is in the purified state. The purified receptor is currently being used for preparation of monoclonal and polyclonal antibodies as well as for biochemical studies. We had previously demonstrated that the phorbol ester binding, like the kinase, showed an absolute requirement for phospholipids, i.e., the binding protein is actually an apo-receptor and phospholipids are an essential cofactor. We have now characterized, in detail, the phospholipid requirements for binding, with particular emphasis on the contribution which the phospholipid makes to the properties of the complex as a whole. We find that different phospholipids vary markedly in their ability to form an active complex. Among active phospholipids, the dose-response curves depend dramatically on the presence or absence of  $Ca^{++}$ . In addition to determining whether or not binding can be reconstituted, the nature of the phospholipids also plays a major role in determining the binding affinity of the complex. As would be predicted from this result, reconstitution into mixtures of liposomes of different compositions yields curved Scatchard plots, indicative of heterogeneity in binding affinity. Our current model is that localization of protein kinase C into different lipid environments may help account for the evidence from both biological and binding experiments that suggests receptor heterogeneity.

The high evolutionary conservation of the phorbol ester receptor had suggested the existence of an endogenous phorbol ester analog. Based on the postulated physiological role of protein kinase C, diacylglycerol derivatives appeared to be attractive candidates for the endogenous analogs. We have now demonstrated that diacylglycerols indeed inhibit phorbol ester binding in a competitive fashion. Furthermore, under appropriate experimental conditions, the ratio of diacylglycerol to receptor approaches 1:1, again consistent with competitive

binding. Comparison of the relative affinities for phorbol and glycerol derivatives with homologous side chains indicates that the phorbol esters show only moderately (20- to 80-fold) greater affinity. A prediction from the *in vitro* studies is that elevated diacylglycerol levels *in vivo* should also competitively inhibit phorbol ester binding. This prediction has been confirmed, in collaborative studies with the IVP Section. Treatment of keratinocytes with phospholipase C to endogenously generate diacylglycerol or the exogenous addition of appropriate diacylglycerols competitively inhibits phorbol ester binding.

An initial issue in interpreting phorbol ester structure-activity relations has been whether the phorbol ester receptor recognized that fraction of the phorbol ester which was present in aqueous solution or that which had partitioned into the lipid bilayer. To distinguish these possibilities, we synthesized a series of highly lipophilic phorbol esters. These derivatives were highly active if first incorporated into liposomes, whereas they showed little activity when added to the aqueous phase. The results indicate that the membrane-dissolved form of the phorbol ester is recognized by the receptor. A further implication of these findings is that, at least for lipophilic phorbol esters, the kinetics of transfer of the ligand to or between membranes may play a dominant role in determining activity.

Identification of the enzymatic activity associated with the phorbol ester receptor has made it possible to analyze the coupling between binding and subsequent response. An important issue has been whether all phorbol esters, upon binding, activate protein kinase C to the same maximal extent. We have found that they do so, confirming pharmacological evidence that the phorbol esters function as complete agonists. Secondly, we have begun to examine the effect of agents other than the phorbol esters on protein kinase C activation. Compounds of particular interest are structurally unrelated classes of tumor promoters and inhibitors of tumor promotion. Although most skin tumor promoters did not activate protein kinase C, activation was found for unsaturated fatty acids at high concentrations. Whether similar behavior can be observed *in vivo* is currently being explored. In addition, inhibition was observed with TMB-8, an "intracellular calcium antagonist" reported to inhibit phorbol ester effects.

Considerable data suggest heterogeneity of biological responses to the phorbol esters in mouse skin. Similarly, we have obtained evidence for three classes of binding sites in mouse skin particulate preparations which differ in their binding affinities and structure-activity relations. To further explore the functioning of the phorbol ester receptors in the biologically relevant system of mouse skin, we are currently characterizing the binding of phorbol esters to intact keratinocytes in collaborative studies with the IVP Section. We find one major binding site in keratinocytes grown under low  $Ca^{++}$  conditions. Induction of cellular differentiation leads to appearance of binding heterogeneity as well as the rapid increase in total binding sites. Cell lines resistant to  $Ca^{++}$ -induced differentiation similarly retain the binding characteristics of cells maintained under low  $Ca^{++}$  conditions.

Coupling between receptor occupancy and biological response has been examined in greatest detail in two cell lines (D16 and GH<sub>4</sub>C<sub>1</sub>) which possess receptors for hormones thought to act through induction of phosphatidylinositol turnover and activation of protein kinase C. D16 cells show rapid desensitization

( $T_{1/2} = 0.9$  hr) following phorbol ester treatment although they retain responsiveness to other hormones. Comparison of control and desensitized cells showed no difference in receptor properties on fractionation, in distribution, or in phospholipid requirements. Several inhibitors of protein kinase C were found in cell lysates of D16 cells. These also did not differ. The characterization of the inhibitors has, however, facilitated quantitation of protein kinase C in crude and partially purified preparations. Analysis of phospholipid requirements identified certain phospholipid mixtures which differentially inhibited protein kinase C activity at limiting  $Ca^{++}$  concentrations without inhibiting binding. These results suggest a possible role for phospholipid metabolism in regulation of the coupling of phorbol ester binding and activation of protein kinase C. This possibility is being further explored. The effects of hormonally induced generation of diacylglycerol in  $GH_4C_1$  cells was examined following treatment with TRH (thyrotropin releasing hormone). No effect on phorbol ester binding or receptor localization was observed. In contrast, generation of diacylglycerol by treatment of the intact cells with phospholipase C mimicked phorbol ester effects, namely competitive inhibition of phorbol ester binding, redistribution of receptors from the cytosol to the membrane compartment, decreased binding of epidermal growth factor, and increased secretion of prolactin.

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. At the biochemical level our laboratory has demonstrated that retinol regulates membrane glycosylation reactions, possibly through its involvement as the lipid moiety of the mannosylated retinyl phosphate mannose. Such biochemical involvement is consistent with the reported alterations in glycosylation of  $\alpha_2\mu$ -globulin and  $\alpha_1$ -macroglobulin in vitamin A-deficient livers, and it might explain altered oligosaccharide composition of fibronectin secreted by chick sternal chondrocytes cultured in excess retinoic acid. In the past year we pursued our investigation of the retinyl phosphate-dependent glycosylation system.

Retinyl Phosphate Mannose: Structural and Functional Studies: A major consideration in understanding the function of vitamin A is that retinoic acid can replace retinol in growth and differentiation. Since retinoic acid can also reverse alterations in glycoprotein biosynthesis due to vitamin A-deficiency, it is important to consider that this compound might be metabolized to a retinoid phosphate mannose derivative which could display chromatographic characteristics of standard retinyl phosphate mannose.

Recent development of soft ionization procedures, such as fast atom bombardment mass spectrometry has made it possible in the past year to confirm the putative structure of retinyl phosphate mannose synthesized in vitro from exogenous

retinyl phosphate and guanosine diphosphate mannose. This technique will be applied to the endogenous mannosyl lipid to determine whether intact retinol or a derivative of retinol and/or retinoic acid constitute the lipid moiety of the endogenous mannosyl lipid.

From the functional point of view we considered that the enzyme phospholipase-C, which is unable to cross the membrane bilayer, can fully inhibit the mannosyl-transferase activity responsible for the synthesis of retinyl phosphate mannose, without considerable effect on the biosynthesis of the lipid intermediate dolichyl phosphate mannose. These findings suggested a location for retinyl phosphate mannose at the cytosolic side of the endoplasmic reticulum and a function for this molecule as a carrier of mannose across the membrane, possibly to generate guanosine diphosphate mannose. Consistent with this hypothesis is our recent finding that retinyl phosphate mannose synthesis is a reversible process which regenerates guanosine diphosphate mannose in the presence of excess guanosine diphosphate in microsomal membranes.

In vivo we probed the effects of vitamin A deficiency upon the oligosaccharide assembly process in hamster liver. Deficiency resulted in accumulation of free mannose and a decrease of 95% in the amount of guanosine diphosphate mannose and dolichyl phosphate mannose. Moreover the prevalence of shorter oligosaccharide chains on dolichyl pyrophosphate and in glycoproteins was clearly demonstrated. Therefore it appears that retinyl phosphate or a closely related structure might function in vivo as a regulator of mannosylation reactions associated with the endoplasmic reticulum.

Studies on the Control of Differentiation by Retinoids: Our recent findings have suggested that the condition of retinol deficiency might be common in hepatocellular carcinomas, whether primary or transplanted. Such vitamin A deficiency condition might be either the result of the tumor or a permissive condition for the establishment of the tumor by providing a new phenotype not available under normal conditions of differentiation.

In the respiratory tract such a phenotype would be the squamoid keratinizing cell which arises under conditions of vitamin A deficiency.

In an attempt to study the biological sequence of events which permit the emergence of keratinocytes under conditions of vitamin A deficiency of the respiratory tract, we used the well-characterized system of hamster trachea in organ culture. Our initial effort was directed to identifying keratin molecules synthesized in the respiratory tract in vitamin A deficiency conditions. The use of monoclonal antibodies directed against human epidermal callus acidic keratins and basic keratins made it possible to demonstrate the presence and synthesis of keratin molecules in tracheas cultured in the absence of vitamin A. Similar results were obtained using the polyclonal antibodies directed against mouse epidermal keratins.

This work permits the conclusion that epidermal keratin molecules are synthesized in cultured vitamin A-deficient tracheas.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04504-12 CCTP

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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

COOPERATING UNITS (if any) Uniformed Services Univ. of Health Sciences, Bethesda, MD (J. Stanley); Univ. of Washington, Seattle, WA (Karen Holbrook); Microbiological Assoc., Bethesda, MD (E. F. Spangler); Princeton Univ., Princeton, NJ (M. Steinberg); Baylor College of Medicine, Houston, TX (James Clark)

## LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

## SECTION

In Vitro Pathogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

11.5

## PROFESSIONAL

7

## OTHER:

4.5

## CHECK APPROPRIATE BOX(ES)

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

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

Cellular and molecular aspects of chemical carcinogenesis in lining epithelia are studied in mouse epidermis by in vivo and in vitro techniques. Normal epidermal growth and differentiation are regulated by extracellular calcium. Calcium may exert its effects indirectly via regulation of intracellular concentrations of sodium and potassium. This control appears to involve phospholipid turnover and new protein synthesis and may involve activation of protein kinase C. Carcinogens alter the regulation of epidermal differentiation. This change is highly correlated to the initiating event in carcinogenesis. An activated *ras* oncogene also alters epidermal differentiation, but this effect is conditional and may be modulated by exposure to a tumor promoter. Initiated cells or mouse epidermal tumors do not transcribe unusually high levels of *ras* or other known retroviral oncogenes. Cloning and sequencing of keratin genes, a family of genes coding for the major cytoskeletal and differentiation proteins of epidermis, has revealed common structural features for keratins coordinately expressed during differentiation. The coordinate regulation of these genes can be altered by transformation or exposure to tumor promoters. Promoters markedly accelerate epidermal differentiation, but this can be blocked by antipromoting retinoids which induce a unique transglutaminase possibly counteracting the transglutaminase induced by promoters. The biological effects of tumor promoters can be reproduced by exposing cells to phospholipase C, suggesting that phospholipid turnover and activation of protein kinase C are essential pathways in promotion. While initiation and promotion both involve changes in epidermal differentiation, another genetic change is required prior to carcinoma formation. This change can be accomplished by genotoxic agents but not by tumor promoters.

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

S. Yuspa	Chief	LCCTP	NCI
H. Hennings	Senior Chemist	LCCTP	NCI
M. Poirier	Research Chemist	LCCTP	NCI
D. Roop	Expert	LCCTP	NCI
J. Strickland	Research Chemist	LCCTP	NCI
U. Lichti	Expert	LCCTP	NCI
H. Kawamura	Visiting Fellow	LCCTP	NCI
R. Toftgaard	Guest Researcher	LCCTP	NCI
K. Elgjo	Guest Researcher	LCCTP	NCI
T. Kreig	Guest Researcher	LCCTP	NCI
L. Zwelling	Senior Scientist	DCT	NCI
P. Steinert	Visiting Scientist	DCBD	NCI
A. Jeng	Expert	LCCTP	NCI
P. Blumberg	Senior Scientist	LCCTP	NCI
S. Jaken	Expert	LCCTP	NCI
S. Thorgeirsson	Chief	LEC	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 which may be causative to the transformation process. Specific markers of the transformed phenotype of epithelium are also being sought and mechanisms to prevent or reverse transformation are being studied.

Methods Employed:

This laboratory has developed and utilized mouse epidermal cell culture as an appropriate model to approach the stated objectives. Previous studies have shown that this model functions biologically in a fashion highly analogous to mouse skin in vivo. Human epidermal cells obtained from neonatal foreskins have also been adapted to growth in vitro. In vivo studies utilizing the initiation-promotion model for mouse skin carcinogenesis and grafts of human or mouse skin onto nude mice are also employed. A number of laboratory techniques are required to pursue the objectives. Morphology is followed by light and electron microscopy and histochemical staining. Macromolecular synthesis and growth kinetics are studied by biochemical and autoradiographic procedures and flow cytometry. Intracellular ion changes are assayed by atomic absorption spectrometry. 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 techniques 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 of cells into nude or newborn mice. A number of immunologic techniques including monoclonal and polyclonal antibody production, fluorescent staining, immunoblotting, immunoprecipitation, and radioimmunoassay are being performed to recognize the normal or altered phenotype and to study specific molecules. Isolation of specific mammalian genes is performed through the preparation of epidermal mRNA, reverse transcription, and cloning of transcripts in plasmid pBr322 and the screening of genomic libraries. Cloned genes are characterized by hybridization-selection assays, blot analysis and sequencing.

#### Major Findings:

The pursuit of this project has led to major new findings in four pertinent areas: 1) factors controlling normal epithelial differentiation; 2) quantitation, selection and characterization of carcinogen-altered epidermal cells; 3) biochemical and molecular genetic characterization of specific marker molecules and assessment of their regulation in normal and transformed epidermal cells; 4) understanding of the process of preneoplastic progression and the mechanisms of tumor promotion and anti-promotion.

Much of the progress in this project has developed from the discovery that ionic calcium is a critical regulator of epidermal growth and differentiation. At low ionic calcium concentrations in culture medium (0.02 - 0.09 mM), epidermal cells maintain a monolayer growth pattern with a high proliferation rate. Such cells do not form desmosomal attachments. Essentially 100% of the attached cells are in the proliferating cell pool. Our studies have shown that these cultures have low transglutaminase activity, virtually no cornified cells in the substrate-attached population and synthesize the bullous pemphigoid antigen, all three characteristic of basal cells. The pemphigus vulgaris antigen, a marker for epidermal cells in a more advanced state of differentiation, is not expressed.

When cells maintained under low calcium growth conditions are switched to medium with calcium content above 0.1 mM (standard commercial culture media are 1.2 - 1.8 mM), differentiation is induced. Desmosomes form within minutes of exposure to high  $\text{Ca}^{++}$  medium. These cells vertically stratify, form cornified cell envelopes and slough from the culture dish. This program of differentiation is characterized by a high transglutaminase activity and the synthesis of the pemphigus vulgaris antigen. The synthesis of bullous pemphigoid antigen ceases during  $\text{Ca}^{++}$ -induced differentiation. Following the addition of  $\text{Ca}^{++}$  to culture medium, DNA synthesis, as measured by thymidine incorporation or autoradiography, remains unchanged for 10 hours and then decreases rapidly to less than 10% of control by 24 hours and less than 5% by 48 hours. The mitotic index drops sharply to less than 10% of control values within 2 hours of  $\text{Ca}^{++}$  addition, but then rises to a peak equivalent to the low  $\text{Ca}^{++}$  control group by 10 hours before declining to very low levels again by 24 hours. These results imply that addition of  $\text{Ca}^{++}$  is associated with a specific rapid but transient block in progression from  $\text{G}_2$  to M. Cells completing DNA synthesis accumulate

in G<sub>2</sub>, eventually progress through M and commit to their final loss of proliferative potential in G<sub>1</sub>. Flow cytometry studies are in progress to confirm these findings.

Earlier studies from this laboratory had indicated that retinoids could modulate epidermal differentiation. We have proposed that this effect of retinoids is important in their anticarcinogenic activity. We were surprised to discover that retinoids were potent inducers of a transglutaminase activity in epidermal cells. Epidermal transglutaminase is responsible for cornified envelope cross-linking during terminal differentiation. Our studies also indicated that retinoids were inhibitors of envelope formation which could be stimulated by Ca<sup>++</sup> or the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). Recent studies have provided an explanation for this paradox. The retinoic acid-induced transglutaminase differs in several important aspects from the normal epidermal enzyme responsible for cornified envelope formation. Transglutaminase induced by retinoic acid is soluble in aqueous buffers, is thermolabile at pH 9.5, 37°C, and elutes from an anion exchange column at 0.4 M NaCl. In contrast the epidermal enzyme, which is stimulated by Ca<sup>++</sup> and TPA and is responsible for envelope crosslinking, is particulate and requires detergent for solubilization, is stable at pH 9.5, 37°C, and elutes from the anion exchange column at 0.25 M NaCl. Furthermore, the retinoid-induced enzyme has a lower K<sub>m</sub> for putrescine than the normal enzyme. Therefore the retinoic acid-induced transglutaminase is either a different enzyme or a markedly altered form of the epidermal enzyme normally associated with epidermal differentiation. Its subcellular localization may allow it to act on cornified envelope precursors before their proper assembly at the cell periphery, thus interfering with cornified envelope formation. This mechanism of action may account, in part, for the inhibition by retinoids of epidermal differentiation and the promotion phase of mouse skin tumorigenesis. Our studies suggest that promoter-stimulated accelerated terminal differentiation of a subpopulation of epidermal cells is a requirement for promotion.

We have also identified a soluble epidermal protein of MW 64-70 kd which specifically reacts with antibodies raised in rabbits by injecting purified cornified envelopes. Further studies will be directed to characterizing this epidermal protein and determining whether it also may be modified by retinoids.

The regulation of epidermal differentiation by Ca<sup>++</sup> in vitro suggested that a calcium binding protein might play a physiological role in epidermal differentiation in vivo. Several years ago a 12 kd protein was isolated in France from rat skin and described as a skin Ca<sup>++</sup> binding protein (SCaBP). Antibodies supplied to us from France indicated that antigens of 12, 11, and 10 kd could be precipitated from <sup>35</sup>S-methionine-labeled cultured epidermal cells and that the synthesis of these antigens was regulated by Ca<sup>++</sup>. However, attempts during the last year to purify SCaBP from newborn mouse epidermis failed to yield copurification of antigenic and calcium binding activity. Subsequent studies using a muscle parvalbumin antibody revealed identity of parvalbumin and SCaBP by immunoblotting. Additional purifications were performed from skin components which showed the major 12 kd antigen in rat skin was obtained from the muscular hypodermis, while the 10 and 11 kd and some 12 kd antigens were localized on the epidermis. Since the latter antigens were likely the same as those modulated



by  $\text{Ca}^{++}$  in cultured basal cells, monoclonal and polyclonal antibodies are being produced to these. These results indicate that the major SCaBP antigen is probably parvalbumin from hypodermis and the antibody also recognizes several epidermal proteins which may be specifically synthesized in basal cells but which lack  $\text{Ca}^{++}$  binding activity.

Further studies to characterize differentiation markers have focused on the synthesis and assembly of desmosomes in epidermis. In collaboration with Akihiro Kusumi and Malcolm Steinberg at Princeton University, we have studied the basis for the appearance of desmosomes which occurs within minutes of switching keratinocytes from low to high  $\text{Ca}^{++}$ . Adding antibodies against the 100 kd and 150 kd desmosomal glycoproteins to culture medium blocked the formation of desmosomes in high  $\text{Ca}^{++}$  and caused loss of cell-cell contact in low  $\text{Ca}^{++}$ . These antigens are most likely used for intercellular adhesion even in the absence of desmosomes. Preliminary data has not shown a change in synthesis of any desmosomal proteins upon switch to high  $\text{Ca}^{++}$ , nor is there a change in the phosphorylation pattern of the 240 kd/210 kd plaque proteins by  $\text{Ca}^{++}$  changes. Thus the assembly of desmosomes must be mediated by additional post-translational changes.

The mechanism by which  $\text{Ca}^{++}$  induces terminal differentiation in keratinocytes has been under study for several years. We have reported that intracellular ion changes appear to be regulatory in the process. In recent published studies we have suggested that both  $\text{Ca}^{++}$  and TPA may induce epidermal differentiation via a common pathway involving protein kinase C, the phorbol ester receptor. If this is the case, one would expect certain common patterns of change after calcium or TPA treatment of epidermal cells. In collaboration with Drs. Peter Wirth and Snorri Thorgiersson of the Laboratory of Experimental Carcinogenesis, we have computer analyzed 2-D gel electrophoresis patterns of newly synthesized proteins from cells treated with  $\text{Ca}^{++}$  or TPA. From 600-1200 spots are visible on autoradiographs of proteins prepared from cultured mouse epidermal cells labeled with a  $^{14}\text{C}$ -amino acid mixture and separated in two dimensions over a pH range of 5-8 and a molecular weight range of 10-130 kd. The pattern of protein synthesis 1-4 hours after TPA treatment in low  $\text{Ca}^{++}$  medium was compared to the pattern after switch to high  $\text{Ca}^{++}$  medium. In the first hour after the  $\text{Ca}^{++}$  switch, changes in protein synthesis of two-fold or more (compared to the low  $\text{Ca}^{++}$  DMSO control) were noted in 63 proteins. With TPA, the synthesis of 122 proteins changed by two-fold or more. Of these proteins, the rate of synthesis of 11 were altered by both  $\text{Ca}^{++}$  and TPA; seven were increased and four were decreased, with changes in the same direction for both effectors. This result suggests that a common program of differentiation is induced by both  $\text{Ca}^{++}$  and TPA. The protein most significantly increased (six- to eight-fold) by both  $\text{Ca}^{++}$  and TPA had a molecular weight of 78-82 kd and a pI of approximately 6.0. The results of 2-D gel analysis at 4 hours after TPA or  $\text{Ca}^{++}$  were similar to those found at 1 hour. In this case TPA-treated cells showed a change of three-fold or more in the synthesis of 82 proteins. Of these, 14 were in common with  $\text{Ca}^{++}$  of which 8 increased and 6 decreased. Again, the synthesis of a protein of molecular weight 80-84 kd and a pI of about 6.0 was increased seven- to eight-fold by both  $\text{Ca}^{++}$  and TPA. In addition to the protein synthesis alterations caused by both TPA and calcium, many other changes are specific to one effector or the other.

For example, 4 hours after TPA treatment, the synthesis of a protein with an approximate molecular weight of 90 kd and a  $pI$  of 6.1 is increased nearly 55-fold to become the fourth most intensely synthesized protein on the gel. The synthesis of this protein is unaffected by the  $Ca^{++}$  switch. Patterns of protein phosphorylation in the first hour after the  $Ca^{++}$  switch or after TPA treatment are being analyzed to determine possible alterations common to the two effectors.

The emphasis we have placed on understanding the regulation of normal epidermal differentiation evolves from our discovery that carcinogen exposure in vivo or in vitro yields keratinocytes whose response to differentiation signals is altered. In the cell culture model where  $Ca^{++}$  induces differentiation, such cells are selectable since they produce expanding colonies under high  $Ca^{++}$  growth conditions. This observation has now provided a standard quantitative assay for comparing the potency of various agents as initiators in vivo and focus inducers in vitro. Eight skin initiators of varying potency and from different chemical classes and ultraviolet light were studied for their ability to induce this alteration in cultured epidermal cells from BALB/c newborn mice. There was an excellent positive correlation for the potency of these agents as initiators in vivo and as inducers of altered differentiation in vitro. Ultraviolet light was particularly effective when given in split dosages. The induction of resistant foci was independent of the relative cytotoxic effects of each agent except where cytotoxicity was extensive and reduced the number of foci. The tumor promoter, TPA, produced no foci by itself and failed to enhance focus number when administered after carcinogen exposure. The results support the hypothesis that initiation of carcinogenesis in skin results in an alteration in the program of epidermal differentiation. Additional evidence for this association was derived from studies in which adult mice were initiated in vivo by increasing doses of 7,12-dimethylbenz[a]anthracene (DMBA) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and epidermis removed and cultured in vitro under conditions selecting for foci altered in their differentiation program. The number of foci increased with carcinogen dose. DMBA, a more potent initiator, consistently gave more foci than MNNG (2.5 foci/ $\mu$ mole vs. 0.4 foci/ $\mu$ mole, respectively). Of particular importance was the finding that differentiation-altered foci were formed even if a 9-week interval existed between carcinogen exposure and plating of epidermal cells in culture. This is consistent with the irreversibility of initiation.

Quantitative studies were performed to correlate focus number with carcinogen binding to DNA. Exposure to benzo[a]pyrene (BaP) or its anti-diol epoxide (BPDE) at concentrations producing low cytotoxicity yielded frequencies of differentiation-altered foci proportional to the dose of the compound used and to the number of DNA adducts formed. The anti-diol epoxide was approximately five times more effective than BaP in inducing foci, and this difference correlated directly with the quantity of DNA adducts formed. Adduct levels associated with induction of altered foci in the hydrocarbon-exposed cultured cells were low (1-10 fmoles/ $\mu$ g DNA) and similar to those found in mouse epidermal DNA after exposure to initiating doses of BaP. Additional studies showed striking similarities between mouse epidermis in vivo and in vitro in the formation and removal of BaP adducts, and indicated that in skin an alteration in normal biological programming can be associated with relatively low levels

of BaP-DNA adduct formation. Interestingly, when N-acetoxyacetylaminofluorene was used as initiator, many more DNA adducts formed but only a few foci were produced. On a molar basis, BPDE is 500-fold more effective in producing foci, suggesting that the nature of the DNA adduct may be crucial to biological effect.

The production of foci with altered differentiation characteristics is analogous to initiation of carcinogenesis in a number of characteristics as has been described. Thus the development of cell lines from such foci could be considered to provide an *in vitro* population of initiated cells. We have extensively characterized eight lines derived from differentiation-altered foci but failed to find a common distinguishing marker beyond the ability to grow in high  $Ca^{++}$ . Three of these lines, which are nontumorigenic, have been further analyzed for unique responses to phorbol ester tumor promoters. TPA failed to induce transglutaminases or increase the number of cornified cells in any line. In two of the three lines, TPA stimulated thymidine incorporation into DNA and increased colony size in a clonal growth assay. The third line was not stimulated by TPA and higher doses of the promoter inhibited growth. The failure of these lines to terminally differentiate in response to phorbol esters, while normal keratinocytes are induced to differentiate, would produce a selective advantage to such cells during tumor promotion.

The monoclonal origin of papillomas in skin tumorigenesis suggests they represent a clonal expansion of initiated cells. We have been able to culture cells from chemically induced papillomas by using enzymatic dissociation and plating in fibroblast-conditioned medium. Papilloma cells grow more rapidly in low  $Ca^{++}$  medium, and their proliferation slows considerably in high  $Ca^{++}$ , although they do not cease proliferating nor do they slough from culture. Nevertheless, their cornification rate increases in high  $Ca^{++}$ . Interestingly, TPA reverses the growth inhibition in high  $Ca^{++}$  and depresses cornification upon prolonged exposure although short exposures enhance differentiation. These results suggest that some initiated cells can respond to  $Ca^{++}$  by growth inhibition, but TPA can reverse this response, thereby providing a growth advantage. Such cells may comprise TPA-dependent papillomas while  $Ca^{++}$  resistant foci may comprise TPA-independent (autonomous) papillomas.

The isolation of cellular homologues of retroviral oncogenes from human tumors and the demonstration of their transforming activity in 3T3 cells has stimulated us to study the role of activated ras oncogene in our culture model. Previous studies have indicated that infection with Harvey or Kirsten murine sarcoma virus enhances basal cell proliferation but such cells stop proliferating when exposed to high  $Ca^{++}$  medium. This seemed inconsistent with the ras gene being sufficient to transform cells. Further studies, however, have revealed new features of the virus infected high  $Ca^{++}$  cells. While the cells do not proliferate in high  $Ca^{++}$ , they continue to synthesize pemphigoid antigen and do not switch on pemphigus antigen, suggesting a block very early in differentiation. These cells maintain their responsiveness to TPA in high  $Ca^{++}$  where exposure results in an induction of ornithine decarboxylase activity and a decrease in transglutaminase activity. Kirsten virus-infected keratinocytes in high  $Ca^{++}$  can revert to a proliferative population when switched back to low  $Ca^{++}$ , unlike

normal keratinocytes. Virus-infected keratinocytes also synthesize a new cytoskeletal protein of 58 kd in high  $Ca^{++}$ , but this protein is not yet identified. These characteristics of Kirsten virus-infected cells imply that the cells are blocked in an early stage of differentiation which is reversible and that most importantly they are responsive to TPA. In this respect they resemble papilloma cells. Thus an active ras gene could render keratinocytes responsive to phorbol esters even after they are committed to differentiate. If TPA could restore their proliferative capabilities, tumor formation could be possible.

Since it is known that infection of fibroblasts with sarcoma viruses leads to growth factor production by the infected cells, it seemed possible that at least some of the biological effects of infection might be explained by growth factors. The unusual kinetics of the virus effects have been difficult to explain. High virus titers are required, and relatively small dilutions of virus result in loss of the biological effects. The effects occur throughout the culture in an all or none fashion rather than becoming focal at lower virus titers. Measurement of epidermal growth factor (EGF) binding demonstrated much decreased binding by virus-infected epidermal basal cells compared to uninfected controls. When infected cells were induced to differentiate by raising  $Ca^{++}$  levels in the media, EGF binding was almost zero. This is consistent with our earlier demonstration that EGF binding capacity decreases in differentiating epidermal cells and bound EGF is not metabolized. Thus, EGF or other EGF-like growth factors would rapidly saturate the receptors. Uninfected epidermal basal cells exposed to 10-100 ng/ml EGF are stimulated to proliferate in a manner similar to that of viral infection. When such cells are induced to differentiate by high  $Ca^{++}$ , the process is delayed but ultimately such cells completely differentiate and slough from the dish. This suggests that EGF-like growth factor elaboration by virus-infected cells could explain the proliferative influence of such an infection but an additional activity which prevents completion of the differentiation program must also be involved.

In an attempt to probe functional changes in gene expression in epidermis which may occur in carcinogen- or promoter-treated normal cells or in malignant cells, cDNA clones for keratin proteins, the major cytoskeletal component and differentiation product of epidermal cells, have been isolated. Initially, a cDNA library was constructed to total poly(A) RNA isolated from newborn mouse epidermis. From this library clones corresponding to the 55, 59, and 67 kd keratins, the major keratins synthesized in this tissue, were isolated. Using these cloned cDNAs as specific probes to detect transcripts of these keratin genes, we have been able to show that transcripts for these genes are abundant in RNA isolated from newborn epidermis but absent in RNA isolated from primary epidermal cell cultures. These results suggest that this subset of keratin genes is only expressed in differentiating cells within the epidermis. We have also isolated cDNA clones corresponding to the keratins expressed in primary epidermal cell cultures, which are 50, 55 (called 55b) and 60 kilodaltons. Transcripts for these genes are present in RNA isolated from newborn mouse epidermis but at a concentration lower than that observed for the 55, 59 and 67 kd keratin genes. We assume that our primary epidermal cell cultures are representative of the basal (proliferating) cells found in intact epidermis and that the transcript levels observed for the 50, 55b and 60 kd keratin genes in newborn mouse epidermis reflect the relative contribution of basal cells, which consists of

one cell layer, to total epidermis, which contains many cell layers at different stages of differentiation. Therefore, the cDNA clones which we have isolated provide probes for proliferating and differentiating epidermal cells.

The expression of keratin genes has been studied in the epithelium of the vagina in collaboration with Jim Clark (Baylor College of Medicine). This tissue is of interest because the degree of keratinization in this epithelium is hormone-dependent and it allows the orderly induction of keratin gene expression to be studied in vivo in ovariectomized rats after exposure to estradiol. Two important observations have resulted from this study. First, the keratin genes expressed in epidermis are also expressed in the internal keratinizing epithelium of the vagina. Second, the temporal induction of these genes after exposure to estradiol correlates well with the observed morphological changes in this epithelium. Resting vagina shows little evidence for expression of any of the keratin genes expressed in epidermis. There is a dramatic induction of the keratin genes expressed in proliferating cultured epidermal cells (50, 55b, 60 kd proteins) within 24 hours after exposure to estradiol and this correlates with the onset of proliferation in the vaginal epithelial cells. By 48 hours after estradiol these transcripts decrease, presumably reflecting a decreased stability of these mRNA's as vaginal cells begin to terminally differentiate. The transcription of the keratin genes expressed in differentiating epidermal cells (55, 59, 67 kd proteins) is sequential during hormone induced differentiation, with 55 kd keratin mRNA expressed first followed by the 59 kd and finally the 67 kd transcript. Transcripts for these genes appear to be stable and accumulate throughout this time course. The expression of these three keratin genes correlates with stratification and keratinization in this epithelium.

The orderly expression of keratin genes following a physiological stimulus such as estradiol is markedly contrasted to the influence of pharmacological agents on keratin gene expression in epidermis. Mice were treated topically with TPA or other hyperplasiogenic agents such as mezerin, retinyl phorbol acetate or ethyl phenyl propiolate and RNA was isolated at various times and probed by slot hybridization. All agents caused a decrease in the relative levels of RNA corresponding to the 55, 59 and 67 kd keratins. At 12 and 24 hours after TPA, less than 20% of the level for these mRNAs in untreated epidermis was observed. By 48 hours mRNA for the 67 kd keratin had returned to control values, while the 55 kd keratin remained low. mRNA for the 59 kd keratin remained lower than control for the first 72 hours after treatment but returned to normal after 7 days. The mRNAs for the 50, 55b and 60 kd keratins were elevated above control at 12, 24, 48 and 72 hours after treatment. The decrease in mRNAs corresponding to the differentiation-associated keratins may reflect a promoter-mediated acceleration of terminal cell maturation and rapid loss of differentiating cells from the skin. The priority return of the 67 kd mRNA before the 59 and 55 kd mRNA is reversed compared to that observed normally and suggests that an altered differentiation program results from promoter treatment, perhaps by accelerating the transit time to the upper layers where the 67 kd keratin is synthesized. The increase in the 50, 55b and 60 kd keratins indicates expansion of the basal cell compartment by compensatory hyperplasia or retardation of maturation in a subclass of basal cells. The 55, 59 and 67 kd keratin mRNAs are very low in

epidermal carcinomas, whereas in papillomas they are essentially the same as in untreated epidermis. The mRNAs for the 50, 55b and 60 kd keratins are apparently not altered in either papillomas or carcinomas.

In collaboration with Peter Steinert (Dermatology Branch, NCI, Z01CB03656-110), the DNA sequence of these cDNA clones has been determined to deduce their amino acid sequence. All of these subunits contain a central domain of about 300 residues which forms a coiled-coil  $\alpha$ -helical structure. Variation in both size and sequence are observed in the non  $\alpha$ -helical amino acid carboxy-terminal domains of these subunits. The 50, 55b and 60 kd keratin subunits expressed in undifferentiated cells contain conserved sequences enriched in serines, whereas subunits expressed in terminally differentiating cells contain numerous repeat sequences enriched in glycines. Variations in these sequences in keratins that are expressed at different states of differentiation implicate their involvement in changes in keratin filament properties and function during differentiation. These glycine and serine-rich ends have been shown to be located on the periphery of filaments and are believed to be sites of modifications as well as regions where filaments may interact with other cell constituents. In addition to these structural features, the C-terminal amino acid sequences of these keratin subunits were found to be unique. Synthetic peptides corresponding to these sequences have been used to produce antisera that are highly specific for the 55b, 59, 60, and 67 kd keratins. These antisera have been used to localize these keratin subunits within newborn mouse skin by indirect immunofluorescence. The results obtained agree with the expression data obtained with the cDNA probes. The 59 and 67 kd keratin subunits were only present within the differentiated layers of the epidermis and not the basal layer. However, the 55b and 60 kd keratins were detected within the basal and suprabasal layers. The utilization of these monospecific antisera in combination with the cDNA probes provides a very specific means to monitor the expression of individual keratin genes at both the level of transcription and translation. In addition, the assembly of keratin subunits into filaments can be followed with the antisera.

In an attempt to determine the structure and organization of these keratin genes within chromosomes, genomic sequences corresponding to these keratin cDNA clones have been isolated. The complete sequence of the 59 kd keratin gene has been determined. This gene contains seven intervening sequences. Of most interest is the presence of an intervening sequence within the 3' untranslated portion of this gene. Although the 3' untranslated portions of all of the keratin genes have highly diverged during evolution, they are highly conserved for a given gene between species. This may suggest as yet undefined function for this region of keratin mRNAs. Genomic sequences for other keratin genes are being sequenced to determine if common regulatory sequences are shared by the genes coordinately expressed during differentiation.

The localization of these keratin genes to individual mouse chromosomes is being determined by restriction fragment length polymorphisms in collaboration with Celia Blatt (Agoaron Institute). To date the 60 and 67 kd keratin genes have been assigned to chromosome 15. The exact linkage has not been determined. Other keratin genes show a different segregation pattern and map to other chromosomes.

Other markers have been explored at the level of gene expression to determine their relevance to epidermal differentiation or tumorigenesis. The report that an active Harvey-*ras* gene can be recovered from epidermal tumors and our findings that *ras<sup>H</sup>* and *ras<sup>K</sup>* affect epidermal differentiation and proliferation steered us toward a study of the expression of other viral oncogenes under various conditions of cell growth and tumorigenesis. RNA was isolated from control adult back epidermis, or epidermis at 12, 24, 48, 72 hours and 7 days after treatment with the tumor promoter, TPA. RNA was also isolated from papillomas and carcinomas and cultured cells grown under low and high  $Ca^{++}$  conditions or treated with TPA.  $Ca^{++}$  resistant cell lines were also studied. The RNA was analyzed by the slot-blot technique using probes corresponding to the following oncogenes: *ras<sup>H</sup>*, *ras<sup>K</sup>*, *myc*, *myb*, *fes*, *fos* and *abl*. Only minimal changes (less than two- to three-fold) in transcript levels for these genes were observed after TPA treatment at any time point. Similar results were obtained for RNA from papillomas and carcinomas as compared to controls and in all cultured cells. These results suggest that TPA exposure or the hyperplasia which ensues is not associated with marked alterations in expression of these oncogenes. Similarly the acquisition of the neoplastic phenotype does not require a large increase in transcription of these genes. If the oncogenes assayed, other than *ras<sup>H</sup>*, are involved in the induction of papillomas and carcinomas, an altered gene product rather than altered expression is more likely.

Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Previous studies from this laboratory have provided a cellular basis for selection. Subpopulations of epidermal basal cells respond heterogeneously to phorbol ester tumor promoters in that some cells are induced to differentiate while others are stimulated to proliferate. Additional studies have indicated that more mature basal cells respond in the differentiative pathway. In another section of this report the lack of a differentiative response for putative initiated cells was described. Thus promotion could be based on selective normal cell loss and clonal expansion of initiated cells.

The molecular basis for this pharmacological heterogeneity may be clarified in studies conducted in collaboration with the Molecular Mechanisms of Tumor Promotion Section of LCCTP. Measurements of [ $^{20-3}H$ ]phorbol 12,13-dibutyrate ([ $^3H$ ]PDBu) binding to mouse skin particulate preparations yielded curvilinear Scatchard plots which best fit a model of three phorbol ester binding components with affinities for PDBu of 0.7 nM (PBS-1), 10.3 nM (PBS-2) and 52.3 nM (PBS-3). Binding of [ $^3H$ ]PDBu to intact cultured mouse primary keratinocytes was analyzed in low (0.07 mM)  $Ca^{++}$  and high (1.2 mM)  $Ca^{++}$ . [ $^3H$ ]PDBu binding to proliferating keratinocytes in low  $Ca^{++}$  yielded a linear Scatchard plot consistent with a single binding component which had a  $K_D$  of  $13.7 \pm 1.6$  nM and was present at  $1.3 \pm 0.3$  pmol/mg protein ( $n = 4$ ). The data span a range of receptor occupancies from 2% to 98% of the total bound. The binding parameters for the intact proliferating keratinocytes closely resemble those for PBS-2 in membrane preparations from epidermis. In contrast, specific binding of [ $^3H$ ]PDBu to differentiating keratinocytes (cultured in the presence of 1.2 mM  $Ca^{++}$ ) yielded a curvilinear Scatchard plot similar to that observed in skin particulate preparations. Thus the characteristics of binding of [ $^3H$ ]PDBu to keratinocytes depend on their state of differentiation, further supporting the idea that

differentiation may modulate the pleiotropic response of this tissue to phorbol esters. Interestingly, putative initiated cell lines display only a single binding component most similar to PBS-2, in either high or low  $\text{Ca}^{++}$ .

Epidermal cell responses to TPA can be modulated by the  $\text{Ca}^{++}$  content of the culture medium. While TPA induces significant cornification under all  $\text{Ca}^{++}$  conditions, cornified cells remain attached to the cell layer in high  $\text{Ca}^{++}$ , whereas they detach in low  $\text{Ca}^{++}$ . In the latter case, a selective detachment of polyploid cells appears likely. While DNA synthesis is inhibited rapidly in both high and low  $\text{Ca}^{++}$  cells exposed to TPA, it remains low only in high  $\text{Ca}^{++}$  cells while low  $\text{Ca}^{++}$  cells regain proliferative capacity. Increasing  $\text{Ca}^{++}$  alone causes a rise in intracellular  $\text{Na}^+$  and  $\text{K}^+$  associated with differentiation. TPA causes a dramatic decrease in  $\text{K}^+$  while  $\text{Na}^+$  increases substantially. These ion changes occur in both high and low  $\text{Ca}^{++}$  medium and are not significantly altered by large changes in the  $\text{Na}^+$  or  $\text{K}^+$  concentration of the medium. Thus, while  $\text{Ca}^+$  and TPA appear synergistic in certain responses, there can be dissociation of response as well.

The induction of terminal differentiation by TPA in a subset of basal cells is associated with the accumulation of DNA strand breaks in this subpopulation. In studies performed in collaboration with Dr. Leonard Zwelling of the Division of Cancer Treatment (LMPH, Z01CM06150-04) we have shown that DNA breaks, as measured by alkaline elution, occur as a consequence of differentiation rather than as causative. Only differentiating cells display breaks after TPA exposure, and cells resistant to the differentiation inducing affects of a single TPA exposure are resistant to breaks after subsequent exposure. Other phorbol ester-like promoters induce breaks in proportion to their potency as promoters and as inducers of differentiation. Breaks occur more rapidly when epidermal cells are exposed to both TPA and high  $\text{Ca}^{++}$ , a condition which enhances the differentiation response. DNA breaks may be a physiological consequence of differentiation in epidermis associated with nuclear breakdown.

Many of the biochemical effects of the tumor-promoting phorbol esters appear to result from interactions of these compounds with specific cellular receptors, namely protein kinase C. Since diacylglycerols, which are produced enzymatically from phospholipids by phospholipase C, appear to be physiological ligands for protein kinase C, primary cultures of mouse epidermal basal cells have been treated with phospholipase C and examined for several biochemical and biological responses associated with phorbol ester treatment. These studies were conducted in collaboration with the Molecular Mechanisms of Tumor Promotion Section of LCCTP. In every case studied thus far, treatment with phospholipase C resulted in responses similar to those seen when cells were treated with tumor-promoting phorbol esters. A 30-minute treatment with phospholipase C at 0.05 U/ml led to the morphological changes characteristic of phorbol ester treatment and to 90% reduction in EGF binding. Continuous treatment at the same dose led to the induction of the enzymes, transglutaminase and ornithine decarboxylase, with a time course and extent similar to the inductions by TPA. Higher concentrations (0.3 U/ml) were required for 50% suppression of phorbol dibutyrate binding without reduction in total number of binding sites, consistent with the production of a competitive inhibitor of phorbol ester binding by phospholipase C. Treatment of cells with phospholipase C after prelabeling with [ $^3\text{H}$ ]arachidonic



acid led to a dose-dependent release of radioactive diacylglycerols, indicating that the enzyme acted on the cells as expected. The results of this collaborative study on a single cell system support the hypothesis that diacylglycerols and tumor-promoting phorbol esters act through a common pathway, namely protein kinase C to produce the biochemical and biological responses we have reported.

While pursuing mechanistic studies on epidermal carcinogenesis *in vitro*, *in vivo* carcinogenesis experimentation is actively pursued under contract CP1-5744. Data obtained previously have indicated a three-stage requirement for carcinoma formation after skin painting. Initiation appears to require a genotoxic carcinogen while promotion by phorbol esters results in a large number of benign tumors. Continuous promoter exposure does not influence the carcinoma yield but subsequent exposure of papilloma-bearing mice to genotoxic agents markedly accelerates and enhances carcinoma yield (malignant conversion).

More recent experiments have confirmed that a papilloma stage is required prior to any carcinoma formation since initiation followed by treatment with urethane or 4-nitroquinoline-N-oxide does not produce malignancies. However, MNNG alone when given repeatedly can lead to carcinomas and therefore is not an optimal agent for these studies. Additional studies on CD-1 mice, where papilloma regression is a significant occurrence when TPA exposure is terminated, confirms the three-stage requirement for carcinoma formation. Most interesting is the observation that carcinoma formation after initiation and promotion was equal in groups receiving continuous TPA or just acetone in the conversion stage. Since the number of papillomas was smaller in the acetone group, it appears that TPA-dependent papillomas are at very low risk for carcinoma formation. Additional studies are underway to determine the dose requirements for genotoxic agents to be effective in converting papillomas to carcinomas and to search for possible inhibitors of the conversion step.

#### Significance to Biomedical Research and the Program of the Institute:

The majority of human cancers are associated with environmental exposures, and most of the tumors are of epithelial origin. The development of a cell culture model system for epithelial carcinogenesis has been a major requirement for understanding the specific cellular and molecular alterations associated with malignant change in these specialized cells. The epidermal cell culture system has provided a required model. In previous years we have demonstrated a strong parallel in the biologies of epidermis *in vitro* and *in vivo*. Our current studies have focused on control mechanisms for normal growth and differentiation, on alterations produced by initiators and promoters, and on markers associated with the transformed phenotype. We have discovered a primary regulation of epidermal differentiation in our calcium studies. Analogous work has subsequently been pursued in other laboratories and it now appears that calcium is an important regulator of differentiation in esophagus, bronchus, bladder and mammary epithelium. Cell culture model systems for these other important epithelial target organs which share characteristics of our model are being developed in other laboratories. Our current studies in this project are defining the biochemical pathways crucial for differentiation to proceed normally in epidermis. Presumably parallels will exist in a variety of models. The importance of understanding normal differentiation, aside from its inherent value,

is emphasized by our findings that an early event in chemical carcinogenesis is an alteration in differentiation control. We have used this alteration to develop an assay system capable of categorizing potency of initiators, a first for epithelial cells, and for primary cultures. Furthermore we have isolated a number of preneoplastic cell lines and have sought markers to characterize their altered biology. The definition of such markers could be useful for recognizing preneoplastic cells *in vivo* and thereby facilitating early diagnosis and chemoprevention efforts. The availability of models in which discrete changes can be induced by carcinogens, in which cell lines can be followed for progressive transformed phenotype, and in which conversion to malignancy can be monitored provide valuable resources to analyze the role of oncogenes in tumorigenesis. Similar importance is associated with the cloning and study of the keratin gene family. These genes code for the major cytoskeletal elements of all epithelial cells and the major differentiation proteins of many epithelia which are common targets for carcinogenesis in humans and experimental animals. Their expression is altered in most epithelia during carcinogenesis, but heretofore the relevance of this finding has not been understood. The regulation of expression of these genes is now amenable to study. Experimentally tumor promotion is the major influence in determining latency period and an important factor in determining target site for carcinogenesis. It is likely that the promotion phase has a similar importance in human carcinogenesis. Our studies have provided a biological basis for tumor promotion in skin, and the pharmacological mechanisms involved are becoming clarified. The concept of selective clonal expansion of initiated cells by tissue-specific agents has applicability to all model systems where promotion has been demonstrated. Our mechanistic studies have provided insight for other investigators to pursue tissue-specific mechanisms of promotion in other models. Furthermore the insights obtained from these studies have guided our experiments on the mechanism of action of promotion inhibitors, in particular retinoids, steroids and protease inhibitors. *In vivo* studies from this project have defined a discrete, promotion-independent step of malignant conversion. This is likely a genetic change and the genes involved are under study. Since tumors become life threatening once malignant conversion occurs, molecular understanding and prevention of this change could have profound effects on cancer mortality.

#### Proposed Course:

This project represents an integrated, comprehensive approach to understanding the biological changes associated with initiation and promotion of carcinogenesis and their underlying molecular mechanisms. Future studies are a logical extension of each component of the overall approach. In order to understand the regulation of normal epidermal differentiation, the calcium-modulated culture model will continue to be studied in detail. Ionic changes, which appear to be important in differentiation, will be analyzed by additional studies on ion flux and measurements of intracellular pH changes. Since these changes may involve NaK-ATPase, the activity of this enzyme will be studied under various  $Ca^{++}$  conditions and after TPA exposure. If antibodies can be obtained, the phosphorylation state of NaK ATPase will be assessed under conditions of induced differentiation. Since our studies suggest that activation of protein kinase C is common to both  $Ca^{++}$  and TPA-induced differentiation, protein phosphorylation patterns will be analyzed in detail by 2-D gel analysis

assisted by computer. These studies will be performed in collaboration with Drs. Snorri Thorgeirsson and Peter Wirth of the Laboratory of Experimental Carcinogenesis, NCI. Direct isolation of protein kinase C from normal and altered epidermal cells, activity measurements and subcellular localization will be studied. New proteins which are synthesized during differentiation will be categorized, and modulated proteins (synthesis or phosphorylation) which change similarly for both inducers (TPA or  $Ca^{++}$ ) will be studied in depth in the hope of identifying their functions. Since activation of protein kinase C often involves phospholipid turnover, changes in phosphatidyl inositol, phosphatidyl choline and fatty acids will be evaluated after  $Ca^{++}$  or TPA. Studies will be carried out using phosphate label or labeled precursor. Further efforts to clarify the role of the calcium binding proteins or associated antigens in epidermis will be made through studies at the protein level and through cloning of the genes to study their expression at the molecular level. Monoclonal and polyclonal antibodies to the basal cell proteins which copurify with ScaBP will be raised in rabbits and mice and used to isolate these proteins. The results obtained with normal keratinocytes will be compared to results of similar studies performed on preneoplastic and neoplastic keratinocytes.

Transformation studies utilizing resistance to induced differentiation will be expanded. Additional chemicals of varying initiating activity and ionizing radiation will be tested. Modification of the target cells at the time of carcinogen exposure will be utilized to attempt to enhance or inhibit the transforming event. Modifiers will be chosen which are known to alter initiation in mouse skin *in vivo*. Modifiers which can alter the extent or pattern of carcinogen binding to DNA will also be utilized to determine the effect on initiation. Immunological assays, developed in this laboratory (see project Z01CP05177-01 LEP), will be used to monitor binding. The effects of split doses of UV will be studied in greater detail to determine the mechanism of enhancement of focus production by this treatment protocol. Analysis of cell cycle changes, persistence of sensitivity to additional exposures after a single exposure and DNA repair kinetics will be analyzed and a variety of split dose regimens will be tested. Further attempts to enhance focus formation with tumor promoters will be made.

Mouse skin will be exposed to a variety of treatment regimens *in vivo*, and epidermal cells from treated skin will be isolated and studied *in vitro*. Progression from the initiated cell to the malignant cell will be systematically studied *in vitro* to elucidate the temporal sequence of this change and to examine the capability of additional carcinogen or promoter treatments to accelerate progression. The development of clonal transformation assays will continue with the testing of epidermal cell lines with a high cloning efficiency and the use of feeder layers to enhance clonal growth of primary cells.

The mechanism by which ras gene expression and the p21 transforming protein can alter proliferation and differentiation will be explored at the molecular level. Virus-altered cells will be characterized by a number of epidermal markers to determine the differentiation state these cells represent. Other viruses will be studied in an effort to find oncogenes which might block epidermal cells in the particular differentiation state characteristic of chemically altered cells.

Combined treatments with chemicals and viruses will be performed under conditions where each treatment alone is insufficient to transform cells to tumorigenicity. Parallel studies using labeled DNA probes to known oncogenes will be conducted to analyze for expression of these genes in various states of altered differentiation and transformation. Transfection of DNA from altered cells into normal basal keratinocytes will be performed followed by high  $\text{Ca}^{++}$  selection in order to isolate genetic information which can impart differentiation resistance to normal keratinocytes. Human tumor DNA will be used to facilitate the isolation of transfected genes. In addition cloned oncogenes derived from tumors or cloned genes from oncogenic viruses will be tested by transfection methodology using differentiation resistance as a selectable marker.

The  $\text{Ca}^{++}$  resistance marker selects for a preneoplastic property. Our in vivo studies indicate that at least two genetic changes are required for malignancy. We will attempt to develop an in vitro assay for the conversion (benign to malignant) step using repeat treatments with carcinogens on differentiation-altered foci obtained after a single exposure. Retreated foci will be tested in vivo for tumorigenicity. If successful, an analysis of the genetic changes involved will be performed by transfecting DNA from the tumorigenic cells into non-tumorigenic  $\text{Ca}^{++}$  resistant cell lines as recipient.

The observation that retinoids induce a unique epidermal transglutaminase which may be causally related to the modulating effect of retinoids on epidermal differentiation and tumor promotion will be pursued at the molecular level. Both the normal transglutaminase and the retinoid-induced enzyme will be purified and antibodies will be produced. Furthermore, using cells with high activity for each enzyme, attempts to clone the two genes involved (assuming two gene products) will be made. If successful the cDNA probes generated will be used to analyze the level of regulation involved in each induction and ultimately to facilitate characterization of the transglutaminase gene family. Probes will be used to characterize changes in expression in transformed cells. Experiments will also be performed to identify and characterize transglutaminase substrates and examine their modulation in transformation and under the influence of retinoids.

The availability of cDNA clones for keratin genes provides unique markers for a gene family whose expression may characterize specific differentiation states. Individual keratin mRNAs will be localized to specific layers of the epidermis by in situ hybridization of radiolabeled keratin cDNA probes in histological sections of normal epidermis and epidermis treated with initiators and promoters. If this is successful, this technique may provide a means of identifying cells with altered patterns of keratin gene expression which may result after exposure of cultured epidermal cells or mouse skin to carcinogens or promoters and in tumors. Additional genomic keratin sequences will be isolated to better understand the structure, localization and regulation of this family. Sequencing of cDNA and genomic sequences will be completed in an attempt to correlate structure with function. The specific antibodies raised to unique keratin peptides will be used to analyze expression changes in cells under various pharmacological conditions. Expression vectors will be constructed using cDNA or genomic fragments and these engineered sequences will be transfected into cells normally not expressing keratins or into cells in which certain

normal keratin genes are not expressed (such as malignant keratinocytes). Biological consequences will be monitored and filaments which form by these methods will be analyzed.

Our studies on phorbol ester effects on epidermal cells will continue to focus on the molecular mechanism of induced differentiation. The phospholipase results suggest that phospholipid turnover and protein kinase C are critically involved in the differentiative response. Future studies, already described, will examine the effects of promoters on protein synthesis and phosphorylation, phospholipid turnover and activation and localization of protein kinase C. Changes in lipid environment under different maturation states will be explored to assess the effects on TPA binding to its receptor. The effect of TPA on ion fluxes and NaK ATPase will be studied. Reconstruction experiments with normal and initiated cells or normal and papilloma cells will assess the ability of phorbol esters to select initiated cells from a mixed population. Similar studies will be performed with agents such as mezerein and teleocidin.

In vivo experiments will be designed to extend our observations of the requirements for carcinoma formation. Treatment schedules will be reversed to test for obligate sequences of various stages in cancer development. Inhibitors will be used in the promotion stage to prevent papilloma formation and these animals will be monitored for carcinoma formation with subsequent treatment with carcinogens. Dose response and treatment duration experiments will analyze the sensitivity of the conversion step to carcinogen exposure. Inhibitors of malignant conversion will be sought and promoting agents other than phorbol esters will be examined to determine their potential to convert papillomas to carcinomas. These results will be useful for establishing the relevant biology in vivo which can then serve as a guide to conducting mechanistic experiments on a conversion assay established in vitro.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1CE04798-14 CCTP

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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
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Others:	K. Creek	Staff Fellow	LCCTP	NCI
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	D. Rimoldi	Visiting Fellow	LCCTP	NCI
	U. Lichti	Expert	LCCTP	NCI
	F. Bonelli	Visiting Fellow	LCCTP	NCI

COOPERATING UNITS (if any) Howard Univ., Wash., DC (K. Olden); National Institute of Environ. Health Sciences, Research Triangle Park, NC (A. M. Jetten); Univ. of Rome, Italy (S. Adamo); Natl. Institute of Dental Research, Bethesda, MD (J. R. Hassell); Microbiological Associates, Bethesda, MD ( R. Curren and E. F. Spangler).

## LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

## SECTION

Differentiation Control Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

7

## PROFESSIONAL:

5

## OTHER:

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.)

Maintenance of epithelial differentiation is one of the biological functions of vitamin A and explains current interest in this nutrient as a chemopreventative agent of epithelial cancer. Therefore, current research efforts center on the elucidation of possible sites and mechanisms of action of the vitamin. Retinoic acid can modify the composition of the carbohydrate moiety of the collagen-binding domain of fibronectin in cultured chicken sternal chondrocytes. This and other data are consistent with a role of the vitamin in glycoprotein biosynthesis, possibly as a result of its participation as a structural component of the membrane-associated retinyl phosphate mannose. This mannosyl lipid, whose structure was confirmed by fast atom bombardment and collisional activation mass spectrometry, may be functioning in the membrane to generate guanosine diphosphate mannose. In support of this proposal are the in vivo results which demonstrate a decreased pool of guanosinediphosphate mannose and the lipid intermediate dolichyl phosphate mannose and the accumulation of shorter oligosaccharide chains on lipids and proteins in vitamin A-deficient hamster liver microsomal membranes. A similar condition of vitamin A deficiency was found in primary and transplanted minimally and maximally deviated hepatocellular carcinomas, suggesting the possibility that deficiency of vitamin A may be either a consequence of cell selection during carcinogenesis or a permissive condition for the development of the tumor from initiated cells. In cultured hamster tracheas vitamin A deficiency causes the predominance of the higher molecular weight species of keratin at the same time as squamous metaplasia and epithelial keratinization become visible. Current studies attempt to define whether changes in epithelial differentiation caused by deficiency of vitamin A may be the result of a direct action of the vitamin on gene expression or the effect of alterations in cell populations due to altered glycosylation reactions and incorrect positioning or function of adhesive molecules such as fibronectin at the cell surface.



## PROJECT DESCRIPTION

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

Luigi M. De Luca	Research Chemist	LCCTP	NCI
Kim Creek	Staff Fellow	LCCTP	NCI
Freesia Huang	Expert	LCCTP	NCI
Donata Rimoldi	Visiting Fellow	LCCTP	NCI
Ulrike Lichti	Expert	LCCTP	NCI
Fabrizio Bonelli	Visiting Fellow	LCCTP	NCI

Objectives:

Vitamin A and some of its synthetic analogs (retinoids) have been shown to act as chemopreventative agents during the promotion phase in experimentally induced epithelial cancer in several animal model systems. Therefore an investigation of their biochemical mode of action may reveal key points in our understanding of the biochemical steps involved in the control of tumor promotion.

1. Vitamin A as retinyl phosphate has been shown to act as acceptor of the sugar mannose from guanosinediphosphomannose. Our immediate objective was to understand the role of this mannanlipid in protein glycosylation. This objective may be of potential relevance to carcinogenesis since we have recently found that membranes from hepatocellular carcinomas are deficient in vitamin A and possess a decreased pool of retinyl phosphate mannose at the same time as the pool of dolichylphosphate mannose remains unaltered compared to normal surrounding or regenerating liver tissue.

2. It is now established that retinoids in pharmacological doses enhance adhesive properties of mesenchymal cells and it is reasonable to expect that the condition of vitamin A deficiency may cause poor adhesion and result in the shedding of mucociliary cells. Thus defects in vitamin A-dependent glycosylation may alter adhesion of mesenchymal cells and their interaction with epithelial cells. We probed the hypothesis that retinoic acid may alter the glycosylation of the collagen binding domain of fibronectin.

3. Finally we were interested in defining the nature of the macromolecules synthesized during keratinization of hamster tracheas cultured in a vitamin A-deficient medium.

Methods Employed:

The function of retinyl phosphate in glycosylation. This project utilizes a variety of chemical and biochemical techniques to study the involvement of retinyl phosphate in mannosylation reactions. A main technical breakthrough has been the employment of bovine serum albumin as a carrier of exogenously added retinyl phosphate to the microsomal membranes of rat liver in "in vitro" studies of glycosylation utilizing guanosine diphosphate mannose as the glycosyl donor. The biosynthesis of retinyl phosphate mannose and the polyisoprenoid

derivative, dolichyl phosphate mannose, is then followed by thin layer chromatography and/or high pressure liquid chromatography on anion exchangers. In particular, a new method employing the anion exchanger, mono Q, from Pharmacia has given highly reproducible results for the separation and determination of retinyl phosphate mannose and dolichyl phosphate mannose. This method has been used for in vivo and in vitro studies.

Structural Studies. Retinyl phosphate mannose structure was studied by fast atom bombardment mass spectrometry (FAB MS). FAB MS was conducted with a ZAB instrument (VG Micromass) using xenon (Xe) and an ion gun at 8 kV and 1 mA discharge current. To apply the sample, 1  $\mu$ l (5-8  $\mu$ g) of Ret-P and Ret-P-Man and appropriate blanks in MeOH were placed on the probe; the MeOH evaporated and 1  $\mu$ l of glycerol:dimethyl formamide (1:1:v/v) was added as matrix. The ion yield from Xe at 9 kV was 5 times greater with negative than positive ion MS.

Techniques for the Study of Keratin Gene Expression in Tracheal Organ Culture. These comprised, in addition to established techniques of organ culture in the presence and absence of vitamin A, the use of various mono- and polyclonal antibodies against purified specific keratins from mouse skin or from human skin. These antibodies were used in immunofluorescence and immunoblotting studies.

#### Major Findings:

##### 1. Retinyl Phosphate Mannose Structure.

Retinol and its derivatives are physiological intermediates for maintenance of differentiation of epithelial tissues and growth, as well as the reproductive and visual functions. They also modify growth and adhesive properties of transformed cultured cells in the direction of the normal phenotype. At the biochemical level the vitamin A status regulates the incorporation of the monosaccharide mannose into glycoproteins. This incorporation is greatly reduced in vitamin A deficiency and enhanced by excess vitamin A. Retinoic acid is as active as retinol in restoring normal levels of mannosylation. Recent studies have demonstrated that rat hepatocytes from deficient animals fail to incorporate mannose into the specific glycoproteins,  $\alpha$ 2U-globulin and  $\alpha$ 1-macroglobulin even though synthesis of the protein remains unaltered. These findings suggest a specific glycosylation defect in A deficiency.

Similar studies have been conducted in a variety of tissues in the past decade to support the concept that in normal physiology the vitamin is somehow involved in the glycosylation of specific mannose-containing glycoproteins.

A study of transplanted and primary rat hepatoma tissue showed that these tumor cells are in a status of vitamin A deficiency. Therefore some of the reported alterations in glycoprotein patterns of hepatoma cells might well be a consequence of the vitamin A-deficient status of the tumor cell.

In 1970, we first reported that rat liver microsomal membranes can synthesize a lipid compound containing retinol, phosphate, and the sugar mannose. This was the first hint into a possible mannose carrier function for vitamin A in microsomal membranes. As our techniques improved, we found that we were really

dealing with two mannlipids: the major compound was a derivative of the polyisoprenoid dolichol, and the second product was the derivative of vitamin A. The development of the bovine serum albumin based technique for the synthesis of retinyl phosphate mannose (Ret-P-Man) permitted an investigation of the endogenous amounts of these lipid intermediates. The possibility that a retinoid derivative of retinoic acid might be involved as a mannosyl carrier was also considered since RA is as active as retinol in restoring normal mannosylation in hamster liver. It was therefore necessary to develop unequivocal techniques for the determination of the structure of the putative Ret-P-Man made in vitro from retinyl phosphate (Ret-P) and guanosine diphosphate mannose in order to eventually elucidate the structure of the retinoid phosphate mannose made in vivo. FAB and collisional activation (CAD) MS were used to analyze Ret-P and Ret-P-Man. Ret-P was made by chemical synthesis. Ret-P-Man was synthesized in vitro and isolated by HPLC on an anion exchanger using a 0 to 50 mM gradient of ammonium acetate in 99% methanol. Positive ion FAB MS of Ret-P showed a short-lived but observable spectrum with a highest mass ion at m/z 366 [M+H-H2PO4]+. Negative ions FAB MS of Ret-P showed an intense spectrum with a parent ion at m/z 365 corresponding to [M-H]-. FAB MS of Ret-P-Man showed a parent ion at m/z 527 [M-H]- and a fragment at m/z 259 [M-H-mannose-1-phosphate]-. Metastable ion- and CAD-spectra of the [M-H]- ion of Ret-P-Man showed daughter ions at m/z 509 [M-H-H2O]-, 458, 436 [M-H-C3H7O3]-, 407 [M-H-C4H8O4]-, 365 [M-H-C6H11O5]- and 241 [C6H11PO8-H]-. These data confirm the structure of the in vitro-made product as retinyl phosphate mannose. We are now aiming at isolating and purifying sufficient amounts of the in vivo synthesized mannlipid having similar chromatographic properties to the in vitro-made product to determine its structure.

## 2. Reversibility of Retinyl Phosphate Mannose Synthesis.

In previous work we have shown that hamster liver microsomes catalyze the in vitro synthesis of Ret-P-Man from GDP-mannose and Ret-P and that maximal Ret-P-Man synthesis occurs at 20-30 min, followed by a subsequent loss of mannose from Ret-P-Man, suggestive of an intermediary function of Ret-P-Man and/or Ret-P-Man hydrolysis (Shidoji et al. *Biochem. J.* 208: 865-868, 1982; Creek et al. *Biochem. J.* 210: 541-547, 1983). To carefully monitor Ret-P-Man synthesis in the microsomal incubation system, we have developed a chromatographic procedure in which mannose, Ret-P-Man, mannose 1-phosphate and GDP-mannose are separated in a single analysis on a Pharmacia Mono Q column eluted with a linear gradient of 0 to 300 mM NaCl in 70% methanol over 30 min. Using this chromatographic system, we have determined that greater than 90% of the Ret-P-Man made in vitro at 30 min from GDP-[14C]mannose and Ret-P is recovered in the pellet with the microsomes upon centrifugation. Subsequent incubation of Ret-P-Man loaded microsomes for 1 hr at 37°C results in an 80% loss of [14C]-mannose from Ret-P-Man to form primarily [14C]mannose 1-phosphate. However, the incubation of Ret-P-Man loaded microsomes for 1 hr at 37° in the presence of excess GDP results in a 90% loss of [14C]mannose from Ret-P-Man to form primarily GDP-[14C]mannose. We conclude that the mannosyl transferase reaction catalyzing the synthesis of Ret-P-Man is clearly reversible. This finding is consistent with the possibility that a function of retinyl phosphate mannose may be to generate guanosine diphosphate mannose within the endoplasmic reticulum by functioning as a carrier across the endoplasmic reticulum membrane.

3. Deficiency of Vitamin A Causes a Depletion in the Pool of Guanosine Diphospho mannose and the Accumulation of Shorter Oligosaccharides on Lipid Intermediates.

If a function of Ret-P-Man is to generate GDP-mannose in the membrane, vitamin A deficiency should cause a decrease in GDP-Man, Dol-P-Man, and membrane glycoproteins. We studied the effect of vitamin A deficiency on the incorporation of [2-<sup>3</sup>H]mannose into hamster liver glycoconjugates 20 min after intraperitoneal injection of the label. Extracts of liver microsomes were analyzed by high performance liquid chromatography (HPLC) with different solvent systems in order to quantitate respectively the amount of radioactively labeled GDP-mannose and dolichyl phosphate mannose (Dol-P-Man). We found a decrease of more than 90% in the pool of GDP-mannose and an equivalent decrease in Dol-P-Man in microsomes from vitamin A deficient animals compared to normal. The products of mild acid hydrolysis of the lipid oligosaccharide fraction and the oligosaccharide chains released by  $\beta$ -endo N-acetyl glucosaminidase-H treatment of membrane-associated glycopeptides were also analyzed by HPLC and showed an accumulation of smaller molecular weight radioactive oligosaccharide products in Vitamin A deficiency. Therefore it appears that the condition of vitamin A deficiency affects the entire dolichol mediated pathway of mannosylation, possibly due to the depletion of GDP-mannose.

4. Retinoic Acid Causes a Modification in the Carbohydrate Moiety of the Collagen-Binding Domain of Chondrocyte Fibronectin.

The mechanism of the retinoic acid-induced increase in adhesion of chicken sternal chondrocytes was investigated. Fibronectins from control and from retinoic acid-treated, cultured sternal chondrocytes were obtained by immunoprecipitation after radioactive labeling with [2-<sup>3</sup>H]mannose. The fibronectin derived from RA-treated cultures displayed a slightly higher molecular weight by SDS-PAGE.

The collagen-binding domain was prepared by collagen-Sepharose chromatography after thermolysin or chymotrypsin cleavage. Polyacrylamide gel electrophoresis of the domain showed an increase in MW caused by RA treatment over control. This increase in molecular weight was abolished when the cells were treated with tunicamycin, an inhibitor of glycosylation.  $\beta$ -Endo N-acetyl glucosaminidase H treatment of the collagen-binding domain showed a relative prevalence of susceptible over resistant oligosaccharide chains in fibronectin from the control cells, thus permitting the conclusion that RA treatment caused a relative increase in the complex type oligosaccharide chains compared to control cultures.

5. Vitamin A Deficiency and the Expression of Keratins in Cultured Hamster Tracheas.

Squamoid metaplasia and keratinization of the surface of the respiratory epithelium are common responses to exposure to chemical carcinogens and the condition of vitamin A deficiency. Although this is a well-known morphological response, macromolecules arising under conditions of vitamin A deficiency had never been identified. The well-characterized system of hamster tracheal organ

culture was used for studies to attempt to characterize molecular species of keratin in this system. Cross-reactive keratins were detected in vitamin A-deficient tracheas, using monoclonal antibodies directed against human epidermal callus acidic keratins (AE<sub>1</sub>) and basic keratins (AE<sub>3</sub>). The antibodies were obtained from Dr. T. T. Sun of the Department of Dermatology and Pharmacology, New York University School of Medicine, New York, N.Y. Strong cross-reactivity was detected by indirect immunofluorescence with the AE<sub>3</sub> antiserum. Sections from vitamin A-treated cultures showed little reactivity. Additional data were obtained by immunoblotting of the electrophoretically separated keratin species. AE<sub>1</sub> brought out molecular species at 50, 48, 46.5, and 45 kD and AE<sub>3</sub> at 58, 56, 52, and 46 kD. Immunoblotted keratin bands could not be detected in control tracheas, except for a 45 kD band which was immunoblotted with AE<sub>1</sub>.

Using polyclonal antibodies (K2) against mouse epidermal keratins, [<sup>3</sup>H]methionine-labeled keratins were immunoprecipitated and then separated by electrophoresis. Radioactive bands were detected at 62, 58, 50, 48, 46.5, and 45 kD in vitamin A-deficient tracheas, with little material detected in normal tracheas.

These data confirm that the condition of depletion of vitamin A causes replacement of the normal mucociliary epithelium of the respiratory tract with a squamoid metaplastic cell type which is active in synthesizing keratins of various MW.

#### Significance to Biomedical Research and the Program of the Institute:

It is the aim of this project to investigate the mechanism(s) by which vitamin A functions in the body. Since vitamin A and its derivatives, the retinoids, are active as preventive agents of certain epithelial cancers, such investigation may yield useful information on mechanisms whereby normal tissue-specific phenotypic expression is maintained by retinoids.

A substantial body of work has shown a biochemical involvement of vitamin A at the level of the biosynthesis of glycoproteins. The phosphorylated vitamin appears to function in mammalian membranes as a carrier of mannosyl residues across the hydrophobic environment of the membrane to partake in glycoprotein biosynthesis. Some glycoproteins mediate cell to cell recognition and adhesion (e.g., histocompatibility antigens, fibronectins); others have hormonal functions in tissue growth and development (e.g., the gonadotropins); others display epithelioprotective functions (e.g., secretory mucins); thus, it is reasonable to propose that the involvement of the phosphorylated vitamin in glycoprotein biosynthesis may be related to its effect on mucus secretion, adhesion, and the maintenance of normal phenotypic expression.

The reported findings demonstrate that retinyl phosphate mannose synthesis is a reversible process and therefore open up the possibility that the activated sugar nucleotide, guanosine diphosphate mannose, may be generated by reversal of retinyl phosphate mannose synthesis in the endoplasmic reticulum. This would offer a mechanism to allow the hydrophilic glycosyl moiety to cross the membrane bilayer and become available on the cisternal side of the membrane for glycoprotein synthesis. Although this mechanistic proposal remains to be verified in detail, data on vitamin A deficient hamster liver tissue have shown a decrease

(90%) in the amount of [2-3H]mannose incorporated into guanosine diphosphate mannose, dolichyl phosphate mannose, and oligosaccharide fractions of oligosaccharide lipids and glycopeptides. Moreover, a prevalence of relatively shorter size oligosaccharides was noted in the membrane fraction from A-deficient hamsters compared to controls.

If this biochemical mechanism of action of vitamin A is proven correct, it may be that cell to cell communication and other adhesive and receptor-mediated mechanisms may be interrupted by the condition of nutritional deficiency of the vitamin.

Conversely, conditions of excess vitamin A may alter intercellular adhesive molecules. We have shown that in a system of chicken sternal chondrocytes in culture excess retinoic acid alters the proportion of high mannose to complex type oligosaccharide chains. Whether these changes in carbohydrate structure are responsible for the observed morphological change to a more fibroblastic phenotype remains to be determined.

The antagonistic actions of retinoids and certain tumor promoters at the biological and biochemical levels are consistent with the concept that tumor promoting substances may interfere with essential functions at the target site causing a deficiency of such functions. Therefore we have put forward the concept that under conditions of depletion of the essential element (e.g., vitamin A) or of its functions as caused by the tumor promoter (e.g., TPA), the mutation present in the initiated cell (and maintained latent by the vitamin or the expression of the vitamin's function within that cell or surrounding cells) is now expressed as a result of essential function deficiency and permits the establishment of a cell population that can survive in a more self-sufficient state, i.e., in a state in which the particular substance is no longer essential for survival. This concept, if proven correct, may suggest novel approaches to "chemoprevention" and it may prove useful in the chemotherapeutic management of the tumor.

#### Proposed Course:

1. Attempts at Determining the Structure of the Endogenous Mannolipid with Chromatographic Properties of Retinyl Phosphate Mannose.

The function of vitamin A in growth and differentiation was clearly distinguished from the visual and reproductive functions when it was found that retinoic acid can support growth, but not vision and reproduction. Therefore to those of us interested in the growth function of the vitamin, two possibilities were open for consideration: either that retinoic acid might be the truly active metabolite of retinol or that one of its metabolites might mimic retinol in its growth function. In either case, molecular manifestations of vitamin A deficiency would have to be reversed if either compound was administered to the vitamin A-deficient animals.

Both retinol and retinoic acid are active in restoring the normal appearance of the mucociliary epithelium, growth in the whole animal, and in the induction of adhesive phenomena in transformed mouse fibroblasts.

Therefore we will attempt to answer the question as to whether a metabolite of retinoic acid may act on the same mannosylation pathway as retinyl phosphate. The technique of FAB-MS has permitted, in this past year, the confirmation of the putative structure of retinyl phosphate mannose synthesized from exogenous retinyl phosphate. Since we have reported that retinoic acid is able to reverse the effects of vitamin A deficiency on the incorporation of 2[<sup>3</sup>H]mannose into glycolipids and glycoproteins, the possibility exists that the endogenous mannolipid with chromatographic properties of authentic retinyl phosphate mannose may contain a common metabolite of retinol and retinoic acid. Therefore, an attempt will be made to isolate sufficient amounts of the endogenous mannolipid to attempt FAB MS analysis. If such a common metabolite is found, a long-sought answer as to why retinoic acid replaces retinol in some of its biological functions may well be obtained. The metabolic fate of retinoic acid in culture of mouse epidermal cells will be pursued. S. H. Yuspa and U. Lichti have recently reported an effect of RA on the enzyme, transglutaminase, which may be a key enzyme in the formation of cornified envelopes and perhaps other structures of keratinocytes. The retinoid may alter the activity, synthesis, or subcellular distribution of epidermal transglutaminase. To maintain high enzyme activity, frequent additions of RA are necessary, suggesting high rates of metabolism of the retinoid. The metabolism of radioactively labeled retinoic acid will be pursued in cultures of primary mouse epidermal cells. Our reverse phase high pressure chromatography system will be used in addition to thin layer chromatographic techniques to study the rate of disappearance of RA and its conversion to other products. Overall these studies should permit the isolation of biologically important metabolites of retinoic acid.

2. To test the hypothesis that retinyl phosphate functions at the membrane level as a mannosyl carrier to generate guanosine diphosphate mannose.

This hypothesis was suggested by the findings that retinyl phosphate mannose synthesis is reversible to generate guanosine diphosphate mannose on endoplasmic reticulum membranes and that the condition of vitamin A deficiency causes a 90% decrease in the amount of GDP-mannose. Therefore retinylphosphate or a similar retinoid derivative may be involved as the first carrier of mannosyl residues across the bilayer of the endoplasmic reticulum. We will test this hypothesis utilizing microsomal membranes prelabeled with [2-<sup>3</sup>H]mannose in vivo to preserve the natural orientation of the mannolipid. The microsomal vesicles will be prepared in the presence or absence of exogenous guanosine diphosphate and the ability of the system to generate luminal GDP-[2-<sup>3</sup>H]mannose and dolichyl phosphate-[2-<sup>3</sup>H]mannose will be tested. These studies will be complemented with in vitro studies in which exogenous retinyl phosphate and/or preformed retinyl phosphate [2-<sup>3</sup>H]mannose will be used to study their ability to generate luminal GDP-[2-<sup>3</sup>H]mannose. Membrane intactness will be monitored by using specific markers such as mannose-6-phosphatase activity, usually a latent enzyme in intact membranes. The condition of severe vitamin A deficiency will offer an additional control for this experiment in that retinol depleted membranes should be incapable of generating cisternal GDP-[<sup>3</sup>H]mannose.

### 3. Vitamin A Deficiency Status of Hepatoma Tissue and Investigation of the Function of Cellular Retinol Binding Protein.

An investigation of the vitamin A status of hepatoma tissue has indicated a virtual vitamin A deficiency status of the tumor. This vitamin A deficiency was accompanied by a marked decrease in the concentration of cellular retinol binding proteins (CRBP) of approximately 15,000 m.w. as determined by polyacrylamide gel electrophoresis. Therefore it appears reasonable to propose that the condition of vitamin A depletion of the tumor might be the result of defective transport of the vitamin and that CRBP might be involved in retinol transfer at the membrane level.

### 4. Gene Expression and its Control by Retinoids.

A main action of retinol, retinoic acid, and derivatives is at the level of the maintenance of normal function in epithelial tissues. Under conditions of vitamin A deficiency mucociliary epithelia, such as in the respiratory tract, undergo profound focal changes which include squamoid type of differentiation with production of keratinizing epithelium.

We will continue our work on the characterization of the keratin molecules in the respiratory tract from hamster tracheas maintained in culture for various periods of time in a vitamin A-depleted medium. A variety of techniques will be employed in collaboration with the In Vitro Pathogenesis Section of the LCCTP, where cloning of the genes for keratin peptide has been accomplished. The technique of in situ hybridization will also be used to permit investigations with small amounts of tissue.

In addition to tissues derived from respiratory tract, keratin profiles will be probed in mouse epidermal cells cultured in vitamin A-depleted medium, which should permit the production of the 67,000 Mr keratin, which is a normal differentiation product in vivo, but is not usually made in cultured keratinocytes.

### 5. Evaluation of Vitamin A Deficiency as a Promoter of 7, 12 Dimethylbenzanthracene (DMBA)-Initiated Skin Papillomas in Female Mice.

The hypothesis that vitamin A deficiency may function as a promoting stimulus in skin tumorigenesis will be tested. Previously employed procedures involving two-stage skin carcinogenesis in SENCAR mice will be used. There will be four experimental groups: two on Purina and two on Vitamin A-deficient diet. All will be initiated with DMBA. One group of 30 mice will serve as a negative promotion control; one as a positive promotion control, a third group as test group for vitamin A deficiency to function by itself as a tumor promoting agent, and a fourth group to study the combined effect of using a suboptimal dose of TPA and vitamin A deficiency on tumor promotion.

### Publications

Bernard, B. A., De Luca, L. M., Hassell, J. R., Yamada, K. M. and Olden, K.: Retinoic acid alters the proportion of high mannose to complex type oligosaccharides on fibronectin secreted by culture chondrocytes. J. Biol. Chem. 259: 5310-5315, 1984.



De Luca, L. M.: Deficiency of vitamin A in hepatocellular carcinoma tissue: Considerations on its establishment. In Roe, D. A. (Ed.): Diet, Nutrition and Cancer: From Basic Research To Policy Implications. New York, Alan Liss, Inc., pp. 111-115, 1983.

De Luca, L. M., Brugh, M. and Silverman-Jones, C. S.: Retinylpalmitate, retinyl phosphate and dolichyl phosphate of postnuclear membrane fraction from hepatoma, host liver, and regenerating liver: marginal vitamin A status of hepatoma tissue. Cancer Res. 44: 224-232, 1984.

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De Luca, L. M., Silverman-Jones, C. S., Creek, K. and Rimoldi, D.: Retinyl phosphate mannose and dolichyl phosphate mannose: Biosynthesis and separation. Methods in Enzymology. (In Press)

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Shidoji, Y., Silverman-Jones, C. S., Noji, S. and De Luca, L. M.: Interactions between retinyl phosphate and divalent cations. Biochem. J. 216: 727-735, 1983.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05177-04 CCTP

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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)

PI:	M. C. Poirier	Research Chemist	LCCTP	NCI
Others:	S. H. Yuspa	Chief	LCCTP	NCI
	E. Reed	Clinical Associate	LCCTP	NCI
	H. Huitfeldt	Visiting Fellow	LCCTP	NCI

## COOPERATING UNITS (if any)

LMPH, LMCP and MB, NCI (L. Zwelling, C. Litterst, R. Ozols); MIT (S. Lippard); Univ. of Texas Medical School (J. M. Hunt); Univ. of North Carolina (D. Kaufman and R. Paules); NCTR (F. A. Beland and J. Young)

## LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

## SECTION

In Vitro Pathogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER

## CHECK APPROPRIATE BOX(ES)

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

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

Antibodies specific for carcinogen-DNA adducts have probed the nature, extent and consequences of in vitro and in vivo DNA modification. Biological samples of DNA substituted with 2-acetylaminofluorene (AAF), benzo(a)pyrene (BP) or cis-dichlorodiammineplatinum (II) (cis-DDP) were subjected to immunological localization and quantitative immunoassays able to detect one adduct in one hundred million nucleotides. In hepatic DNA of rats fed a carcinogenic level of AAF for 4 weeks, adduct accumulation reached a plateau at 2-3 weeks. During 4 subsequent weeks on control diet, adduct removal was biphasic with a rapid initial phase followed by a slow second phase. A pharmacokinetic model consistent with this data proposes that adducts are formed in two DNA compartments, one from which adducts are removed rapidly and another from which adducts are removed slowly. Persistent adducts accumulate in the slow-repairing compartment, but constitute less than 7% of the total adducts formed. In contrast to the high levels of AAF adducts in liver DNA, binding of BP to deoxyguanosine in DNA of mouse epidermis and cultured epidermal cells was more than 50-fold lower. Binding levels were similar in epidermis and epidermal cells, subsequent to dosages known to induce papillomas in vivo and differentiation-altered foci in the cultured keratinocytes. The kinetics of repair for BPdG in vivo and in vitro were biphasic (as in liver) but much more rapid, with 50% removal by 1-2 days. Thus adduct accumulation and removal seem to be characteristic of interaction between a particular target tissue and an individual carcinogen, and may not be quantitatively related to efficiency of tumorigenesis or transformation. Antisera specific for cis-DDP-DNA (bidentate N-7 dideoxyguanosine intrastrand adduct) have been used to measure adducts in DNA of nucleated blood cells from cancer patients on platinum drug chemotherapy. Adducts appear to accumulate both as a function of total cumulative dose and increasing cycle number in individuals who have not received previous platinum drug therapy. The relationship between ability to form adducts, and disease response, appears promising.

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

M. C. Poirier	Research Chemist	LCCTP	NCI
S. H. Yuspa	Chief	LCCTP	NCI
E. Reed	Clinical Associate	LCCTP	NCI
H. Huitfeldt	Visiting Fellow	LCCTP	NCI

Objectives:

To develop specific and sensitive quantitative and morphological immunoassays, and utilize them to probe the interaction of carcinogens with DNA. Studies are directed toward quantification of the extent of in vivo covalent DNA adduct formation and removal in experimental models and in exposed humans. Of particular importance is investigation of the biological consequences of DNA adduct formation in these model systems and in the human population.

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 patients given cancer chemotherapeutic agents are also utilized. The chemical synthesis of radiolabeled and unlabeled DNA-carcinogen adducts and their purification by column chromatography are currently performed. Isolation of macromolecules for carcinogen binding and repair studies utilize density gradient centrifugation. Antibodies are produced by injection of purified antigens into rabbits. A variety of immunological techniques are employed including the qualitative procedures of immunofluorescence and immunochemical electron microscopy and the quantitative radioimmunoassay (RIA) and enzyme-linked radioimmunoassay (ELISA). High performance liquid chromatography (HPLC) is being established for characterization of specific adducts.

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 section. Rabbit antibodies have been elicited against protein-conjugated carcinogen-nucleoside adducts or methylated-BSA-complexed modified DNAs. The high-affinity antisera obtained have been used to develop radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) able to detect as little as one adduct in  $10^8$  nucleotides. Morphological procedures such as immunofluorescence and immunoelectron microscopy have the potential to demonstrate carcinogen-DNA adducts localized within subpopulations of cells in a complex tissue or within unique sites of the DNA molecule. The three 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) comparison of DNA adduct formation and removal profiles in mouse epidermis subsequent to topical application of initiating BP doses, and in cultured mouse keratinocytes which form differentiation-altered foci in response to benzo[a]pyrene (BP) or BPanti-diol epoxide (BPDE I) exposure; (3) investigation of cis-diamminedichloroplatinum (II) (cis-DDP)-DNA adducts in the nucleated blood cells of testicular and ovarian cancer patients receiving cis-DDP chemotherapy and in tumor bearing animal models.

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 with [<sup>3</sup>H]G-8-AAF and [<sup>3</sup>H]G-8-AF tracers to assay for liver DNA adducts during chronic AAF feeding. By feeding nonradioactive and radioactive AAF for varying periods of time, it has been possible to compare adduct accumulation both at the beginning and the end of a 4-week period of carcinogen administration. In addition, adduct removal and persistence could be monitored during a subsequent 4 weeks of feeding control diet. These studies have previously been performed in collaboration with Drs. B. Laishes and J. Hunt, McArdle Laboratories, and have recently culminated in a pharmacokinetic analysis generated by analog-digital hybrid computer in collaboration with Drs. J. Young and F. Beland of the National Center for Toxicological Research. The experimental data were consistent with a model in which DNA adducts accumulated into two liver compartments, one amenable to rapid removal ( $K = 0.213 \text{ days}^{-1}$ ) and a second from which adduct removal was much slower ( $K = 0.026 \text{ day}^{-1}$ ). At early and late times during a month of AAF feeding, the rate of adduct formation remained essentially unchanged yielding a total accumulation of about 1300 fmol adducts/ $\mu\text{g}$  DNA. Adduct removal from the fast-repairing region was complete after a subsequent month on control diet, while the persistent adduct, which was about 100 fmol/ $\mu\text{g}$  DNA under these conditions, was apparently located in the slow-repairing compartment. The model generated by these parameters is able to explain the steady-state adduct plateau of 250-400 fmol/ $\mu\text{g}$  DNA observed at 2-3 weeks of chronic feeding in several experiments in this and other laboratories. Overall, adduct formation and removal remain stable and quite efficient in the liver during 4 weeks of chronic AAF ingestion since  $\geq 93\%$  of the adducts formed were removed by a subsequent 4 weeks on control diet.

Since the kinetic data suggested that accumulation of AAF adducts in liver DNA might not be uniform, experiments were performed with immunofluorescence and RIA to survey adducts in various liver lobes. Using both techniques, the highest levels of adducts were observed in the left lateral lobe and the lowest levels were seen in the triangular lobe after 3 days of AAF feeding, indicating that adduct accumulation was not uniform. In addition, immunofluorescence revealed a lack of adduct accumulation in the periportal areas of liver lobules. After 28 days of AAF feeding, overall adduct levels were about two-fold higher, but the relative proportions in each lobe remained similar. In addition, relatively large adduct-free areas were visible in livers from animals fed AAF for 28 days. These studies are being extended, in collaboration with Dr. J. Hunt at the University of Texas, to include comparison of  $\gamma$ -glutamyl-transpeptidase positive regions with adduct-positive and -negative areas.

Antisera developed against DNA substituted with the 7,8-diol, 9,10 epoxide of benzo[a]pyrene (BPDE-I, the anti-isomer), such that the only adduct was trans-(7R)-benzo[a]pyrene-N<sup>2</sup>-deoxyguanosine (BPdG), have a higher affinity for BP-substituted DNA than for the isolated BPdG adduct (suggesting antibody recognition of the DNA backbone). The ability of this antiserum to recognize BPdG in intact (nonhydrolyzed) DNA makes it particularly useful for morphological studies. In collaboration with Drs. D. Kaufman and R. Paules of the University of North Carolina, electron microscopic visualization of BPdG adducts on DNA fibers has been achieved using the specific antiserum, calf thymus DNA modified with BPDE-I in vitro and ferritin-conjugated goat anti-rabbit IgG. The most recent investigations, using monovalent Fab fragments, have yielded quantitative detection by EM when values obtained by measurement of approximately 1500 DNA fragments per sample were compared with those determined by ELISA. In addition, adducts in DNA from C3H 10T 1/2 cells exposed to BPDE-I in culture were determined by EM and ELISA as virtually identical. Thus, the EM immunotechnology will be feasible for use with DNA samples generated by in vivo exposure.

In studies designed to compare mechanisms of BPdG adduct formation and removal in mouse epidermis and cultured mouse epidermal cells, ELISA has been used to quantitate DNA adducts after topical application of initiating BP doses in vivo, and foci-inducing BP or BPDE-I exposure in vitro (see Project Z01CE04504-12 CCTP). A remarkable feature both in vivo and in vitro is that similar levels of DNA binding were associated with initiation in mouse skin (2-6 fmol/ $\mu$ g DNA) and induction of differentiation-altered foci in cultured mouse keratinocytes (0.4-6 fmol/ $\mu$ g DNA). In addition, the kinetics of BPdG removal were similar ( $T_{1/2} = 24-48$  hours) both in epidermis and in the cultured keratinocytes. Experiments in which DNA turnover was monitored by prelabeling suggested that in the epidermal cultures, adduct removal by differentiating cells was more rapid than that exhibited by basal cells. If a similar relationship exists in vivo, these experiments suggest that cells which are the least likely to persist in the epidermis have the most efficient repair capacity. In any case, both in vivo and in vitro the quantity of BPdG remaining on the DNA after the initial removal and during the period of oncogenic expression is only about 400 adducts per cell. Overall, striking similarities have been observed between the processing of genomic damage during BP-induced initiation in vivo and the production of differentiation-altered foci in vitro.

Rabbit antisera have been elicited against DNA modified with the chemotherapeutic drug, cis-DDP, in collaboration with Dr. S. Lippard of MIT. Studies in collaboration with Dr. L. Zwelling of NCI (LMPH, Z01CM06150-04) constituted the first evidence that some cis-DDP DNA adducts formed in vivo are recognized by the rabbit antibody elicited against DNA modified in vitro. Competitive ELISAs performed with DNAs and synthetic polymers modified by a variety of platinum drugs indicate that primary antigenic specificity is directed toward a bidentate adduct on adjacent deoxyguanosines (dG) formed with the dG-N<sup>7</sup> position by displacement of the cis chloride groups. We have found that this adduct is formed in human patients since DNA extracted from circulating nucleated cells in individuals receiving cis-DDP chemotherapy (patients of Dr. R. Ozols, Medicine Branch, NCI) competed in the ELISA, whereas DNA from normal volunteers and individuals on other chemotherapy did not. An analysis of 130 blood samples from 63 individuals has shown that the assay is very

specific for cis-DDP exposure since no false positives were observed among the controls. In addition, in most individuals who had received no previous platinum drug chemotherapy, an accumulation of cis-DDP DNA adducts was observed with increasing cumulative cis-DDP dose. A course of platinum drug chemotherapy generally consists of 3 to 4 cycles in which drug is given for 5 days, followed by a 16- to 23-day drug-free period. In these studies the accumulation of adducts with cycle suggested that enzymatic removal of the cis-DDP DNA adducts may be very slow if it occurs at all. Similar conclusions have resulted from studies performed with animal models in collaboration with Dr. C. Litterst (LMCP, Z01CM07119-06). Adducts were measured in kidneys and tumors of rats bearing a subcutaneous Walker 256 sarcoma, and adduct levels, though similar in both organs, were found to vary with the nutritional status of the individual animal. A continuation of both the human and animal studies will explore the relationship between adduct formation and tumor response.

#### Significance to Biomedical Research and the Program of the Institute:

The development of immunological procedures for the investigation of carcinogen-DNA interactions has provided a powerful tool for the study of this phenomenon. Experimentally, antibodies are more specific and sensitive, and less costly than the conventional radiolabeled probes used for such studies. Standard carcinogenesis protocols need not be modified for quantitation of DNA-binding, and prolonged sequential administration can be monitored. Morphological approaches can be employed to determine inter- and intracellular distribution of adducts, and even intramolecular localization is possible. Our most recent results suggest that antibodies may be useful to probe for adducts in humans exposed to DNA damaging agents both in prospective epidemiological studies and in assisting the clinician to judge dosage on individuals exposed to DNA-interacting chemotherapeutic agents.

#### Proposed Course:

Intensive investigations of specific experimental models of tumor induction such as AAF-induced tumors in rat liver and BP-initiation in mouse epidermis have provided an opportunity to evaluate tissue-specific and carcinogen-specific aspects of carcinogen-DNA interactions. We expect that intensive investigation of the manner in which a particular tissue processes a specific short-term or long-term genomic insult may lead us to new insights regarding mechanisms of carcinogenesis. In an attempt to discover why tumorigenesis is associated with many DNA adducts in liver and very few DNA adducts in skin, studies have been initiated using the keratinocyte differentiation-altered focus assay. A preliminary experiment has shown that a small number of adducts are associated with a large number of BPDE-I-induced foci, but many more adducts are formed with exposure to nontoxic doses of N-acetoxy-2-AAF, a compound which induces only a few foci. Thus, the *in vitro* system provides a unique opportunity to explore such relationships, and yield insights which can be interpreted relative to data obtained *in vivo*. We will continue to investigate localization of AAF adducts in the liver by immunofluorescence, but adduct levels in skin have been too low to localize by immunohistochemistry. In the liver it should be possible to compare adduct accumulation in preneoplastic foci with that in surrounding

liver. In attempts to elucidate the nature of the fast- and slow-repairing liver DNA components, we will continue to employ both quantitative and morphological procedures.

Collaborative studies utilizing electron microscopic immunohistochemistry (ferritin labeling) to detect specific localization of BP adducts on DNA will focus on 10 T 1/2 cells exposed *in vivo* and on a search for binding to initiation sites in replicons in SV40 and regenerating rat liver nuclei. We would like to utilize this approach to determine if gaps in nascent DNA are localized opposite adducts after exposure of cells to BPDE-I. A more theoretical extension of the adduct localization studies would be an attempt to correlate the location of adduct formation with structural or functional features of the genome. Such studies could be performed using the mouse keratinocytes which form foci consistently as a result of exposure to BP or BPDE-I. A combination of techniques involving cloned DNA probes specific for certain portions of the murine genome and molecular DNA fractionation procedures would be employed in these investigations.

The human studies with anti-*cis*-DDP-DNA will be extended to investigate the relationship between formation of the *cis*-DDP bidentate intrastrand adduct recognized by the antibody, and patient response. It is our anticipation that this assay may become a useful predictor of which individuals are most likely to respond to platinum drug therapy, and may allow lower drug doses to be given if adduct quantities can be associated with tumor remission. In addition, the possibility exists that *cis*-DDP may induce secondary malignancies in the young testicular cancer patients currently undergoing cures. If so, we will have accumulated valuable information concerning dose-response in the etiology of human carcinogenesis. Hopefully, the animal model studies will elucidate the nature of platinum drug resistance and, in addition, allow us to become familiar with dietary manipulations that will improve drug efficacy.

#### Publications

Lippard, D. J., Ushay, H. M., Merkel, C. M. and Poirier, M. C.: Use of antibodies to probe the stereochemistry of antitumor platinum drug binding to DNA. Biochemistry 22: 5165-5168, 1983.

Nakayama, J., Yuspa, S. H. and Poirier, M. C.: Comparison of benzo(a)pyrene-DNA adduct formation and removal in mouse epidermis *in vivo* and mouse keratinocytes *in vitro* and the relationship of DNA keratinocytes *in vitro* and the relationship of DNA binding to initiation of skin carcinogenesis. Cancer Res.

Paules, R. S., Poirier, M. C., Mass, M. J., Yuspa, S. H. and Kaufman, D. G.: Quantitation by electron microscopy of the binding of highly-specific antibodies to benzo(a)pyrene-DNA adducts. Carcinogenesis. (In Press)

Perera, F. P., Santella, R. M. and Poirier, M. C.: Biomonitoring of workers exposed to carcinogens: Immunoassays to benzo[a]pyrene-DNA adducts as a prototype. Proceedings of the Conference on Medical Screening and Biological Monitoring for the Effects of Exposure in the Workplace, Cincinnati, Ohio, July 1984. (In Press)

Perera, F. P., Santella, R. M. and Poirier, M. C.: Potential methods to monitor human populations exposed to carcinogens: Carcinogen-DNA binding as an example. In Hoel, D., Merrill R. and Perera, R. (Eds.): Banbury Report 19. Risk Quantitation and Regulatory Policy. New York, Cold Spring Harbor Laboratory (In Press)

Poirier, M. C. Review: The use of carcinogen-DNA adduct antisera for quantitation and localization of genomic damage in animal models and the human population. Environ. Mutagen. (In Press)

Poirier, M. C. The use of antibodies to detect carcinogen-DNA adducts in vivo and in vitro. In Mirand, E. A., Hutchinson, W. B. and Mihich, E. (Eds.): 13th International Cancer Congress, Part B, Biology of Cancer (1). New York, Alan R. Liss, 1983, pp. 289-298.

Poirier, M. C., Hunt, J. M., True, B. and Laishes, B. A.: Kinetics of DNA adduct formation and removal in liver and kidney of rats fed 2-acetylaminofluorene. In Rydstrom, J., Montelius, J. and Bengtsson, M. (Eds.): Extra-hepatic Drug Metabolism and Chemical Carcinogenesis. Amsterdam, Elsevier, 1983, pp. 479-488.

Poirier, M. C., Hunt, J. M., True, B. A., Laishes, B. A., Young, J. F. and Beland, F. A.: DNA adduct formation, removal and persistence in rat liver during one month of feeding 2-acetylaminofluorene. Carcinogenesis (In Press)

Poirier, M. C., Nakayama, J. M. Perera, F. B., Weinstein, I. B. and Yuspa, S. H.: Identification of carcinogen-DNA adducts by immunoassays. In Millman, H. and Sell, S. (Eds.): Application of Biological Markers to Carcinogen Testing. New York, Plenum Press, 1983, pp. 427-440.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05178-04 CCTP

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## TITLE OF PROJECT (90 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  
 D. Roop Expert LCCTP NCI  
 H. Kawamura Visiting Fellow LCCTP NCI  
 R. Toftgard Guest Worker LCCTP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

## SECTION

In Vitro Pathogenesis Section

## INSTITUTE AND LOCATION

NIH, NCI Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

2.4

## PROFESSIONAL:

2.4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

SENCAR mice are markedly more susceptible to skin carcinogenesis by initiation and promotion than are other mouse strains. The observation that SENCAR susceptibility is a property of the skin itself suggests the utility of in vitro studies to elucidate the mechanism. Experiments with adult SENCAR and BALB/c (a resistant mouse strain) epidermis have selected cell foci resistant to Ca-2+-induced terminal differentiation following treatment with initiating doses of the carcinogens, dimethylbenz[a]anthracene (DMBA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Such cells have several properties expected of initiated cells. The number of foci was higher at higher doses for each carcinogen. DMBA was a far better inducer of foci than was MNNG in both strains, in agreement with in vivo papilloma incidence data. There was no consistent difference between mouse strains in incidence of induced foci. However, spontaneous foci were routinely found in control dishes of untreated SENCAR cells but not BALB/c. Cell lines established from differentiation-resistant foci have been characterized, and no consistent difference has been found between strains. All lines have epithelial morphology, have a keratin cytoskeleton, and synthesize the keratins expressed by normal keratinocytes in culture. Several lines, however, synthesize keratins not expressed by normal cells. All lines tested were negative for gamma glutamyl transpeptidase, growth in soft agar, and, generally, for tumorigenicity, though a few tumors have developed which seem not to be derived from the cells tested. There are diploid and tetraploid lines as well as mixtures. Transglutaminase was not induced by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), but was induced by retinoic acid. Using the dot-blot technique, surveys of RNA from SENCAR epidermis, both control and TPA-treated, as well as papillomas and carcinomas, have been made for altered expression of a number of oncogenes and only minimal changes in transcript levels have been found.

## 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
Dennis Roop	Expert	LCCTP	NCI
Hideki Kawamura	Visiting Fellow	LCCTP	NCI
Rune Toftgard	Guest Worker	LCCTP	NCI

Objectives:

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

Methods Employed:

The SENCAR mouse was developed by a selective breeding protocol for enhanced susceptibility to skin tumors produced by initiation-promotion protocols. In order to elucidate the basis for this susceptibility, skin of SENCAR mice or SENCAR epidermal cells in culture are exposed to carcinogens and tumor promoters. Comparisons are made with BALB/c as a representative resistant strain. For culture of adult mouse epidermal cells for in vitro carcinogenesis experiments, the epidermis can easily be separated from the dermis after flotation of the skin, dermis side down, on a solution of 1 g trypsin per 100 ml phosphate-buffered saline (without calcium and magnesium) for 1 hr at 37°. Epidermal cells are plated on dishes coated with fibronectin and collagen and are cultured in low-calcium medium conditioned by dermal fibroblasts. After treatment with initiating doses of carcinogens, selection for cells resistant to calcium-induced terminal differentiation can be made. Receptor binding studies with cells in culture have used radioactively labeled epidermal growth factor. Effects of various agents on epidermal cell growth kinetics have been determined in culture by cell counts and thymidine incorporation. The presence of keratins was determined by fluorescent antibody staining and by polyacrylamide gel electrophoresis of radioactively labeled cell proteins precipitated by anti-keratin antibodies. Gamma glutamyl transpeptidase was determined by histochemical staining. Transglutaminase and ornithine decarboxylase were measured in cell lysates by standard enzyme assays using radioactively labeled substrates.

Major Findings:

The SENCAR mouse was developed by a selective breeding protocol for increased sensitivity to skin carcinogenesis by initiation and promotion. Subsequent studies have shown that the enhanced sensitivity exists for initiators with a variety of different chemical structures, including some not requiring metabolic activation, and does not result from differences in metabolism of polycyclic aromatic hydrocarbon carcinogens. Indeed, the SENCAR mouse is more sensitive to skin carcinogenesis by a single dose of ultraviolet (UV) radiation than the

more resistant CD-1 strain. The sensitivity of the SENCAR mouse is a property of the skin itself, as shown by skin graft studies, rather than a systemically mediated phenomenon. Epidermal cells from SENCAR mice do not differ from other strains in their ability to repair DNA damaged by UV radiation or chemical carcinogens. SENCAR epidermal cells are comparable to BALB/c in the extent to which they bind epidermal growth factor (EGF) and respond to modulators of EGF binding such as the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), retinoic acid, and  $\text{Ca}^{2+}$ -induced terminal differentiation. Langerhans cells, bone marrow-derived cells normally present in the epidermis, are present in similar numbers in SENCAR and BALB/c and are functionally indistinguishable from such cells in skin of mice resistant to carcinogenesis.

It is still unclear whether the sensitivity of SENCAR epidermis to carcinogenesis is due to a greater susceptibility to initiation or promotion or to elements of both. This laboratory has provided both in vivo and in vitro data supporting the existence of a constitutively initiated cell population in SENCAR epidermis. Additional studies were undertaken to determine whether SENCAR epidermal cells are more susceptible to initiation by the carcinogens dimethylbenz[a]anthracene (DMBA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) than cells from the resistant BALB/c strain. In vivo studies with these initiators had indicated that more tumors developed in SENCAR epidermal cells with either initiator when followed by TPA promotion. For our studies, initiation frequency was defined as the number of differentiation-altered foci which formed from epidermal basal cell cultures in which unaltered cells were induced to terminally differentiate by shifting from medium with 0.05 mM  $\text{Ca}^{2+}$  to medium with 0.5 mM  $\text{Ca}^{2+}$ . Primary cultures of adult BALB/c and SENCAR epidermal basal cells were treated with initiating doses of DMBA or MNNG. Primary cultures were also derived from skin of each strain which had been initiated by topical application of either carcinogen in exactly the same manner as in carcinogenesis studies. Adult epidermal cells were isolated and plated in 0.05 mM  $\text{Ca}^{2+}$  medium which had previously been conditioned by dermal fibroblasts. For each carcinogen, the number of foci was higher at higher doses of initiator, whether exposure occurred in vivo or in vitro. On a molar basis, DMBA was a far better inducer of foci than was MNNG (2.5 foci/ $\mu\text{mole}$  and 0.4 foci/ $\mu\text{mole}$ , respectively), in agreement with in vivo papilloma incidence data. There was no consistent or significant difference in incidence of induced foci for either strain. However, spontaneous foci were routinely found in control dishes of untreated SENCAR cells but almost never in BALB/c. This is consistent with data from carcinogenesis studies in these strains showing significant papilloma formation in SENCAR cells, but not BALB/c mice treated with promoter alone. The role of the endogenously initiated cell population in the sensitivity of SENCAR cells is not clear. After treatment of BALB/c and SENCAR epidermal cells with initiating doses of carcinogens, the number of differentiation-resistant colonies was similar for both mouse strains. However, since there is evidence that two types of papillomas exist which vary in potential for conversion to carcinomas, we cannot rule out the possibility that papillomas resulting from endogenously initiated cells have higher potential for such conversion or that these cells are more responsive to promoters and thus form papillomas more readily than some chemically initiated cells. Cell lines were established from differentiation-resistant foci of both SENCAR and BALB/c cells, and characterization has begun. All cell lines have epithelial morphology and

have a keratin cytoskeleton as demonstrated by immunofluorescent staining of cells. Immunoprecipitation with antikeratin antisera and polyacrylamide gel electrophoresis (PAGE) of [ $^{35}\text{S}$ ]methionine-labeled cell extracts revealed that all lines synthesized the 60, 55, and 50 kd keratins expressed by normal keratinocytes in culture, but in addition several were synthesizing a 67 kd keratin and several lower molecular weight keratins not expressed by normal cells. No consistent difference among BALB/c or SENCAR lines was established. Flow cytometric measurement of the DNA content indicates that two lines are diploid, six lines are tetraploid, and three lines are a mixture of diploid and tetraploid populations. All 12 lines tested to date (11 SENCAR, 1 BALB/c) have been negative for gamma glutamyl transpeptidase by histochemical staining. This enzyme is reported to be elevated in epidermal carcinomas and some benign papillomas. Tumorigenicity was tested by subcutaneous implantation of cells on plastic inserts into nude mice. Seven lines were negative at 20 weeks in five of five test animals while one line (SENCAR) was positive in one of five recipients and a second line (SENCAR) positive in two of five recipients. The tumors in these three cases were fibrosarcomas rather than carcinomas, and thus may have resulted from the plastic inserts rather than the implanted cells. Plastic inserts are known to cause tumors occasionally in mice.

None of the SENCAR or the BALB/c cell lines tested so far forms colonies in soft agar. TPA fails to induce the high levels of transglutaminase in these lines that are seen in normal primary epidermal cells. Transglutaminase activity is induced, however, by retinoic acid treatment of the cell lines. Some cell lines also bind higher levels of epidermal growth factor (EGF) than normal and this binding is less suppressed by TPA treatment than normal and further suppressed by retinoic acid. These properties could result in a growth advantage over normal cells in the presence of tumor promoter.

It has been reported that papillomas and squamous cell carcinomas induced in mouse skin by chemical carcinogens contain an activated cellular homolog of the Harvey ras ( $\text{ras}^{\text{H}}$ ) oncogene which can transform NIH/3T3 fibroblasts. We have surveyed RNA from SENCAR control adult back epidermis, epidermis at various times after TPA treatment, papillomas, and carcinomas for altered expression of several oncogenes. Using the dot-blot technique with cDNA probes for  $\text{ras}^{\text{H}}$ ,  $\text{ras}^{\text{K}}$ ,  $\text{myc}$ ,  $\text{myb}$ ,  $\text{fes}$ ,  $\text{fos}$ ,  $\text{abl}$ , and  $\text{raf}$ , we have found only minimal changes (<3-fold) in transcript levels of these genes for the samples tested. These results suggest that neither TPA exposure and the resultant hyperplasia nor benign or malignant tumor formation is associated with marked alterations in expression of these oncogenes. From our present data we cannot rule out expression of one or more of these oncogenes at normal levels but with an altered gene product in the cells tested.

#### Significance to Biomedical Research and the Program of the Institute:

Epidemiological and medical genetic data have indicated major individual differences in cancer risk in humans. Increased risks are associated both with overall susceptibility to cancer or susceptibility in a particular target organ. In some cases, specific genetic changes have been associated with increased risk, but in many examples, polygenic influences appear more likely. To date biochemical epidemiological studies have focused only on genetic differences in

carcinogen metabolism. In the complex and multistage evolution of cancer, it seems unlikely that carcinogen metabolism is solely responsible for enhanced risks. In fact, it seems likely that factors associated with the expression of neoplastic change would play an important role in ost susceptibility. The development of animal strains through selective breeding with high susceptibility at a particular organ site provides an excellent model for the study of susceptibility determinants. In vivo studies have indicated that carcinogen metabolism is unlikely to explain the sensitivity of SENCAR cells, and grafting experiments indicate the target tissue itself is somehow more susceptible. This validates the use of in vitro techniques to explore susceptibility. If this model reflects susceptibility determinants in human cancer, it will provide important insights and potential assays for studies in human populations. Furthermore, understanding determinants of susceptibility is likely to yield information concerning the molecular mechanisms of carcinogenesis.

#### Proposed Course:

In addition to appearing earlier and in larger numbers in SENCAR than in resistant mouse strains, papillomas from SENCAR mice do not regress upon cessation of promoter treatment as occurs in resistant strains such as BALB/c, i.e., SENCAR papillomas are largely promoter independent. It is these papillomas which appear to be most likely to progress to carcinomas. Although promoter-independent papillomas occur in resistant strains, they are much fewer on a percentage basis. Identification of the mechanism of promoter independence of papillomas may thus offer major insight into SENCAR susceptibility.

We do not know whether the so-called endogenously initiated cells in SENCAR have the property of resistance to terminal differentiation before they are removed from the animal. An alternative explanation is that a genetic instability of some type favors development of this property. There is evidence from some unpublished chromosome studies of SENCAR and BALB/c cells that spontaneous chromosomal abnormalities are more numerous in SENCAR than in BALB/c epidermal cells after several weeks in vitro. Such an instability may contribute to the formation of cells resistant to terminal differentiation as well as papilloma cells which more readily become promoter independent. If the "endogeneously initiated" cells primarily develop in culture, we should see increased numbers of resistant foci with longer culture times in low calcium before the high calcium selection pressure is applied. Such experiments will be done.

We presently have no lines of terminal differentiation-resistant SENCAR cells derived from foci in epidermal cell control cultures not treated with carcinogen. It will be important to obtain such lines and to compare them with SENCAR and BALB/c lines derived from carcinogen-treated cells. In vivo experiments to repopulate denuded areas of mouse epidermis with cells from these three types of lines and to examine TPA effects on these cells may help to clarify the role of SENCAR endogenously initiated cells in susceptibility as well as the mechanism of promoter independence. Such questions as whether cells from the SENCAR spontaneous foci are more responsive to proliferative effects of TPA than cells from carcinogen-derived foci of SENCAR and BALB/c cells will be investigated. Both in vivo studies to examine the biological responses of these cells to TPA

and in vitro studies to examine the biochemical responses on proliferative and differentiative pathways will be done. Comparative studies will be done with terminal differentiation-resistant cell lines of BALB/c and SENCAR and cell lines derived from papillomas from these strains.

SENCAR skin is clearly more sensitive to the toxic effects of TPA than is BALB/c skin. In in vivo studies it was necessary to reduce the TPA treatment from twice weekly to once weekly because of ulcerative lesions that developed in SENCAR but not BALB/c skin. Although we have no in vitro evidence of differential TPA dose-response sensitivity to any parameter we have examined, we plan to conduct in vivo experiments to determine whether the gap between strains in time of appearance and number of papillomas can be reduced by increasing levels of TPA applied to BALB/c skin during promotion.

If the endogenously initiated cells of SENCAR skin are responsive to tumor promoters as expected, we should be able to expand clones of these cells in vivo by applying TPA to SENCAR skin and then selecting in vitro. Since foci from untreated BALB/c skin are quite rare, TPA treatment should not change the number of foci in controls. Preliminary experiments with SENCAR cells have been unsuccessful, but we will continue to pursue this area.

Finally, with the development in our laboratory of the capability to transfect genes into recipient primary epidermal cells, it may be possible to identify the gene or genes responsible for resistance to  $Ca^{2+}$ -induced terminal differentiation. Attempts will be made to transfect SENCAR cell sensitivity using the end point of spontaneous resistant foci in cells not treated with carcinogens.

#### Publications:

Kawamura, H., Strickland, J. E. and Yuspa, S. H.: Inhibition of 12-O-tetradecanoylphorbol-13-acetate induction of epidermal transglutaminase activity by protease inhibitors. Cancer Res. 43: 4073-4077, 1983.

Strickland, J. E. and Strickland, A. G.: Host cell reactivation studies with epidermal cells of mice sensitive and resistant to carcinogenesis. Cancer Res. 44: 893-895, 1984.

Strickland, J. E., Jetten, A. M., Kawamura, H. K. and Yuspa, S. H.: Interaction of epidermal growth factor with basal and differentiating epidermal cells of mice resistant and sensitive to carcinogenesis. Carcinogenesis 5: 735-740, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05270-03 CCTP

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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)

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## LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

## SECTION

Molecular Mechanisms of Tumor Promotion Section

## INSTITUTE AND LOCATION

NIH, NCI Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

4.75

## PROFESSIONAL:

3.75

## 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.)

The efforts of the Molecular Mechanisms of Tumor Promotion Section are directed at 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. Novel protocols have been developed for its purification that are more efficient and afford higher yields than is possible with current methods. Purification of monoclonal and polyclonal antibodies to the receptor is in progress. The role of lipids in reconstitution of the receptor has been characterized in detail. Phospholipids differ in whether or not they can reconstitute, in the amounts required for reconstitution, and in the phorbol ester binding affinities of the resultant complex. Diacylglycerols competitively inhibit phorbol ester binding in vitro, consistent with their being the postulated endogenous phorbol ester analogs. Comparison with the homologous phorbol esters yields differences in affinities of only 20- to 80-fold. As expected from the in vitro assays, treatment of intact cells with phospholipase C to generate diacylglycerol endogenously or the exogenous addition of appropriate diacylglycerols likewise inhibits phorbol ester binding competitively in vivo. The inhibition of binding by diacylglycerols suggested that the phorbol ester receptor could recognize the membrane-dissolved form of the phorbol esters. Analysis of the behavior of a series of highly lipophilic phorbol esters confirmed this prediction. Identification of the enzymatic activity association with the phorbol ester receptor has made it possible to analyze the coupling between binding and subsequent response. Most mouse skin tumor promoters, structurally unrelated to the phorbol esters, did not activate protein kinase C in vitro. Unsaturated fatty acids at high concentrations did activate, however. Multiple phorbol ester receptors have been implicated in the heterogeneity of phorbol ester responses. The binding characteristics of intact, cultured keratinocytes change from homogeneous to heterogeneous as differentiation proceeds.

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

Peter M. Blumberg	Research Chemist	LCCTP	NCI
Arco Jeng	Expert	LCCTP	NCI
Karen Leach	Staff Fellow	LCCTP	NCI
Bernhard Konig	Visiting Fellow	LCCTP	NCI

Objectives:

The early events in the interaction of the phorbol esters with cells and tissues are being characterized. Specific aims are as follows: 1) purification and biochemical characterization of phorbol ester receptors; 2) determination of the role of phospholipids in phorbol ester receptor function; 3) analysis of the interaction of diacylglycerols, the putative endogenous phorbol ester analogs, with the phorbol ester receptors; 4) determination of the activity of phorbol esters, structurally unrelated tumor promoters, and modulators of tumor promotion on protein kinase C activity; 5) understanding of the pharmacology of highly lipophilic phorbol esters; and 6) characterization of phorbol ester binding and response in intact cells. The major phorbol ester receptor is protein kinase C. This enzyme is postulated to mediate one of the two pathways activated by a large class of hormones for which receptor occupancy is associated with rapid 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 to identify biochemical steps suitable for intervention.

Methods Employed:

This Section uses a wide range of techniques to pursue the above aims. Phorbol and derivatives are isolated from natural sources. Semisynthetic derivatives for affinity labeling, structure-activity analysis, and binding studies are prepared and radioactively labeled as necessary. Binding studies are carried out using the ligands and methodology developed by us. Analysis of receptors utilizes both photoaffinity labeling and standard biochemical membrane methodology. The systems analyzed are chosen as optimal for the specific questions being examined. Brain homogenates, because of their richness in receptors, are being used for receptor purification and biochemical analysis. Mouse skin and cultured keratinocytes are being used to dissect subclasses of receptors. Intact cells are being utilized to determine the relationship between receptor occupancy and biological responses. Importance is placed upon the ability to relate the answers obtained to the biological system of mouse skin promotion and to coordinate effectively in exploiting the systems being studied by the other Sections of the Laboratory.



### Major Findings:

An obstacle to the biochemical and immunological analysis of the major phorbol ester receptor, protein kinase C, has been the unavailability of an efficient purification procedure. Published methods afford yields of 0.5-5%, provide only small amounts of product, and are often difficult to scale up. Taking advantage of modern advances in chromatographic technology, we have developed two independent procedures for purification. We have used mouse brain cytosol as our source of receptors, since brain is the tissue with the highest specific binding activity and the mouse is the best studied model system. Purification is routinely monitored by phorbol ester binding rather than by assay of kinase activity because of the presence in cytosol of substances which interfere with the kinase assay. Both purification procedures involve three chromatographic steps. For the initially developed procedure, the cytosol is fractionated on a DE52 column using a NaCl gradient. The peak of activity is then chromatographed by fast protein liquid chromatography (FPLC) using a strong anion exchange (Mono-Q) column eluted with a salt gradient in the presence of adenosine 5'-triphosphate (ATP). The peak of activity is rechromatographed under the same conditions in the absence of ATP. The basis for the procedure is that the ATP causes a shift in elution position of the receptor but not of the contaminating proteins. The yield from the two Mono-Q columns is approximately 30%. Purity is estimated at 80-90%. The problems with the procedure are the limited capacity of the first Mono-Q column (25 mg) and the presence of ATP in the final preparation. The alternative purification procedure replaced the two Mono-Q columns with a Blue Sepharose column followed by a column of a high-resolution hydrophobic support, Bio-Gel TSK-Phenyl-5-PW, eluted with a reverse salt gradient. The yield from these two columns is 20%. Purity is estimated at 80-90%. Because the two procedures fractionate on different bases, the combination of the two should prove of value for purification of protein kinase C from starting material of lower specific activity.

A major difficulty in studies on the purified receptors has been its rapid loss of activity upon storage. The decrease in activity within 24 hr may be as much as 80%. This loss contrasts with stability for several months of the receptor which has been partially purified over DE52. It is, therefore, of considerable importance that we have now found stabilization conditions for the purified receptor, making it readily available for the other studies in the laboratory.

We had previously shown that the cytosolic form of the phorbol ester receptor was, in fact, an apo-receptor, which requires the addition of phospholipids for activity. We are in the process of characterizing this phospholipid requirement in detail. Different phospholipids vary markedly in their ability to reconstitute binding. In the absence of  $\text{Ca}^{++}$ , the anionic phospholipids phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA) reconstituted binding to equal maximal extents. Half-maximally effective concentrations ( $\text{ED}_{50}$  values) were 2-7  $\mu\text{g}/\text{ml}$ . Phospholipid mixtures containing anionic phospholipids could also reconstitute binding fully, albeit at higher concentrations. The  $\text{ED}_{50}$  for the phospholipid mixture corresponding to that in red blood cells, for example, was 180  $\mu\text{g}/\text{ml}$ . In contrast, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin were ineffective

at concentrations up to 1 mg/ml. Although binding was not  $\text{Ca}^{++}$  dependent,  $\text{Ca}^{++}$  had a dramatic effect on the  $\text{ED}_{50}$  values for reconstitution by phospholipids. For PS, for example, the  $\text{ED}_{50}$  in the presence of excess  $\text{Ca}^{++}$  was decreased 30-fold to 0.2  $\mu\text{g}/\text{ml}$ . Differences in intracellular  $\text{Ca}^{++}$  might, therefore, be able to regulate protein kinase C reconstitution.

In addition to determining whether or not phorbol ester binding activity can be reconstituted, phospholipids also play a major role in determining the binding properties of the reconstituted complex. Thus, the apo-receptor reconstituted into phosphatidic acid or the red blood cell phospholipid mixture has  $K_D$  values for PDBu binding of 30 and 22 nM, respectively, in contrast to a  $K_D$  of 0.8 nM for the apo-receptor reconstituted into PS. Similarly, the dose-response curves for activation of protein kinase C by PDBu are shifted, depending on the phospholipid. These results suggest that heterogeneity in phorbol ester binding or response may reflect differences in the lipids associated with the receptor rather than the existence of other receptors in addition to protein kinase C. Indeed, reconstitution into mixed populations of liposomes of different compositions yields curved Scatchard plots indicative of heterogeneous binding.

The high degree of conservation of the phorbol ester receptors over evolution strongly argued for the existence of an endogenous analog, which normally interacted at the phorbol ester binding site. The postulated role of diacylglycerol as a natural modulator of protein kinase C activity suggested that it might be the postulated endogenous analog. Binding experiments indeed confirmed that the diacylglycerol derivative diolein inhibited PDBu binding in a competitive fashion. As in the case of the phorbol esters, the phospholipid environment in which the apo-receptor is reconstituted affected the affinity of the diacylglycerol. This result is important because it suggests that the nature of the phospholipids associated with the protein kinase could modulate its responsiveness. To determine how potent diacylglycerols are relative to phorbol esters, we compared phorbol and glycerol diesters having identical side chains. For the lauroyl and oleoyl diesters, the glycerol derivatives were only moderately (20- to 80-fold) less potent.

For diacylglycerol to be a competitive inhibitor, it should interact stoichiometrically with the phorbol ester binding sites. Because of the hydrophobic nature of the diacylglycerols, this prediction is quite difficult to test. Nonetheless, by optimization of the binding conditions, a 1:1 ratio of diolein to receptor can be demonstrated at the  $\text{ID}_{50}$ .

The inhibition of phorbol ester binding *in vitro* by diacylglycerols is corroborated by *in vivo* studies. First, treatment of intact cells with phospholipase C should cleave phospholipids and generate diacylglycerol *in situ* in the membranes. The endogenously generated diacylglycerol in turn should lead to a decreased PDBu binding affinity with no change in the number of binding sites. This expectation was confirmed for keratinocytes treated with phospholipase C in collaborative studies with the *In Vitro* Pathogenesis Section. Secondly, sufficiently hydrophilic diacylglycerols added to the aqueous phase should be able to transfer to the cell membranes of intact cells and inhibit phorbol ester binding. This result was likewise obtained using the intact mouse keratinocytes.

The identification of enzymatic activity associated with the phorbol ester receptor has made it possible to examine, *in vitro*, the coupling between phorbol ester binding and response. Three questions were of particular interest. First, a number of the dose-response curves for biological responses to the phorbol esters are biphasic. Did this reflect inhibition of protein kinase C by supra-optimal phorbol ester concentrations? *In vitro* activation of protein kinase C revealed no evidence for such inhibition. Secondly, did all active phorbol esters activate protein kinase C to the same maximal extent? For the derivatives examined, which included the two second-stage promoters, mezerein and phorbol 12-retinoate 13-acetate, as well as the inflammatory, weak tumor promoter, 12-deoxyphorbol 13-acetate, similar maximal levels of activation were found. Thirdly, what was the correspondence between the dose-response curves for PDBu binding and activation of protein kinase C? Agreement was found, provided that binding and activation assays were carried out under identical conditions.

In addition to examining the effects of the phorbol esters, we have also examined the effect of structurally unrelated tumor promoters and of inhibitors of tumor promotion on protein kinase C activity in the absence and presence of the phorbol esters. Most of the structurally unrelated tumor promoters were inactive, suggesting that they function either through distinct pathways or else through later stages in the protein kinase C pathway. On the other hand, unsaturated fatty acids, e.g., palmitoleic, oleic, and linoleic, were able to stimulate protein kinase C in the absence of phorbol ester and to further stimulate protein kinase C activity in the presence of the phorbol ester. The unsaturated fatty acids have been reported to be weak mouse skin tumor promoters and to induce some biological responses, e.g., partial loss of fibronectin in chicken embryo fibroblasts, similar to those induced by the phorbol esters. Among inhibitors of tumor promotion, the retinoids were without activity at concentrations below 10  $\mu$ M. The intracellular  $\text{Ca}^{++}$  antagonist, TMB-8, which has been reported to inhibit a number of phorbol ester effects, inhibited both phorbol ester binding and protein kinase C activation *in vitro*. The mechanism of inhibition is unclear, however, since inhibition could be blocked either by the addition of  $\text{Ca}^{++}$  or of phospholipid. The  $\text{ID}_{50}$  for inhibition of protein kinase C activity *in vitro*, moreover, is approximately 10-fold the *in vivo* effective concentration, raising the issue of whether its effect in protein kinase C is a primary or a secondary one.

An ongoing uncertainty in the analysis of phorbol ester structure-activity relations has been whether the receptor recognizes those phorbol ester molecules which are dissolved in the lipid bilayer or those which are free in aqueous solution. The indirect evidence so far available has provided some support for both viewpoints. On the one hand, derivatives more lipophilic than PMA, e.g., phorbol 12,13-didecanoate, are less potent, although they should partition more strongly into the membranes. On the other hand, since diacylglycerols, which are insoluble, compete for phorbol ester binding, the simplest model would be that the phorbol esters also are recognized after they dissolve in the lipid bilayer.

An experimental approach to this question was to synthesize a series of highly lipophilic phorbol derivatives--phorbol 12,13-dimyrystate, phorbol 12,13-distearate, and phorbol 12,13-dioleate. Based on aqueous solubilities of  $2 \times 10^{-6}$  and  $5 \times 10^{-8}$  M for PMA and phorbol 12,13-didecanoate, respectively, these derivatives would be expected to show aqueous solubilities too low to achieve an effective inhibitory concentration. Like diolein, however, these derivatives can be mixed in organic solvents with phosphatidylserine and be incorporated directly into the liposomes used to reconstitute the apo-receptor. The results with all three derivatives were similar. Incorporated directly into the liposomes, they displayed apparent  $K_D$  values between 7 and 35 nM. Added to the aqueous phase of the binding assay, they only inhibited with apparent  $K_D$  values above 4  $\mu$ M. For the more soluble phorbol 12,13-didecanoate, in contrast, identical values were obtained by either route of addition.

The low activity of the highly hydrophobic phorbol esters when added to the aqueous phase presumably reflects their slow rate of transfer from phorbol ester micelles to the PS liposomes. Addition of low concentrations of detergent would be expected to accelerate the rate of transfer. In fact, incubation with 0.03% Triton X-100 overnight reduced the  $K_i$  for phorbol 12,13-distearate (i.e., increased its apparent affinity) to 14 nM. In analogy with phospholipid exchange proteins, the phorbol ester binding protein from serum described by Shoyab and Todaro (J. Biol. Chem., 257:439-445, 1982) may be able to function similarly to promote equilibration.

One implication of the studies is that for highly lipophilic phorbol esters, activity may predominantly reflect pharmacokinetic factors rather than equilibrium binding affinities. Secondly, the phorbol esters cause an apparent transfer of protein kinase C from the cytosolic to the membrane fraction. Recognition by the receptor of phorbol ester dissolved in the membranes suggests that the "cytosolic receptor" is either loosely or transiently membrane associated.

Considerable evidence suggests heterogeneity in the response of cells and tissues to the phorbol esters. In mouse skin, for example, phorbol esters have been found to differ in their relative inflammatory and tumor-promoting activities. The process of tumor promotion itself has been subdivided into two stages distinguishable by their structure-activity relations. Binding analysis using particulate preparations from mouse skin likewise demonstrates heterogeneous phorbol ester binding, consistent with a minimum of three classes of binding sites. To better understand the relation between phorbol ester binding and response in the skin system, we are collaborating with the IVP Section in the analysis of phorbol ester binding to intact mouse keratinocytes. Under low  $Ca^{++}$  culture conditions, which maintain the keratinocytes in a replicating state, PDBu bound to one predominant class of receptors, giving an affinity of 12 nM and a level of binding at saturation for these sites of 1.4 pmol/mg. Shift to high  $Ca^{++}$  culture conditions, which induces differentiation of the keratinocytes, causes a two-fold increase in the number of these high-affinity sites as well as the appearance of a population of lower affinity sites. Binding to keratinocyte variants (D and 308), which are resistant to calcium-induced differentiation, yielded straight Scatchard plots under either set of conditions.

Significance to Biomedical Research and the Program of the Institute:

Much of human cancer is thought to result from a combination of carcinogenic and tumor-promoting activities. Although considerable progress has been made in elucidating the mechanisms of carcinogens, much less is understood about the mode of action of tumor promoters. One of the most accessible model systems for analyzing this process is that of phorbol ester tumor promotion in mouse skin. The unique value of the phorbol esters in this system lies in their very high potency, which facilitates the distinction between specific and nonspecific effects. The Laboratory of Cellular Carcinogenesis and Tumor Promotion is conducting an integrated study of the skin tumor promotion system at multiple levels of analysis--whole animal, cellular, and biochemical. The focus of the Molecular Mechanisms and Tumor Promotion Section on phorbol ester receptors should identify, unambiguously, the initial biochemical steps which are both necessary and sufficient for tumor promotion by these agents. Identification of such biochemical steps should permit the analysis of their control, modulation, and function in human cells under normal and pathological conditions. Determination of the ability of other less specific tumor promoters to perturb, indirectly, the same processes will shed light on the generality of mechanisms of promotion. Such information is of central importance in attempting to develop better means of detecting tumor promoters and evaluating their potential hazard. In addition, the biochemical analysis should provide both a new avenue for the rational development of inhibitors of promotion as well as shed light on the mechanism of current classes on inhibitors. Particular effort currently is being devoted to the analysis of the major phorbol ester receptor, which is protein kinase C.

The subdivision of tumor promotion *in vivo* into multiple stages implies that cellular or biochemical mechanisms (or indeed *in vitro* assays) will also be stage-specific. The analysis of functional receptor subclasses provides an essential basis for determining which processes belong to which subclass of response. Moreover, emerging evidence suggests that subclasses of receptors may have antagonistic effects. For example, the tumor yield for the complete tumor promoter, PMA, is reduced by co-administration of first- or second-stage tumor promoters. Understanding of the interaction between the processes belonging to each subclass may be essential for predicting biological outcome and may provide an additional means of intervention.

Proposed Course:

The overall aims of the section will remain the same over the coming year. The major objectives will be to clarify the interaction of phorbol esters and diacylglycerols with the protein kinase C-phospholipid complex and to elucidate the further consequences of this interaction. An important issue will be to determine whether protein kinase C in various lipid environments accounts for all or only some of the phorbol ester responses.

Polyclonal and monoclonal antibodies will be prepared against the purified receptor. The antibodies will be used for receptor localization and for identification of receptor modifications generated in intact cells under control and stimulated conditions. Cells will be micro-injected with the catalytic

fragment from the purified receptor to confirm its role in biological activity. Photoaffinity labeling of the purified receptor with appropriately modified phorbol esters and diacylglycerols will be attempted to identify and characterize the modulatory site on the protein. Phosphorylation of specific substrates using purified kinase will be examined and compared with *in vivo* responses.

In an effort to understand the mechanisms by which receptor intracellular localization might be controlled, the interaction between receptor and lipid mixtures resembling those of different cellular organelles will be examined. Phospholipids affect the binding affinity of the receptor. The lipid reconstitution analysis will be extended to determine whether lipid composition also modifies the phorbol ester structure-activity relations. It will need to do so if protein kinase C can account for different receptor sub-classes. Photoaffinity labeling of intact cells using phorbol ester derivatives selective for the lipid portion of the receptor complex will be undertaken to determine if the lipids which are associated *in vivo* correspond to those expected from the reconstitution experiments. Genetic and biochemical techniques will be used to modify cellular membranes, and the consequences of these modifications on phorbol ester binding and response will be explored.

The results with the highly lipophilic phorbol esters raise the possibility that differences in the rate of phorbol ester equilibration between plasma and intracellular membranes might cause differences in the pattern of responses which the phorbol esters induce. The predictions of this model will be evaluated. Structure-activity analysis of phorbol esters will attempt to define the role of the different groups in the molecule with respect to their interactions with protein, phospholipid, and  $Ca^{++}$  in the receptor complex. The objective will be to identify structures which might act as antagonists for the receptor. Other appropriate compounds will be screened to identify additional classes of modulators.

#### Publications

Blumberg, P. M., Delclos, K. B., Dunn, J. A., Jaken, S., Leach, K. L. and Yeh, E.: Phorbol ester receptors and the *in vitro* effects of tumor promoters. Ann. NY Acad. Sci. 407: 303-315, 1983.

Blumberg, P. M., Dunn, J. A., Jaken, S., Jeng, A. Y., Leach, K. L., Sharkey, N. A. and Yeh, E.: Specific receptors for the phorbol ester tumor promoters and their involvement in biological responses. In Sloga, T. J. (Ed.): Mechanism of Tumor Promotion, Vol. 3. Tumor Promotion and Cocarcinogenesis In Vitro. Boca Raton, FL, CRC Press, 1984, pp. 143-184.

Blumberg, P. M., Jaken, S., Konig, B., Sharkey, N. A., Leach, K. L., Jeng, A. Y. and Yeh, E.: Mechanism of action of the phorbol ester tumor promoters: Specific receptors for lipophilic ligands. Biochem. Pharmacol. 33: 933-940, 1984.

Blumberg, P. M., Konig, B., Sharkey, N. A., Jaken, S., Leach, K. L. and Jeng, A. Y.: Analysis of phorbol ester receptors: A biochemical approach to understanding the mechanism of action of tumor promoters. In Tashjian, A. H., Jr. (Ed.): Molecular and Cellular Approaches to Understanding Mechanisms of Toxicity. Boston, Harvard School of Public Health, 1984, pp. 108-127.

Blumberg, P. M., Konig, B., Sharkey, N. A., Leach, K. L., Jaken, S. and Jeng, A. Y.: Analysis of membrane and cytosolic phorbol ester receptors. In The Role of Cocarcinogens and Promoters in Human and Experimental Carcinogenesis. (In Press)

Blumberg, P. M., Sharkey, N. A., Konig, B., Jaken, S., Leach, K. L. and Jeng, A. Y.: Membrane and cytosolic receptors for the phorbol ester tumor promoters. In The Cancer Cell, Proceedings of the Eleventh Cold Spring Harbor Conference on Cell Proliferation and Cancer. (In Press)

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Dunn, J. A. and Blumberg, P. M.: Specific binding of [20-<sup>3</sup>H]12-deoxyphorbol 13-isobutyrate to phorbol ester receptor subclasses in mouse skin particulate preparations. Cancer Res. 43: 4632-4637, 1983.

Leach, K. L., James, M. L. and Blumberg, P. M.: Characterization of a specific phorbol ester apo-receptor in mouse brain cytosol. Proc. Natl. Acad. Sci. USA 80: 4208-4212, 1983.

Sharkey, N. A., Leach, K. L. and Blumberg, P. M.: Competitive inhibition by diacylglycerol of specific phorbol ester binding. Proc. Natl. Acad. Sci. USA 81: 607-610, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05356-02 CCTP

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Phorbol Ester Receptor Occupancy-Response Coupling in Hormone Responsive Cells

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

PI: S. Jaken Expert LCCTP NCI

Others: P. M. Blumberg Research Chemist LCCTP NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

## SECTION

Molecular Mechanisms of Tumor Promotion Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.25

## PROFESSIONAL:

1.25

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

The objective of this project is to define events associated with phorbol ester action, beginning with occupancy of the phorbol ester receptor in hormone-responsive cells. Particular emphasis is placed on the coupling of phorbol ester receptor occupancy with phorbol ester activation of protein kinase C. A model cell culture system has been used to study the process by which cells become refractory to phorbol ester-directed responses. The results indicate that the observed desensitization is not due to a biochemical change in the receptor/protein kinase C. Certain phospholipid mixtures were found to inhibit protein kinase C activity at limiting  $Ca^{++}$  concentrations without inhibiting binding, suggesting a role for phospholipid metabolism in regulation of coupling of phorbol ester binding and activation of protein kinase C. Several factors which interfere with either the binding or the kinase assay have been identified. Although no role for these in regulation of kinase or binding is known, characterization of these factors has facilitated quantitation of the kinase and binding activities in crude and partially purified preparations. The role of diacylglycerol in activation of protein kinase C in intact cells was studied by treating cultures with phospholipase C. This treatment caused a decrease in phorbol ester binding consistent with the diacylglycerol generated being a competitive inhibitor of phorbol ester binding. Phospholipase C-treatment mimicked phorbol ester effects as measured by redistribution of receptors from the cytosol to the membrane compartments, decreased binding of epidermal growth factor, and increased secretion of prolactin.



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

P. M. Blumberg	Research Chemist	LCCTP	NCI
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Objectives:

The overall objective of this project is to characterize the events involved in coupling of phorbol ester receptor occupancy with biological responses in cells with well characterized receptors for hormones thought to act through phosphatidylinositol turnover and activation of protein kinase C. The specific goals include: (1) characterizing the properties of the phorbol ester receptor and protein kinase C in control cells and cells that are refractory to phorbol ester-treatment; (2) characterizing the responses of cells to hormones and other effectors which increase diacylglycerol, the postulated endogenous ligand for the phorbol ester receptor; and (3) characterizing factors, with particular emphasis on phospholipid requirements, that regulate phorbol ester binding and phorbol ester activation of protein kinase C. The intention is to describe mechanisms whereby certain cells modulate their responsiveness to phorbol ester-mediated events.

Methods Employed:

Two cell lines are being used in these studies: a clonal strain of rat anterior pituitary mammothrophs (GH<sub>4</sub>C<sub>1</sub>) and a clonal strain of mouse anterior pituitary corticotrophs (AtT<sub>20</sub>/D16<sub>v</sub>). Both types are maintained by routine passage of cells. Phorbol ester binding to intact cells, isolated cell membranes, and isolated cell cytosol is measured using [<sup>3</sup>H]phorbol 12,13-dibutyrate (PDBu). Cell membrane and cytosol fractions are prepared by centrifugation of cell lysates at 100,000 x g for 60 minutes. Protein kinase C activity is measured by assaying the amount of <sup>32</sup>P transferred from <sup>32</sup>P-ATP to histone using a standard assay procedure.

Major Findings1. Properties of the phorbol ester receptor and protein kinase C in control and desensitized cultures.

The effects of phorbol esters on secretion of ACTH by hormonally responsive pituitary corticotrophs (AtT<sub>20</sub>/D16<sub>v</sub>, referred to as D16 cells) have been studied in detail. Initially, phorbol esters cause a two-fold stimulation of ACTH release. Over a fairly rapid time course (t<sub>1/2</sub> = 0.85 hours), the phorbol ester effect is lost. During this time, no change in phorbol ester binding to intact cells is observed. Furthermore, the cells remain responsive to other hormones that are thought to act via cAMP. These cultures are considered to be desensitized, a pharmacological process common to regulation of cellular responsiveness to a wide variety of natural effector-receptor systems, and are being used

as a model system for studying the process of uncoupling of phorbol ester receptor occupancy from biological responses.

Several approaches have been used to establish that the receptor/protein kinase C from control and desensitized cultures have similar biochemical properties. First, the receptor and protein kinase C from both control and desensitized cultures coelute on fast protein liquid chromatography (FPLC) (Pharmacia) anion exchange chromatography. Furthermore, the activities from control and desensitized cultures elute at the same salt concentration, indicating no large change in biochemical properties of the receptor from desensitized cultures. A second approach was to study the phorbol ester-directed redistribution of receptors and protein kinase C from a predominantly cytosolic location to a predominantly membrane location. Exposure of D16 cells to phorbol esters for brief times consistent with active hormone secretion causes an increase in membrane-associated receptor concentration and a decrease in cytosolic receptor and protein kinase C concentrations. Exposure to phorbol esters for longer times consistent with desensitization causes a quantitatively similar redistribution. Therefore, desensitization is not associated with a defect in the phorbol ester-mediated redistribution process. A third approach was to compare the phospholipid requirements for reconstituting binding and kinase activities of partially purified preparations from control and desensitized cultures. A wide variety of mixed liposome preparations were used for this comparison and no differences were found. Enzyme preparations from control and desensitized cultures were both activated by phorbol esters. These results suggest that at the first level of analysis, the biochemical properties of the receptor/kinase from control and desensitized cultures is the same.

## 2. Responses of cells to hormones and other effectors which increase diacylglycerol.

Diacylglycerol has been identified as a probable endogenous ligand for the phorbol ester receptor based on in vitro studies including: (1) phorbol ester substitution for diacylglycerol for activation of protein kinase C and (2) diacylglycerol inhibition of phorbol ester binding. A hormonally responsive cell line, GH<sub>4</sub>C<sub>1</sub> rat pituitary mammatrophs, has been used to further explore the effects of diacylglycerol on the phorbol ester receptor in intact cells. Thyrotropin-releasing hormone (TRH) stimulates phosphatidylinositol turnover in GH<sub>4</sub>C<sub>1</sub> cells leading to an increase in diacylglycerol. Therefore, TRH-treatment was used to study the effects of hormonally generated diacylglycerol on the subcellular distribution of phorbol ester receptors. As discussed above, phorbol esters mediate a membrane-association of the receptor/kinase, and this process is thought to fulfill the phospholipid requirements for the kinase activity. Under the assay conditions used, TRH did not affect the subcellular distribution of the receptor. At the present time, these results are considered to indicate that the quantity of hormonally-generated diacylglycerol may not be sufficient to measurably affect receptor subcellular distribution. However, additional protocols should be attempted to substantiate this negative result.

An alternative method for generating diacylglycerol in cell membranes, phospholipase C-treatment, was also studied. Phospholipase C was found to yield a concentration-dependent increase in diacylglycerol to a level two- to three-fold

over that of controls. This treatment resulted in a decrease in affinity of phorbol ester binding with no apparent change in receptor number. This is the predicted result for a competitive inhibitor. Furthermore, phospholipase C-treatment caused a change in subcellular distribution of receptors from the cytosol to the membrane compartment. This redistribution was quantitatively similar to that produced by phorbol esters. Phospholipase C-treatment also caused two biological responses caused by phorbol ester-treatment, namely, decreased binding of epidermal growth factor and increased secretion of prolactin. These results provide evidence that diacylglycerol interacts with the phorbol ester receptor in intact cells and further characterizes the role of protein kinase C in mediating cellular responses to phorbol esters.

### 3. Factors regulating phorbol ester binding and phorbol ester activation of protein kinase C.

Several categories of factors that interfere with either the binding or kinase reactions have been found during the process of characterizing the systems described above. Initially, it was observed that the kinase activity, but not the binding activity, in cytosol preparations was masked until samples were chromatographed on anion exchange columns. This suggested the presence of inhibitors of protein kinase C. Mixing experiments have been done to identify peaks of inhibitory activity from the column profiles. Two peaks of inhibitory activity have been detected. Although no regulatory function has yet been ascribed to these inhibitors, their existence stresses potential difficulties in quantitating kinase activity and suggests that binding may be a more reliable approach to quantitating the kinase in crude preparations.

Studies of phospholipid requirements for binding led to the identification of cellular phospholipase C as an interfering activity in the binding assay. Certain mixed liposome preparations caused a large decrease in receptor affinity. This inhibition was not apparent if the assays were performed at 4°C or with limiting  $Ca^{++}$ . Further analysis showed that the samples contained large amounts of phospholipase C activity. These results suggest that endogenous phospholipase C activity, under certain conditions, can lead to generation of diacylglycerol during the phorbol ester binding assay incubation, thus leading to apparent inhibition of binding. This result demonstrates the importance of studying the phospholipid dependence of binding under conditions which limit phospholipase C activity.

As stated above, a major objective of this work is to identify factors which lead to decreased responsiveness of cells to phorbol esters. The initial results from the comparison of the binding and kinase activities from control and desensitized D16 cells suggest that in that case, decreased responsiveness is not associated with a major change in the receptor/kinase molecule. Therefore, the focus of this project has shifted toward analysis of phospholipid regulation of the coupling between binding and kinase activities. Increasing the phosphatidylcholine content of liposomes was found to increase the  $Ca^{++}$  requirement for protein kinase C activity (measured in the presence of phorbol esters); however, it did not decrease the binding activity. The effect was shown to be associated with phosphatidylcholine and not neutral phospholipids in general, because increasing the phosphatidylethanolamine concentration in

mixed liposomes did not increase the  $Ca^{++}$  requirement for enzymatic activity. This change in  $Ca^{++}$  requirement for kinase activity may be sufficient to prevent phorbol ester activation of protein kinase C in intact cells at limiting cytosolic  $Ca^{++}$  levels. These results suggest a potential role for phosphatidylcholine metabolism in regulation of coupling between phorbol ester receptor occupancy and phorbol ester activation of protein kinase C.

#### Significance to Biomedical Research and the Program of the Institute:

This project is designed to use model cell culture systems for detailed biochemical analysis of the mechanism of action of phorbol esters. It is anticipated that the general principles can then be applied to more complex systems, including whole skin or cultured keratinocytes. That is, the cultured cells used in this project should provide a useful model system for designing critical experiments with less readily available tissues in which phorbol esters have been shown to have tumor promoting properties.  $GH_4C_1$  cells are a particularly useful model system because they have well-characterized receptors and responses to a variety of hormones and effectors. They can be used to study the influence of several hormones on responses to phorbol esters.  $GH_4C_1$  cells have receptors for glucocorticoids and compounds that work through the adenylate cyclase system, both of which have been suggested to influence phorbol ester actions. In addition, the effects of other compounds that may also activate protein kinase C, such as thyrotropin-releasing hormone, can also be explored. In this way,  $GH_4C_1$  cells can be used to study the concerted cellular response to a variety of conditions.

Desensitization to tumor promoters is of potential importance in limiting susceptibility to carcinogenesis. For example, hamsters become refractory to phorbol ester-mediated hyperplasia. Based on data indicating that sustained hyperplasia is necessary for tumor promotion in mice, it seems likely that desensitization to phorbol esters in hamster skin is related to the resistance of this tissue to phorbol ester-promoted carcinogenesis. Biochemical analysis of this process in the easily obtainable cultured pituitary cells will provide a model for designing critical experiments to characterize the mechanism of desensitization in the less readily available hamster keratinocytes. Understanding the pathway of desensitization may also lead to pharmacological approaches to limiting cellular sensitivity to tumor promoters.

The overall significance to this project is that it will increase the understanding of the mechanism of phorbol ester action using cell culture model systems. This should provide insight into the biochemical pathways important in carcinogenesis in human tissues as well.

#### Proposed Course:

The overall objectives of this project will remain the same during the next year. The well-characterized model systems will be used to further characterize the events involved in coupling of phorbol ester receptor occupancy with biological responses and in modulation of the coupling process.

Efforts of others in this section are directed towards purification of the receptor/kinase for antibody preparation. The antibodies could be used to compare the receptor/kinase from control, desensitized, and down-modulated cultures for biochemical differences that may be related to the observed changes.

The D16 cells will be used to attempt to identify substrate proteins for protein kinase C that are related to the secretory response. Presumably, phosphorylation events associated with secretion should be absent from the desensitized cultures.

Further definition of the role of the phospholipid environment in coupling of receptor occupancy to kinase activation will be done. Particular emphasis will be placed on phorbol ester-directed changes in phospholipid metabolism that might lead to desensitization or receptor-down modulation.

#### Publications

Jaken, S., Feldman, H., Blumberg, P.M., and Tashjian, A.H., Jr.: Association of phorbol ester receptor down modulation of phorbol ester receptors is associated with a cryptic receptor state. Cancer Res. 43: 5795-5800, 1983.

CONTRACT IN SUPPORT OF PROJECTS:

Z01CE04504-12 CCTP  
Z01CE04798-04 CCTP  
Z01CE05177-04 CCTP  
Z01CE05178-04 CCTP

MICROBIOLOGICAL ASSOCIATES INC. (N01-CP1-5744)

Title: Rodent and Rabbit Facility as a Resource to LCCTP

Current Annual Level: 325,000

Man Years: 3.0

Objectives:

To provide space, care and technical support for the conduct of in vivo experiments designed to correlate, validate and extend the in vitro findings developed in project Z01CE04504-12 CCTP. In addition this contract supports the antibody production work of project Z01CP05177-04 CCTP and the genetic susceptibility studies of project Z01CP05178-04 CCTP and the vitamin research of project Z01CP04798-13 CCTP.

Major Contributions:

Project Z01CE04504-12 CCTP: Factors influencing the progression of benign papillomas to carcinomas were studied in SENCAR mice. After initiation with 7,12-dimethylbenz[a]anthracene and promotion for 10 weeks with TPA, papillomas were the only neoplastic lesions. Continued TPA application for 40 weeks did not alter the carcinoma incidence from that of control animals in which promotion was terminated at 10 weeks. However, administration of a tumor initiator, either urethane (systemically), MNNG (topically) or 4NQO (topically), markedly accelerated and enhanced (four-fold) the conversion of papillomas to carcinomas. The papilloma stage appears to be required in the production of carcinomas since no carcinomas developed when treatment with acetone was substituted for TPA in the promotion stage of the protocol. Inhibitors of tumor promotion by TPA (fluocinolone acetonide and retinoic acid) are currently being tested as potential inhibitors of the progression of papillomas to carcinomas. In another experiment in progress, the conversion of papillomas to carcinomas by treatment of papilloma-bearing mice with a tumor initiator (urethane) has been confirmed in Charles River CD-1 mice and it appears that a single exposure to urethane is sufficient to enhance malignant conversion.

Formaldehyde, a hazardous agent to which humans are exposed, has been reported to induce nasal cancer in rats. In mouse skin, however, repeated topical application caused only a few benign tumors. In SENCAR mice, formaldehyde was

inactive as an initiator but demonstrated promoting activity. Repeated application for 80 weeks following DMBA initiation produced 37 papillomas and 6 suspected carcinomas on 30 mice.

Tumorigenicity testing of cell lines derived from in vitro transformation experiments are being conducted by injection into nude mice. More than 20 lines have been tested, some repeatedly with the results showing that many lines which demonstrate altered response to differentiation signals after carcinogen exposure are initially not tumorigenic. However, progression to malignancy results with prolonged passage of these cell lines in culture. Cell lines derived from chemically induced papillomas and virus-transformed keratinocytes are also being tested in nude mice. Nude mice are also receiving skin grafts of human foreskins and these mice will be subjected to initiation-promotion protocols to assess the response of the human skin.

#### Project Z01CE05178-04 CCTP:

Grafting of SENCAR mouse skin to nude mice and performing carcinogenesis studies on grafted tissue have indicated that susceptibility resides in the target tissue. SENCAR mice are being compared to more resistant strains by dose-response studies for initiators and promoters. Animals receiving treatment in vivo are also transferred to LCCTP to isolate cells for culture and selection of initiated foci.

Project Z01CE05177-04 CCTP: Rabbits are maintained for antibody production. In previous years a number of carcinogen-DNA adduct antibodies were produced under this contract (see project description). New antibodies against specific epidermal differentiation products have been produced. Specific antibodies to cornified envelope proteins are now available and provide a unique opportunity to isolate precursors to that important component of terminally differentiated cells. Of great significance has been the production of antibodies to the 67 kd, 59 kd, 60 kd and 50 kd keratins following injection of 12-amino acid peptide sequences of each protein. These sequences were determined to be unique from the keratin sequencing studies performed under Z01CE04504-12 CCTP. Characterization of each antiserum is currently underway. These appear to be the first completely specific antisera available to individual keratin peptides.

Project Z01CP04798-14 CCTP: Vitamin A-deficient hamsters are routinely supplied for isolation of liver microsomes and as a source for tracheal cultures. Mice are made vitamin A-deficient as weanlings for studies relating to susceptibility to carcinogenesis.

Significance to Biomedical Research and the Program of the Institute: Studies in epithelial target tissues are the most relevant with regard to the pathogenesis of human cancer. While information gained from in vitro experiments on epithelial cells have yielded major insights into mechanisms of carcinogenesis, correlative in vivo experiments are required for validation of results and confirmation of concepts using a tumor endpoint. These in vivo studies have also been invaluable in identifying mechanisms of tumor progression and the genetic basis for cancer susceptibility, both extremely important and relevant research areas in carcinogenesis. The work contributed by this animal support is an integral part of the overall research program of LCCTP.

Proposed Course:

This contract will be continued at the current or slightly increased level to pursue studies on the mechanism of conversion of benign to malignant lesions, the basis for susceptibility to carcinogenesis and to provide rabbit antibodies for various program needs. The nude mouse colony will be used to assess tumorigenicity of cells and for experiments involving heterografts or allografts.



ANNUAL REPORT OF  
THE LABORATORY OF CHEMOPREVENTION  
NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

The problem of the isolation, characterization, and biological role of transforming polypeptide 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, that are the subject of current attempts at amino acid sequencing. 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. These tissues are human placenta, human blood platelets, and bovine kidney. The experimental use of TGF- $\beta$  in wound healing has been a finding of major importance and has provided a great deal of encouragement to proceed further with the entire problem of the molecular biology and molecular genetics of these growth factors. Finally, we are now involved in a major attempt to integrate studies of retinoids into our current program of studies on peptide growth factors.

A major laboratory effort has been the purification of large amounts of TGF- $\beta$  from human platelets. The availability of sufficient quantities of TGF- $\beta$  has enabled our laboratory to progress with the development of antisera to this peptide and with development of receptor binding studies. Human platelets have been extracted with acid-ethanol and platelet-derived TGF- $\beta$  has been purified from the extract by a two-column procedure using sequential gel filtration in the absence and then presence of urea. Purified TGF- $\beta$  is a protein of 25,000-daltons, and it is comprised of two 12,500-dalton subunits held together by disulfide bonds. The purified factor elicits its biological activity at concentrations less than 4pM. Comparative studies showed that platelets contain 100 times more TGF- $\beta$  than do other non-neoplastic tissues. Incubation of TGF- $\beta$  with normal rat kidney cells results in an increased number of cell surface epidermal growth factor receptors. This is an important new finding, since it provides the first evidence for a biochemical mechanism of action of TGF- $\beta$ .

In certain cells, such as NRK fibroblasts (NRK cells), both type  $\alpha$  and type  $\beta$  TGFs must be present to effect phenotypic transformation as evidenced by the acquisition of the ability to form large colonies of cells in semi-solid agar medium. It has been shown previously that NRK cells transformed by sarcoma viruses secrete type  $\alpha$  TGFs and that this secretion is correlated with expression of the transformed phenotype in cells infected with temperature-sensitive mutants of the virus. It has now been demonstrated that these cells also secrete increased amounts of type  $\beta$  TGFs after sarcoma virus transformation and that the levels of TGFs secreted by the cells into the conditioned medium are consistent with an autocrine mechanism of transformation. It also has been shown for the first time that transformed cells secreting type  $\beta$  TGFs have a reduced number of cell surface receptors for that ligand, suggesting that ligand-induced down-regulation has occurred.

A new cell line has been derived from a metastatic, human melanoma. It is producing peptides that can stimulate an untransformed cell line to reversibly express a transformed phenotype. This transformed phenotype is expressed in monolayer culture as a disorganized growth pattern and in an anchorage-independent growth (AIG) assay as colonies forming in soft-agar from single cells. One of the peptides in this mixture contributing to this activity is an EGF-like growth factor that has a molecular weight of approximately 26,000 daltons as determined by gel permeation chromatography on Bio-Gel P-30 in 1 M acetic acid. This EGF-like activity requires a second peptide to efficiently stimulate anchorage independent growth. The second required peptide in this mixture has a modulator activity in that it appears to require the presence of either EGF or an EGF-like activity to efficiently stimulate large colony formation. This TGF- $\beta$  activity has an apparent molecular weight of approximately 21,000 daltons. Both of these peptides have physical properties that are distinctly different than those previously reported for the TGFs. These "ectopic" peptides may play a role in the expression of the transformed phenotype of the tumor cells producing them. Molecular clones have been derived to determine which genes are regulated by these growth and modulating factors. This knowledge will increase our understanding of the role these factors play in the expression of the transformed state.

In an important new project, polyclonal antisera have been raised to the TGFs and development of monoclonal antibodies is in progress. The effects of these antibodies on the anchorage-independent and independent growth of normal and transformed cells are being investigated. Since the first step in the interaction of TGFs with the cell is binding of the growth factor to the cell surface, initial investigations are concentrating on the characterization of cell surface receptors for TGFs. Development of radioreceptor assay for TGF- $\beta$ , a TGF- $\beta$  that depends on a second TGF (EGF or TGF- $\alpha$ ) for activity, has allowed identification and preliminary characterization of a specific high affinity receptor for TGF- $\beta$  on normal rat kidney cells. A similar receptor has been found on all normal and transformed cell lines studied so far. Receptor expression in normal cell lines (NRK and NIH-3T3) is modulated by infection of the cell with acute transforming retroviruses (HSV and MoSV) and by transformation with certain oncogenes (myc, H-ras). Human cell lines and tissues are being screened for an abundant source of the receptor for use as a starting material for receptor purification and as an immunogen for the production of anti-receptor monoclonal antibodies. Further characterization of the role of endogenously-produced TGFs and their interaction with the cell surface receptor should help elucidate the role these molecules may play in the process of carcinogenesis.

Type  $\alpha$  and  $\beta$  TGFs, retinoids, and the peptide products of cellular oncogenes all affect gene expression of cells and all play a role in control of cellular proliferation and neoplastic transformation. We are searching for common elements in the mechanism of action of these three groups of compounds, with emphasis on the determination of the basis for the heterogeneous effects of retinoids and TGFs on cells and for the observation that their interactions are antagonistic in certain situations and synergistic in others. Studies are aimed at three levels: in the whole embryo, in various organs and tissues, and in cell culture.

In NRK cells, retinoids and TGFs were shown to synergize to induce phenotypic transformation of the cells, as measured by the growth of the cells under anchorage-independent conditions. In new studies employing Fischer rat 3T3 cells transfected with the *myc* oncogene (done in collaboration with D.F. Stern and R.A. Weinberg, MIT), it has been shown that type  $\beta$  TGFs synergize with platelet-derived growth factor in the induction of anchorage-independent growth of the cells and that this effect of the peptides is completely blocked by concentrations of retinoic acid as low as one nanomolar. In contrast, when the anchorage-independent growth of these same cells is induced by epidermal growth factor (a type  $\alpha$  TGF), retinoids have no effect. Like the retinoids, type  $\beta$  TGFs can also be shown to have opposite effects on these cells: TGF- $\beta$  stimulates the anchorage-independent growth of the cells when assayed in the presence of PDGF, but inhibits the anchorage-independent growth of the same cells when assayed in the presence of EGF. The molecular basis for the changing effects of both the retinoids and the type  $\beta$  TGFs on these cells is being investigated.

Initial results of the effects of retinoids on embryogenesis in the Japanese quail have shown that normal embryogenesis can be restored to retinoid-deficient embryos by the injection of a single dose of various retinoids, including the retinoidal benzoic acid derivatives, into the eggs. Similar effects can be observed *in vitro* by culture of 24 hr retinoid-deficient embryos on retinoid-repleted agar medium. The most striking early effect of the deficiency is the failure to form a functional vascular system as evidenced by the lack of development of the omphalomesenteric veins and arteries. Examination of thin sections has shown that the chambers of the heart are closed in the developing retinoid-deficient embryo (done in collaboration with U.I. Heine). Investigations of the effects of TGFs on this system are planned, as are hybridizations to study levels of oncogene expression.

The Laboratory of Chemoprevention continues to have a major interest in the role of retinoids in modifying the proliferative activity of cells and in the ultimate clinical use of retinoids as chemopreventive agents. Collaborative studies on the use of retinoids to prevent breast and bladder cancer in rats and mice continue to be pursued, and the Laboratory Chief continues to provide advice and consultation to various clinical groups that are interested in the use of retinoids to prevent skin, breast, and bladder cancer in men and women at high risk. The Laboratory of Chemoprevention is still involved in the screening of new retinoids that hopefully will have better pharmacological properties than existing agents. New agents have been synthesized at the BASF laboratories in Germany which have greatly altered chemical structure, and the cell biology of these agents is being investigated in our laboratories in Bethesda.

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

transformation now becomes more of a reality. The acquisition of a robot DNA synthesizer in our laboratory within the past few months and start-up of work directed at the first chemical synthesis of the human gene for TGF- $\alpha$  now indicates that we have begun meaningful scientific bench-work directed at the important goal of developing new antagonists of peptide growth factors.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05051-06 LC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Isolation of Polypeptide Transforming Factors from Cells

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

PI:	Anita B. Roberts	Staff Scientist	LC	NCI
Others:	Michael B. Sporn	Chief	LC	NCI
	Mario A. Anzano	Visiting Scientist	LC	NCI
	Paturu Kondaiah	Visiting Fellow	LC	NCI
	Sonia B. Jakowlew	Staff Fellow	LC	NCI
	Joseph M. Smith	Biologist	LC	NCI
	Nannette B. Roche	Biologist	LC	NCI
	Ursula I. Heine	Chief, Ultrastructural		
		Studies Section	LCC	NCI

## COOPERATING UNITS (if any)

Massachusetts Institute of Technology, Cambridge, Mass. (R.A. Weinberg and D.F. Stern)

## LAB/BRANCH

Laboratory of Chemoprevention

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

5.5

## PROFESSIONAL:

3.5

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

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

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

The purpose of the project is to determine the relationships between the mechanisms of action of three different classes of molecules each of which has been demonstrated to play a role in control of differentiation and/or neoplastic transformation of cells. The specific classes of molecules under investigation are: 1) polypeptide growth factors with emphasis on type alpha and beta transforming growth factors (TGFs) and platelet-derived growth factor (PDGF), each of which has been demonstrated to play a role in the maintenance of the transformed phenotype; 2) retinoids and other low-molecular weight effectors; and 3) oncogenes and their polypeptide products. Each of these classes of substances is known to affect gene expression of cells. Particular attention is directed at elucidation of the mechanism of action of the type alpha and beta TGFs in control of cell proliferation and phenotypic expression, since these polypeptides have been the focus of our laboratory's research for the past 4 years and since our laboratory is uniquely able to employ diagnostics such as receptor assays, immunoassays, and assays for messenger RNA for these peptides. Studies are aimed at three levels: in the whole embryo, in various organs and tissues, and in cell culture. Specific systems employed for these investigations include: 1) an experimental model for studying the effects of retinoids on avian embryogenesis utilizing retinoid-deficient Japanese quail embryos (Thompson, J.N. Br. J. Nutr. 23: 471-492, 1969); 2) comparison of oncogene and TGF expression in certain tissues of retinoid-deficient compared to normal hamsters; and 3) examination of the effects of retinoids and TGFs on the expression of oncogenes by certain cultured rodent and human cell lines. Electron microscopy and *in situ* hybridization will be used as adjuncts to biochemical determinations in the first two systems.

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

Anita B. Roberts	Senior Scientist	LC	NCI
Michael B. Sporn	Chief	LC	NCI
Mario A. Anzano	Visiting Scientist	LC	NCI
Paturu Kondafah	Visiting Fellow	LC	NCI
Sonia B. Jakowlew	Staff Fellow	LC	NCI
Joseph M. Smith	Biologist	LC	NCI
Nannette B. Roche	Biologist	LC	NCI
Ursula I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI

Objectives:

This project is directed towards a search for common elements or relationships in the mechanisms of action of transforming growth factors (TGFs), retinoids, and oncogenes (or oncogene products) in control of cellular proliferation and differentiation. Investigations are directed at the processes of embryogenesis, tissue repair, and neoplastic transformation. An effort will be made to clarify the mechanistic basis for the heterogeneous effects of both retinoids and TGFs on proliferation and neoplastic transformation and for the observations that interactions between retinoids and TGFs are antagonistic in certain situations and synergistic in others.

Methods Employed:

Our laboratory has purified to homogeneity the type  $\beta$  transforming growth factor and is currently making milligram quantities of this peptide from human platelets. In collaboration with Genentech, Inc., the complete amino acid sequence of type  $\beta$  TGF is being determined and a specific cDNA probe is being cloned. Receptor binding assays and radioimmunoassays for both type  $\alpha$  and  $\beta$  TGFs have been developed. Specific molecular probes have been made from clones provided by various investigators and include cDNA probes for TGFs, for many oncogenes, and for other marker peptides such as actin, collagen, and hemoglobin.

Methodology will include: utilization of classical biochemical assays for growth factor receptor levels and for growth factor production by cells, assays for proliferation of cells in monolayer culture and for growth of cells in semi-solid agar medium (used as a marker for in vitro transformation of fibroblastic cells), molecular hybridization including Northern and Southern blotting techniques, light microscopy and scanning and transmission electron microscopy (in collaboration with U.I. Heine), and the development of techniques for in situ hybridization using cloned cDNA probes.

Major Findings:

In certain cells, such as normal rat kidney fibroblasts (NRK cells), both type  $\alpha$  and type  $\beta$  TGFs must be present to effect phenotypic transformation as evidenced by the acquisition of the ability to form large colonies of cells in semi-solid agar medium. It has been shown previously that NRK cells transformed by sarcoma viruses secrete type  $\alpha$  TGFs and that this secretion is correlated with expression of the transformed phenotype in cells infected with temperature-sensitive mutants of the virus. It has now been demonstrated that these cells also secrete increased amounts of type  $\beta$  TGFs after sarcoma virus transformation and that the levels of TGFs secreted by the cells into the conditioned medium are consistent with an autocrine mechanism of transformation. It has also been shown for the first time that transformed cells secreting type  $\beta$  TGFs have a reduced number of cell surface receptors for that ligand, suggesting that ligand-induced down-regulation has occurred.

In NRK cells, retinoids and TGFs were shown to synergize to induce phenotypic transformation of the cells, as measured by the growth of the cells under anchorage-independent conditions. In new studies employing Fischer rat 3T3 cells transfected with the *myc* oncogene (done in collaboration with D.F. Stern and R.A. Weinberg, MIT), it has been shown that type  $\beta$  TGFs synergize with platelet-derived growth factor in the induction of anchorage-independent growth of the cells and that this effect of the peptides is completely blocked by concentrations of retinoic acid as low as one nanomolar. In contrast, when the anchorage-independent growth of these same cells is induced by epidermal growth factor (EGF) (a type  $\alpha$  TGF), retinoids have no effect. Like the retinoids, type  $\beta$  TGFs can also be shown to have opposite effects on these cells: TGF- $\beta$  stimulates the anchorage-independent growth of the cells when assayed in the presence of PDGF, but inhibits the anchorage-independent growth of the same cells when assayed in the presence of EGF. The molecular basis for the changing effects of both the retinoids and the type  $\beta$  TGFs on these cells is being investigated.

Preliminary results indicate that treatment of NRK cells with type  $\alpha$  and  $\beta$  may lead to altered expression of certain cellular oncogenes. This finding will be extended to the translational level by the use of monoclonal antibodies to specific oncogene products.

Initial results of the effects of retinoids on embryogenesis in the Japanese quail have shown that normal embryogenesis can be restored to retinoid-deficient embryos by the injection of a single dose of various retinoids, including the retinoidal benzoic acid derivatives, into the eggs. Similar effects can be observed *in vitro* by culture of 24 hr retinoid-deficient embryos on retinoid-repleted agar medium. The most striking early effect of the deficiency is the failure to form a functional vascular system as evidenced by the lack of development of the omphalomesenteric veins and arteries. Examination of thin sections has shown that the chambers of the heart are closed in the developing retinoid-deficient embryo (done in collaboration with U.I. Heine). Investigations of the effects of TGFs on this system are planned, as are hybridizations to study levels of oncogene expression.

Significance to Biomedical Research and the Program of the Institute:

It is clear from recent studies demonstrating the identity of PDGF and of the EGF receptor and known oncogene products that growth factors and their receptors occupy a central position in carcinogenic transformation of cells and in other non-neoplastic proliferative states in which oncogene expression has been shown to be transiently increased, such as embryogenesis and tissue repair. Understanding of the mechanisms leading to enhanced synthesis of growth factors and their receptors by cells and to control of the effects of growth factors by low-molecular weight effectors, such as the retinoids, is fundamental to our understanding of both non-neoplastic and neoplastic proliferative states.

Proposed Course:

Future work will continue to focus on relationships between the mechanisms of action of growth factors, oncogenes and retinoids on cells. Specifically, studies will center around the complete definition of the genomic clones for the type  $\alpha$  and type  $\beta$  TGFs with emphasis on the identification of promoter and enhancer elements. Attempts will be made to clone the rodent and chicken homologues to the human cDNA TGF clones so that they might be used as probes in investigations of transformation using rodent tissues and cell lines and in investigations of avian embryogenesis. In the latter system, attempts will be made to define a pattern of oncogene expression in normal embryogenesis and to compare that with the pattern observed in retinoid-arrested embryogenesis.

Publications:

- Anzano, M. A., Roberts, A. B., Smith, J. M., Sporn, M. B. and De Larco, J. E.: Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type  $\alpha$  and type  $\beta$  transforming growth factors. Proc. Natl. Acad. Sci. USA 80: 6264-6268, 1983.
- Assoian, R. K., Frolik, C. A., Roberts, A. B., Miller, C. M., and Sporn, M. B.: Platelet-derived transforming growth factor- $\beta$  controls receptor levels for epidermal growth factors in NRK-fibroblasts. Cell 36: 35-41, 1984.
- Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y. and Goeddel, D. V.: Human transforming growth factor- $\alpha$ : Precursor structure and expression in E. coli. Cell (In Press)
- Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blatcher, R., Pan, Y.-C. E., Stein, S., Lehrman, S. R., Smith, J. M., Lamb, L. C. and Sporn, M. B.: Purification and properties of a type- $\beta$  transforming growth factor from bovine kidney. Biochemistry 22: 5692-5698, 1983.
- Roberts, A. B., Frolik, C. A., Anzano, M. A., Assoian, R. K., Sporn, M. B.: Purification of type  $\beta$  transforming growth factors from non-neoplastic tissues. In Barnes, D., Sato, G., and Sirbasku, D. (Eds.): Methods in Molecular and Cell Biology. New York, Alan R. Liss, 1984, pp. 181-194.



Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M. and Sporn, M. B.: Antagonistic actions of retinoic acid and dexamethasone on anchorage-independent growth and epidermal growth factor binding of normal rat kidney cells. Cancer Res. 44: 1635-1641, 1984.

Sporn, M. B., Anzano, M. A., Assoian, R. K., De Larco, J. E., Frolik, C. A., Meyers, C. A. and Roberts, A. B.: Isolation and characterization of Type  $\beta$  transforming growth factors from human, bovine, and murine sources. In Cancer Cells, Vol 1. New York, Cold Spring Harbor Laboratory, 1984, pp. 1-4.

Sporn, M. B., Roberts, A. B. and Driscoll, J. S.: Growth factors and differentiating agents. In DeVita, V. T., Hellman, S. and Rosenberg, S. A. (Eds.): Cancer: Principles and Practice of Oncology, Second Edition. Pennsylvania, J.P. Lippincott Company (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05267-03 LC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Identification and Action of Platelet-derived Transforming Growth Factor-beta

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

PI: Richard K. Assoian Staff Fellow LC NCI

Others: Dorothea M. Miller Biologist LC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Human platelets were extracted with acid-ethanol and platelet-derived TGF-beta was purified from the extract by a two-column procedure using sequential gel filtration in the absence and then presence of urea. Purified TGF-beta is a protein of 25,000-daltons, and it is comprised of two 12,500-dalton subunits held together by disulfide bonds. The purified factor elicits its biological activity at concentrations less than 4pM. Comparative studies showed that platelets contain 100 times more TGF-beta than do other non-neoplastic tissues. Incubation of TGF-beta with NRK cells results in an increased number of cell surface EGF receptors. IGF-II receptors are not affected.

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

Richard K. Assoian	Staff Fellow	LC	NCI
Dorothea M. Miller	Biologist	LC	NCI

Objectives:

To examine the roles of bioactive peptides in modulating normal and neoplastic cell growth. Emphasis will be placed on 1) the isolation of a transforming growth factor (TGF) from platelets and 2) the mechanism by which platelet-derived TGF elicits a transformed phenotype in NRK-fibroblasts and smooth muscle cells.

Methods Employed:

Clinically outdated human platelets are extracted with acid-ethanol and the soluble peptides are precipitated with ether. The extract is purified by gel filtration, high pressure liquid chromatography and preparative gel electrophoresis. Biological activity is localized by use of an anchorage-independent growth assay with NRK-fibroblasts. Peptides are chemically localized by polyacrylamide gel electrophoresis in conjunction with silver staining and analytical radioiodination. Purified TGF- $\beta$  is incubated with cultures of NRK cells. EGF receptors are measured with a radio-receptor assay.

Major Findings:

Platelets are the major non-neoplastic source of TGF- $\beta$ . The platelet-derived factor has been purified to homogeneity and shown to be a protein of 25,000 daltons comprised of two 12,500 dalton subunits. Disulfide bonds are involved in maintaining the subunits in association. The amino acid analysis and structure of platelet-derived TGF- $\beta$  shows similarities to PDGF, but biologically the two proteins are distinct. TGF- $\beta$  is not a strong mitogen, but it does induce cells to grow in soft agar. Purified PDGF is highly mitogenic, but it has no transforming activity. TGF- $\beta$  in monolayer culture will increase the number of cell surface receptors for EGF.

Significance to Biomedical Research and the Program of the Institute:

The presence of a transforming growth factor in platelets (a non-neoplastic cell fragment) indicates that, in the non-neoplastic situation, stringent controls are operative in limiting the biological effects of this peptide. Comparative studies with non-neoplastic and neoplastic tissues will likely aid in defining these control systems.

Proposed Course:

The mechanism of action and biochemical effects of platelet derived TGF- $\beta$  will be examined with cell cultures of NRK-fibroblasts and smooth muscle. Cytoskeletal proteins and extracellular matrix proteins will be examined for alterations in response to TGF- $\beta$ .

Publications:

Assoian, R. K., Frolik, C. A., Roberts, A. B., Miller, D. M. and Sporn, M. B.: Transforming growth factor- $\beta$  controls receptor levels for epidermal growth factor in NRK-fibroblasts. Cell 36: 35-41, 1984.

Assoian, R. K., Grotendorst, G. R., Miller, D. M. and Sporn, M. B.: Three peptide growth factors from human platelets coordinating phenotypic transformation. Nature (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05396-01 LC

## PERIOD COVERED

February 1, 1984 to September 30, 1984

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

Development of Analogs for Study of Oncogenesis and Development of the Rat

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

PI: Shinichi Watanabe Senior Staff Fellow LC NCI

Other: Eliane M. Lazar Visiting Fellow LC NCI  
Gloria M. Sundaresan Chemist LC NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Chemoprevention

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.25

## PROFESSIONAL:

1.0

## OTHER:

0.25

## CHECK APPROPRIATE BOX(ES)

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

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

We are studying the molecular biology of TGF-alpha and TGF-beta for the relationship between structure and function. The chemically synthesized genes (normal and altered genes) will be expressed in bacteria or cultured cells to study the activity in vitro as well as in vivo. In vitro assays include measurement of soft-agar colony forming activity and receptor binding. The altered genes in retrovirus vectors will be inserted into rat embryos to study the function of these growth factors during normal development.

PROJECT DESCRIPTIONNames, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Shinichi Watanabe	Senior Staff Fellow	LC	NCI
Eliane M. Lazar	Visiting Fellow	LC	NCI
Gloria M. Sundaresan	Chemist	LC	NCI

Objectives:

We are studying the molecular biology of TGF- $\alpha$  and TGF- $\beta$  in order to find the domains of these molecules which are required for their interaction with their receptors. The effects of alterations on TGF- $\alpha$  and TGF- $\beta$  will be examined in both in vitro cell culture systems and in vivo animal systems. The possibility for the use of analogs to reverse and/or prevent transformed (cancerous) cells to normal cells is a major goal.

Methods Employed:

The modification of TGF- $\alpha$  and TGF- $\beta$  genes will be carried out by oligonucleotide mutagenesis on molecularly cloned genes in the M13 vector. The altered genes will be expressed in several systems including animal cell culture. The altered gene products from host cells will be examined for their activity to induce anchorage-independent growth of indicator cells in soft agar, for activity to bind to receptors, and for amino acid composition. Embryo transplantation techniques also will be used to study the role of TGF- $\alpha$  and TGF- $\beta$  during development of the rat.

Major Finding:

The automated DNA synthesizer is being used to construct oligonucleotides in order to synthesize a gene. Early results demonstrate usefulness of this instrument for synthesis of the gene for TGF- $\alpha$ .

Significance to Biomedical Research and the Program of the Institute:

Although the levels of TGF- $\alpha$  and TGF- $\beta$  are elevated in cells transformed by various carcinogens, the role of these growth factors in carcinogenesis is not known. The development of analogs for TGF- $\alpha$  and TGF- $\beta$  will be very useful tools to examine the role of the growth factors during carcinogenesis. The analogs will be useful for understanding the growth factor-receptor interaction at the molecular level. The role of TGF- $\alpha$  and TGF- $\beta$  during developmental processes of an animal is not known. Embryo transplantation after introduction of altered genes will provide valuable information. The overall information obtained with analogs of TGF- $\alpha$  and TGF- $\beta$  will help to develop practical methods to reverse/prevent transformation.

Proposed Course:

The project described here has several objectives in the field of basic molecular biology of growth factor-receptor relationships and growth factors in developmental process of animals as well as the development of antagonists to the TGF- $\alpha$  and TGF- $\beta$  for controlling cancer. The automated DNA synthesizer will be used extensively to mutagenize the gene synthesized from the amino acid sequence or cDNA clones. The mutagenized genes will be expressed in several host systems to study the activity in vitro as well as in vivo. In vitro assay includes the soft-agar colony forming activity and receptor binding assay. In vivo assay will be carried out in rat embryo. The gene in retrovirus vectors will be introduced into rat embryos to study the effect of altered growth factors on developmental processes.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05397-01 LC

PERIOD COVERED  
 October 1, 1983 to September 30, 1984

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
 Role of Ectopic Peptides in Phenotypic Transformation

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

PI:	Joseph E. De Larco	Research Chemist	LC	NCI
Others:	Dennis Pigott	Visiting Fellow	LC	NCI
	Jan E. Lazarus	Biologist	LC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

A cell line was derived from a metastatic, human melanoma. It is producing peptides that can stimulate an untransformed cell line to reversibly express a transformed phenotype. This transformed phenotype is expressed in monolayer as a disorganized growth pattern and in an anchorage-independent growth (AIG) assay as colonies forming in soft-agar from single seeded cells. One of the peptides in this mixture contributing to this activity is an epidermal growth factor-like growth factor that has a molecular weight of approximately 26,000 daltons as determined by gel permeation chromatography on Bio-Gel P-30 in 1 M acetic acid. This appears to be present at concentrations between 210 and 1,000 nanograms/ml. This EGF-like activity requires a second peptide to efficiently stimulate AIG. The second required peptide in this mixture has a modulator activity in that it appears to require the presence of either EGF or an EGF-like activity to efficiently stimulate large colony formation. This TGF-beta activity has an apparent molecular weight of approximately 21,000 daltons. Both of these peptides have physical properties that are distinctly different from those previously reported for the TGFs. These "ectopic" peptides may play a role in the expression of the transformed phenotype of the tumor cells producing them. Molecular clones have been derived to determine which genes are regulated by these growth and modulating factors. This knowledge will increase our understanding of the role these factors play in the expression of the transformed state.



PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

Joseph E. De Larco	Research Chemist	LC	NCI
Dennis Pigott	Visiting Fellow	LC	NCI
Jan E. Lazarus	Biologist	LC	NCI

Objectives:

One of the primary objectives is the production of immunological reagents against the epidermal growth factor-like growth factors and the modulators released by a human melanoma cell. Taking advantage of a melanoma cell line that produces a very large quantity of an unusually high molecular weight EGF-like growth factor that may be analogous to that released in the urine of cancer patients, we will isolate this material from serum-free media conditioned by these melanoma cells in vitro. Enough of this material will be isolated for amino acid sequence determination and the production of immunological reagents. Knowing the amino acid sequence, antisera can be generated against peptide sequences that are specific to this particular EGF-like growth factor. With specific reagents it will be possible to determine if these large molecular weight growth factors are more closely related to human EGF (urogastrone) or the transforming growth factors (TGFs). The possibility of using these reagents as diagnostic tools to determine if there are any qualitative or quantitative differences in the EGF-like peptides expressed in patients having certain malignancies will be examined using urine samples from a variety of patients. The possibility of using these antibodies as therapeutic agents will also be explored. Specific, high titered antibodies against these factors will allow one to examine for the expression of these factors under normal development and physiological conditions. These reagents, along with cell culture systems, will be used to determine if there is a correlation between the expression of these genes, or closely related genes, and the transformed phenotype.

Along similar lines the cellular genes that are regulated by these growth and modulation factors will be isolated using molecular cloning techniques. These isolated clones will be used to study the expression of the transformed phenotype both in vitro and in vivo. They will also be used to study the developmental processes and basic mechanisms for growth control.

Cell clones have been isolated to determine what genes must be expressed for the indicator cells to manifest the transformed phenotype when treated with the growth and modulatory factors released by transformed cells.

### Methods Employed:

Tissue culture methods are used for both the production of growth and modulatory factors as well as for assaying these activities. The tissue culture methods include roller bottles for isolation of both serum-free conditioned media and mRNAs. Mitogenic and soft agar assays are used to quantitate and characterize the ectopic factors released by transformed cells. Standard biochemical methods are used to isolate the factors released by the transformed cells. These include centrifugation, high pressure liquid chromatography column chromatography, gel exclusion chromatography, ion exchange chromatography and polyacrylamide gel electrophoresis. Molecular biological techniques are used to clone the regulated genes. These methods include the purification of poly(A) containing mRNA, the cloning of the poly(A) containing RNA and the characterization of the individual clones using the appropriate probes.

### Major Findings:

Melanoma cells: Single cell clones were picked from a human melanoma cell line that had been established from a surgically isolated metastatic tumor. The individual clones differed from one another with respect to their growth properties and the amount of "ectopic" peptide growth factors they released. The major growth factor released by these cells is an EGF-like peptide that is often referred to as a TGF, TGF- $\alpha$  or TGF-I. Another peptide factor was also isolated from these cells. It had a modulatory activity, but was not mitogenic in these assays. This modulating activity is also referred to as TGF- $\beta$  or TGF-II. The production of TGFs appears to be related to the growth properties of the individual clones. The clones that grew better in agar also released more growth and modulating factors. These cells are producing much more growth factors than previously reported human melanoma lines. In the literature it has been reported that human melanoma cells release between 10 and 20 nanograms of an EGF-like peptide per liter of conditioned media. These cells, however, release between 210 and 1,000 nanograms of a EGF-like peptide per liter of conditioned media. The EGF-like or TGF- $\alpha$  activity from these cells has an apparently larger molecular weight than had previously been reported for melanoma cells. Its apparent molecular weight is approximately 26,000 compared to 7,000 for the TGF- $\alpha$  reported for other melanoma lines. This molecular weight appears to be analogous to the peptides found in the urine samples from cancer patients that have certain types of tumors.

This could be a very useful cell line for the production of immunological reagents to be used as diagnostics in the early detection of malignant tumors. It can supply antigens for the production of antibodies for screening urines of potential cancer patients for the presence of these or similar peptides. Antibodies to these peptide growth factors released by the melanoma cells may be very useful as diagnostic tools for the detection of certain malignancies. This melanoma line also will be used to study the biochemistry and the cellular biology of the transformed cells producing these factors.

These cells produce other modulating factors that have not been characterized as yet. These modulating activities appear to contribute to the transformed phenotype. This can be seen in the indicator cells when they are treated with serum-free conditioned media from these melanoma cells. These modulators are being examined presently for their ability to influence the metastatic potential of transformed cells. An understanding of the mechanism of action of these factors and/or the ability to control their expression would be very useful in the treatment of malignant melanomas. The ability to prevent the migration of tumor cells from the primary tumor would, hopefully, minimize the metastatic potential of tumors in vivo.

Proximal Effectors of the Transformed Phenotype: It has been observed that when serum-free conditioned media from either murine sarcoma virus-transformed cells or human melanoma cells is added to the media in which untransformed indicator cells (49F) are growing, they express the transformed phenotype. This phenotype is expressed if the cells are treated with inhibitors of DNA synthesis. The transformed phenotype, however, is not induced if the cells being treated with the serum-free conditioned media are treated with inhibitors of either RNA or protein synthesis. This suggests that the cells must synthesize mRNA and protein before expressing the transformed phenotype. Similar phenotypic transformation is obtained if the 49F cells are treated with EGF and purified TGF- $\beta$  from platelets. It is assumed that the combination of these two factors turns on the expression of a "silent" gene(s) that is normally not expressed by these cells. This required "silent gene" or series of genes may have the ability to cause the cells to express the transformed phenotype. This property would make the product(s) from this gene(s) a more proximal effector(s) of the transformed phenotype or transformation. The identity of these "proximal effectors of transformation" would therefore be of great interest both from the point of understanding transformation as well as the point of understanding the regulatory mechanisms for growth and differentiation. To accomplish this task the "silent gene(s)" will be cloned from the mRNAs of cells that have been stimulated. These cDNA clones will be used to try to dissect out the steps in transformation. At present a series of cellular clones of the original indicator cells, 49F, have been isolated for this purpose. The cDNA clones of the "proximal effectors" will also be used to examine transformed and untransformed cells from in vitro and in vivo sources. These clones will be helpful in establishing the expression of the regulated genes during development and the progression of the transformed phenotype.

#### Significance to Biomedical Research and the Program of the Institute:

This work is both significant and of high priority because the results from these experiments will give information that will hopefully help in defining the factors responsible for the uncontrolled proliferation and altered social behavior expressed by malignant cells. The data collected from the human melanoma system would appear to be directly applicable for the development of diagnostics for human malignancies. The in vitro system developed will allow the rational evaluation of methods for early detection and screening of possible therapeutic agents for the treatment of these malignancies. Two classes of therapeutic agents can readily be screened using these systems. The first consists of those that suppress the production or release of the factors

responsible for the expression of the transformed phenotype. The second includes those agents that decrease the response of the tumor cells to the "ectopic" factors. These goals are consistent with those of the Laboratory of Chemoprevention, the Division and the NCI. It is hoped the information gained also will add to our basic knowledge of growth control and differentiation. This knowledge will provide methods and rationale that can be applied to patient care for both early diagnosis and treatment.

#### Proposed Course:

This project represents an integrated approach to understanding the roles of the "ectopic" growth and modulatory factors in the expression of the transformed phenotype. Future studies are to proceed in a logical manner using the basic knowledge already accumulated. This affords the opportunity to prepare immunological and molecular biological reagents to study the control mechanisms for the expression of these factors during normal growth and differentiation, as well as during malignant transformation. Immunological reagents developed will be used to monitor the expression of the "ectopic" growth and modulatory factors by malignant tumors in cancer patients before and during treatment. With the cellular clones isolated the cellular and molecular biology of the expression, production and release of EGF and EGF-like peptides will be examined. The development of molecular probes for these peptides should afford insight into the normal expression and function of these potent mitogens.

#### Publications:

Anzano, M. A., Roberts, A. B., Smith, J. M., Sporn, M. B. and De Larco, J. E.: Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type  $\alpha$  and type  $\beta$  transforming growth factors. Proc. Natl. Acad. Sci. USA 80: 6264-6268, 1983.

Carpenter, G., Stoscheck, C. M., Preston, Y. A. and De Larco, J. E.: Antibodies to the EGF receptor-kinase block the biological activities of sarcoma growth factor. Proc. Natl. Acad. Sci. USA 80: 5627-5630, 1983.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1CE05398-01 LC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Functional Characterization of Transforming Growth Factors and Their Receptors

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

PI: Charles A. Frolik Chemist LC NCI

Other: Lalage M. Wakefield Visiting Fellow LC NCI

Linda L. Dart Biologist LC NCI

Diane M. Smith Biologist LC NCI

## COOPERATING UNITS (if any)

Genentech, Inc., South San Francisco, California (C. Benton)

## LAB/BRANCH

Laboratory of Chemoprevention

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

3.4

## PROFESSIONAL:

1.6

## OTHER:

1.8

## CHECK APPROPRIATE BOX(ES)

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

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

Transforming growth factors (TGFs) are acid-stable polypeptides that induce a reversible phenotypic transformation of normal indicator cells such that they will grow in an anchorage-independent manner in soft agar, a property characteristic of transformed cells. The purpose of this project is to determine the role that endogenously-produced TGFs may play in the growth of normal and transformed cells and to characterize their mode of action at a biochemical level. To this end, polyclonal antisera have been raised to the TGFs and development of monoclonal antibodies is in progress. The effects of these antibodies on the anchorage-independent and independent growth of normal and transformed cells are being investigated and will be analysed in terms of the biochemical functions affected. Since the first step in the interaction of TGFs with the cell is binding of the growth factor to the cell surface, initial investigations are concentrating on the characterization of cell surface receptors for TGFs. Development of radioreceptor assay for TGF-beta, a TGF that depends on a second TGF (EGF or TGF-alpha) for activity, has allowed identification and preliminary characterization of a specific high affinity receptor for TGF-beta on normal rat kidney cells. A similar receptor has been found on all normal and transformed cell lines studied so far. Receptor expression in normal cell lines (NRK and NIH-3T3) is modulated by infection of the cell with acute transforming retroviruses (HSV and MoSV) and by transformation with certain oncogenes (myc, H-ras). Human cell lines and tissues are being screened for an abundant source of the receptor for use as a starting material for receptor purification and as an immunogen for the production of anti-receptor monoclonal antibodies. Further characterization of the role of endogenously-produced TGFs and their interaction with the cell surface receptor should help elucidate the role these molecules may play in the process of carcinogenesis.

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

Charles A. Frolik	Chemist	LC	NCI
Lalage M. Wakefield	Visiting Fellow	LC	NCI
Linda L. Dart	Biologist	LC	NCI
Diane M. Smith	Biologist	LC	NCI

Objectives:

The purpose of this project is to examine the role of transforming growth factors (TGF) in the control of normal cell growth and in the process of malignant transformation. Anti-TGF antibodies are being employed to investigate the involvement of endogenously-produced TGFs in growth control in normal and transformed cells. The mechanism of action of these growth factors is being studied at a biochemical level with particular emphasis on the initial interaction of the TGFs with the cell surface receptor; the first step is the cascade of events leading to expression of the transformed phenotype. With a greater understanding of the importance and mechanism of action of TGFs in transformation, 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 with essentially no loss of biological activity. This iodinated material is used in a radioreceptor assay to characterize TGF- $\beta$  binding to a variety of cell lines in culture. Binding data are subjected to Scatchard analysis, where necessary using the "Ligand" computer program of Munson and Rodbard (Munson, P. J. and Rodbard, D. Anal. Biochem. 107: 220-239, 1980). Polyclonal antisera to TGF- $\beta$  have been raised in rabbits and monoclonal antibodies against this growth factor are being developed in collaboration with Dr. C. Benton of Genentech, Inc. Immunoglobulin fractions from the antisera are prepared by affinity chromatography and antibody affinity is determined by radioimmunoassay. The ability of cells to grow is 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 is measured using an Omnicon image analyser. 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 TGFs to the cell membrane initiates the chain of events leading to cell transformation, so TGF-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. The normal rat kidney (NRK) cell line which responds to TGF- $\beta$  (in the presence of epidermal growth factor or TGF- $\alpha$ ) by growth in soft agar was

shown to possess a specific high affinity receptor for TGF- $\beta$ . There are 17,000 such receptors per cell, with a Kd of 25-30 pM. Other growth factors such as platelet-derived growth factor, EGF, TGF- $\alpha$ , insulin and insulin-like growth factors I and II do not compete for binding to this receptor, so unlike TGF- $\alpha$  which binds to the previously characterized EGF receptor, TGF- $\beta$  appears to bind to a distinct receptor. Binding of TGF- $\beta$  is a time and temperature-dependent process and at 37° the bound ligand is rapidly internalized and degraded in the lysosomes. TGF- $\beta$  will down-regulate its receptor to a maximum of 30-50% of the levels initially observed, in contrast to EGF and TGF- $\alpha$  which can fully down-regulate their receptor. Similar high affinity receptors have been found on all cell lines assayed so far with the rodent cell lines, NIH-3T3 and Swiss 3T3, expressing the highest number of receptors, at 60,000-90,000 per cell. The human cell lines studied so far have many fewer receptors (less than 10,000 per cell) and there is no obvious correlation between the number or affinity of the receptor and the tissue source of the cell line (normal or tumor, adult or embryonic) or the ability of the cell to grow in soft agar.

#### Modulation of receptor expression

Transformation of rodent cell lines by acute transforming retroviruses was shown to cause a marked decrease in the number of TGF- $\beta$  receptors. Transformation of NRK cells by MoSV results in a 50% decrease in TGF- $\beta$  binding and transformation of NIH-3T3 by HaSV gives an 80% decrease in the number of receptors, but an increase in receptor affinity from a Kd of 68 to 27 pM. This decrease in receptor number correlates with increased expression of TGF- $\beta$  by the transformed cells and is probably due to receptor down-regulation. A similar effect has been observed in virally transformed cells that secrete TGF- $\beta$  or PDGF, where there is an accompanying loss of assayable cell surface receptors for these ligands. Similarly, transfection of Fischer rat 3T3 cells with the oncogenes myc and ras cause a decrease in TGF- $\beta$  receptor number, with a concomitant decrease in affinity in the case of ras. Thus TGF- $\beta$  receptor levels appear to be modulated by the expression of certain oncogenes and these studies will be expanded to include other oncogenes.

#### Antibodies to TGF- $\beta$

Polyclonal antibodies have been raised against human platelet TGF- $\beta$ . Immunoglobulin fractions have been prepared from this serum by affinity chromatography on protein A-sepharose. These antibodies effectively block binding of iodinated TGF- $\beta$  to its receptor and inhibit the transforming effect of exogenously-added TGF- $\beta$  on NRK cells (in combination with EGF) as measured by the soft agar growth assay. These antibodies are now being studied for an effect on anchorage-dependent and independent growth of cells that synthesize TGF- $\beta$  to determine the role of endogenous TGF- $\beta$  in growth control. Preliminary results suggest higher affinity antibodies may be required for these studies and work is under way to produce new antibodies, both poly- and monoclonal, against TGF- $\alpha$  and TGF- $\beta$ .

Significance to Biomedical Research and the Program of the Institute:

Characterization of the roles of TGFs in initiating or maintaining the transformed phenotype, and their mechanism of action and relationship to normal growth factors in both normal and tumor cells, will give an insight into cellular growth control processes that may provide a rational basis for the development of effective chemotherapeutic agents for the inhibition of carcinogenesis.

Proposed Course:

Future research will continue on two main areas. The first involves the use of anti-TGF antibodies to determine what role endogenously-produced TGFs play in controlling growth and phenotype of normal and transformed cells. It is anticipated that the various TGFs may have different regulatory functions in different cell types. Since the first step in the mechanism of TGF action is their interaction with the cell surface receptor the other major research interest will be to characterize these receptors further. Particular emphasis will be placed on raising monoclonal antibodies against the TGF- $\beta$  receptor for use in an affinity column for the purification of the receptor. The current Principal Investigator has left the NCI and Dr. Lalage Wakefield will be taking over the responsibilities as the Principal Investigator.

Publications:

- Assoian, R. K., Frolik, C. A., Roberts, A. B., Miller, D. M. and Sporn, M. B.: Platelet-derived transforming growth factor- $\beta$  controls receptor levels for epidermal growth factors in NRK-fibroblasts. Cell 36: 35-41, 1984.
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- Frolik, C. A., Roller, P. P., Cone, J. L., Dart, L. L., Smith, D. M. and Sporn, M. B.: Inhibition of transforming growth factor-induced cell growth in soft agar by oxidized polyamines. Arch. Biochem. Biophys. 230: 93-102, 1984.
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ANNUAL REPORT OF  
THE LABORATORY OF COMPARATIVE CARCINOGENESIS  
NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

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 and Achievements: At the beginning of this fiscal year, Dr. Arnold Fowler, Chief, Developmental Biology and Biochemistry Section, resigned his position in order to accept an appointment in the Food and Drug Administration. The two staff members remaining in the Section are being transferred to the Office of the Chief, LCC, to form the nucleus of a newly established Developmental Biology Working Group, and the Section will be disestablished.

Also at the beginning of this fiscal year, the Chemistry Section was established in the LCC. The program of this group, formerly the Analytical Chemistry Section, Laboratory of Carcinogen Metabolism, is primarily devoted to the chemistry and biochemistry of carcinogenic N-nitrosamines. It brings to the LCC state-of-the-art expertise in organic synthesis and structure determination, and will collaborate with the more biologically oriented Sections in projects requiring a high degree of chemical expertise.

Two other events have occurred this year that merit special mention:

"Use of Small Fish Species in Carcinogenicity Testing," the proceedings of a Division of Cancer Etiology-sponsored Symposium held at NIH during December 8-10, 1981, has appeared as NCI Monograph 65 edited by Dr. Karen Hoover of the Nutrition and Metabolism Section.

Dr. Jerrold Ward, Chief, Tumor Pathology and Pathogenesis Section, received the Food and Drug Commissioner's Special Citation, for "exceptional ability in dealing with a highly complex problem of pathology which led to scientifically sound regulatory policy of great public health significance."

Summary Report: The Laboratory of Comparative Carcinogenesis provides a major focus within the Carcinogenesis Intramural Program for studies on the mechanisms of chemical carcinogenesis that involve primary neoplasia in animals as experimental end points. An increasing volume of evidence continues to support the hypothesis that for many, if not most tissues, transient exposure to chemical carcinogens either before or after birth may be necessary but is not sufficient to elicit tumor development. The widely differing patterns of organ specificity

that frequently occur in experimental carcinogenesis in different species, even even in studies with direct-acting agents that are independent of cellular metabolism, are in many cases not explicable on the basis of toxicodynamics, nor on the basis of differential capacity to repair damage in different tissues. These findings complicate efforts at human risk assessment based on the extent of reaction or persistence of binding products of carcinogens in known animal and putative human target tissues. In addition, more and more agents are being identified that cause tumors in experimental rodents but do not react chemically with cellular constituents including DNA, i.e., are not genotoxic. The fact that potential tumor cells may remain latent for large fractions of a lifetime in experimental animals, and that increasing numbers of nongenotoxic agents are being discovered which promote proliferation of such latent cells to form preneoplastic lesions that progress to neoplasia suggest that the phenomenon of tumor promotion may be of great significance for the genesis of human cancer.

There is, at present, no unifying hypothesis for the general mechanism of action of tumor promoters other than the ill-defined concept that such agents reduce intercellular communication. Furthermore, most experimental studies on tumor promotion in specific tissues or organ systems have focused on one, or at most two species, and the empirical data base from which mechanistic hypotheses of tumor promotion will eventually emerge remains very narrow. Accordingly, there is no certainty at present that agents identified as promoters in rodent tissues will have similar effects in other species, including man. In order to expand the limited data base on organ specificity and interspecies correlations in tumor promotion, a major coordinated program has been established in this Laboratory to identify previously unsuspected promoting agents; to establish rigorously the limits of cellular specificity for tumor promotion by specific agents; to compare dose/effect relationships from one species to another, including both rodent and nonhuman primate species; and to utilize these data in mechanistic investigations on the phenomena of tumor promotion.

This is particularly relevant to findings that certain drugs that are prescribed on a long-term basis, among them a number of sedatives, tranquilizers, anticonvulsants, and hypolipidemics, are potent promoters for certain tissues in experimental animals. Both phenobarbital and barbital have now been confirmed to promote not only in the rat and mouse liver but the rat thyroid as well, and barbital is reported this year by the Perinatal Carcinogenesis Section to promote carcinogenesis in rat renal cortical epithelium also. Phenobarbital has been shown by the Primate Working Group to be the first agent to promote carcinogenesis in a nonhuman primate species. In fact, it is a testable hypothesis, now being systematically investigated in this Laboratory, that tumor-promoting activity is associated with long-acting sedative/anticonvulsant activity among barbiturate derivatives, and that a wide spectrum of cellular and organ specificities in tumor promotion by these agents varies with the nature and pattern of substitution at position C-5 on the heterocyclic barbituric acid ring. While prolonged exposure to these agents appears as of now to be required for promotion, this has been shown not to be true of all classes of tumor promoters. The Tumor Pathology and Pathogenesis Section has demonstrated that brief exposures to certain phorbol esters or to dioctyl phthalate are irreversibly promoting in mouse skin and liver, respectively, so that interruption of exposure may not be always a useful strategy for primary cancer prevention, if and when chemical promotion is involved. Accordingly, comparative studies on tumor promotion continue to be a major theme of the research in the different Sections within the LCC.

Major research programs on metabolic determinants of transplacental carcinogenesis in rodents and nonhuman primates, on carcinogenesis by salts of heavy metals, on the chemistry and biochemistry of nitrosamines, and on the role of oncogenes in chemical carcinogenesis 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 Working Group has continued its studies on transplacental carcinogenesis and tumor promotion in nonhuman primates. In two independent series of experiments, it has been conclusively shown that phenobarbital, given in the drinking water after direct or transplacental exposure to N-nitrosodiethylamine (DEN) and after an interval of as long as 4 years, can selectively promote hepatocellular carcinogenesis in the patas monkey. These findings demonstrate that DEN, like the direct-acting alkylating agent, nitrosoethylurea (ENU), is a transplacental carcinogen in this nonhuman primate and that the transformed hepatocytes which result from prenatal exposure to DEN may persist, latent, for years after exposure to the carcinogen has ceased. The findings with phenobarbital also provide a positive control compound for further studies of hepatocellular tumor promotion in the patas monkey or other nonhuman primate species, and strongly suggest the potential importance of tumor promotion during carcinogenesis in epithelial cells of primates in general, including man.

New primary neoplasms continue to appear in patas and in rhesus monkeys exposed transplacentally to ENU, as long as 10 years postnatally (middle-aged adulthood) in patas monkeys and more than 5 years postnatally in rhesus monkeys (young adulthood). It is increasingly evident that in primates as in rodents, neoplastic development can occur late in life as a consequence of transplacental exposure to a chemical carcinogen. Recent identification of primary epithelial tumors of the peripheral lung in patas monkeys prenatally exposed to ENU and surviving to adulthood without further treatment is additional evidence that neoplasms of lining epithelia--characteristically the predominant forms of human cancer--can result from transient prenatal initiation by chemical carcinogens.

The induction of rapidly fatal gestational choriocarcinoma in pregnant patas monkeys given ENU suggests a correlation between choriocarcinoma and pediatric neoplasia which may exist in human populations if environmental carcinogens play a role in causation of these tumors in man and which may be testable by epidemiologic techniques. The frequent occurrence of acute aflatoxicosis in human populations in many areas of the world that are also noted for a high incidence of choriocarcinoma, including subsaharan Africa, the Indian subcontinent, and tropical/subtropical regions in or bordering the Pacific Ocean suggests the experimentally testable possibility that mycotoxins may play an etiologic role in human trophoblastic disease, at least in some regions. In association with the Environmental Epidemiology Branch, DCE, these questions will continue to be explored, initially in the context of the forthcoming World Conference on Trophoblastic Neoplasia.

The Nutrition and Metabolism Section (1) investigates the effects of dietary constituents on target tissue susceptibility to chemical carcinogenesis, (2) carcinogenic processes, and (3) investigates the role of physiologically essential divalent metals in protecting against metal carcinogenesis.

The Section has focused its interest on the role of the lipotropes, methionine, choline, vitamin B<sub>12</sub> and folic acid in chemical carcinogenesis. The chronic administration of diets devoid of methionine and/or choline has been shown to promote the formation of hepatocellular carcinomas in the livers of rats initiated with DEN. Diets devoid of both methionine and choline enhanced tumor formation more than did the diets singly devoid of either methionine or choline. Administration of diets devoid of both methionine and choline led to the formation of metastatic hepatocellular carcinomas and to an abnormal hepatization of pancreatic acinar cells in rats, even in the absence of any further treatment with hepatocarcinogens. Chronic feeding of diets devoid of both methionine and choline, or containing the carcinogenic methionine antagonist ethionine, decreased the hepatic levels of both S-adenosylmethionine and of 5-methyldeoxycytidine in DNA. These results are consistent with the hypothesis that, in rat liver at least, methyl insufficiency can play a major role in carcinogenesis. Further evidence that methyl insufficiency exerts a causative role in hepatocarcinogenesis is obtained with mice. Ethionine demonstrated hepatocarcinogenic activity in three strains of mice. Tumor promotion and causation by phenobarbital in C3H mice were inhibited in animals fed high dietary levels of methionine and choline. Finally, deazaadenosine, an inhibitor of physiological methylation, transformed rat liver epithelial cells in culture.

These results provide reasonable evidence that a physiological insufficiency of methyl donors, possibly acting via hypomethylated DNA, contributes significantly to hepatocarcinogenesis in rodents. Results from this and other laboratories have shown clear associations between methyl insufficiency, DNA hypomethylation and tumor formation, even in humans. Establishment of a causal relation between methyl insufficiency and tumor formation in a variety of tissues and cell types would be of major significance in understanding the etiology of cancer.

Recent results from the Section have shown that the physiological effects of carcinogenic divalent metals are often inhibited by the physiologically essential divalent metals, calcium, magnesium and zinc. Thus magnesium administered in vivo along with the carcinogenic metal inhibits lung adenoma formation in strain A mice by lead and nickel and sarcoma production in rats by nickel and cadmium. The inhibition by magnesium of carcinogenesis by metals appeared to result in part from the reduced uptake and retention of the carcinogenic metal in the target tissue. Calcium injection also inhibits lung adenoma in strain A mice by nickel and lead. High dietary levels of calcium and magnesium exerted no significant effects on the formation of injection site sarcomas and of interstitial cell tumors in cadmium-treated rats. In other studies the chemical binding of cadmium to DNA was found to be inhibited by calcium, and magnesium, as well as by the cadmium antagonist, zinc. These results indicate a frequent, but not universal, inhibition of metal carcinogenesis by the physiologically essential divalent metals. Such inhibition results in part from altered metabolism of the carcinogenic metal and may be associated with a decreased binding of the carcinogen to DNA. In general, the area of metals carcinogenesis can be described as lacking (1) sufficient members of research groups investigating the problem, (2) adequate model biological systems in which the effects of organic

and inorganic carcinogens may be compared, and (3) reasonable evidence regarding the critical site(s) of attack of the carcinogen in the target tissue. In view of the widespread occurrence of metal carcinogens and of the great activity of some, investigations on their mode of action are important. The use of physiological metal antagonists may be expected to help specify the cellular sites of activity of the carcinogenic metals. The present studies will be expanded by examining the effects of essential metal deficiency on metal carcinogenesis.

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 relationship of cellular differentiation to perinatal susceptibility to chemical carcinogens and to the consequent development of neoplasia.

Prenatal exposure to chemical carcinogens, followed by postnatal application of tumor promoters, can result in tumor formation at sites where no tumor would occur in the absence of promotion. Organ specificity in transplacental carcinogenesis may therefore be more apparent than real, as latent tumor cells may persist in many tissues. To investigate this hypothesis, two major biological projects initiated last year have been continued and expanded. Mutation assays for resistance to thioguanine, ouabain, and diphtheria toxin have been successfully applied to primary cell cultures from whole embryos and from specific organs from conceptuses of several rodent species exposed to metabolism-dependent or direct-acting carcinogens at various precisely defined periods during gestation. Mutant recovery has been high enough to allow quantitative comparisons of the mutagenic effects of a given agent in different organs or tissues, and of sequential changes in susceptibility of an organ or tissue during prenatal development. This program will allow comparison of unequivocal genotoxic effects (mutation) with organ-specific carcinogenesis and should serve to identify organs where mutagenesis occurs, but where tumors do not develop.

A second approach is to expose animals given carcinogens prenatally to tumor promoters during postnatal life in order to reveal the presence of potentially latent neoplastic cells in apparently resistant tissues. Exposure of F344 rats to phenobarbital after prenatal initiation by nitrosomethylurea caused multiple thyroid follicular adenomas and carcinomas and multiple preneoplastic foci in the liver, thus radically modifying the apparent organ specificity of the transplacental carcinogen for the nervous system and kidneys by promotion of lesions in tissues known to be susceptible to phenobarbital.

The role of fetal versus maternal metabolic activation of metabolism dependent transplacental carcinogens has been studied using methylcholanthrene (MC) in mice and procarbazine (PCZ) in rats. In a pharmacogenetic study in mice, genetic backcrosses were made to obtain, in the same litter, fetuses which were either inducible or noninducible for the mixed function oxidases that metabolize MC. Inducible fetuses developed a significantly (two to three times) higher incidence of lung tumors than did those of the noninducible genotype. This is the first direct demonstration of a determining role of enzyme inducibility in fetal susceptibility to a carcinogen. Conversely, administration of the transplacental carcinogen, PCZ, to gravid rats resulted in O-6 and N-7-methyl guanine adducts in both fetal and neonatal tissues that could not be detected in the corresponding

tissues of newborn rats given PCZ directly by injection. This observation constitutes evidence that maternal metabolic activation of the carcinogen is crucial for transplacental carcinogenesis by this agent. Systematic investigation of the role of the gene, *nu*, a cause of genetically determined thymic aplasia and T-cell deficiency in mice and rats, has shown that athymic doubly recessive nude (*nu/nu*) mice are approximately one decimal order of magnitude more susceptible than phenotypically normal (*nu/+*) heterozygous littermates with respect to skin carcinogenesis by a variety of carcinogens, with or without tumor promotion.

Finally, an assay for functional repair of DNA damaged by mutagenic and carcinogenic agents or rendered partially apurinic by mild acid hydrolysis has been improved. In vitro repair of lac operon-containing bacteriophage DNA, using 300,000 x g supernatants from wild type *E. coli* plus all four deoxyribonucleoside triphosphates, has been accomplished and is being extended to the use of comparable homogenates of fetal tissues and dissociated specific cell types to explore ontogeny of DNA capacity during prenatal development.

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 National Cancer Institute and other U.S. Federal Government agencies.

The pathology and biology of experimentally induced and naturally occurring neoplasms of rodents are characterized and compared. Pathology and histogenesis of individual tumor types are investigated with the use of serial sacrifice studies, immunocytochemistry, automated image analysis with stereology, conventional light microscopy, ultrastructure and histochemistry. Computerized image analysis of early and late induced focal proliferative lesions was used, together with stereologic techniques, to demonstrate that small focal hyperplastic lesions progressively grew in size to develop into adenomas and carcinomas. Detailed histogenesis investigations were performed for mouse, rat, hamster and monkey liver, rat thyroid gland, mouse and rat lung, and rat pituitary gland. Hepatocellular carcinomas were found to originate in all species from initial focal proliferative hepatocellular lesions. The histochemical and immunocytochemical profiles of hepatocytes in these lesions, however, varied greatly among these species. For example, alpha-fetoprotein was a marker for rat hepatocellular carcinomas only, but was demonstrated in both benign and malignant lesions in mice and patas monkeys.

The avidin-biotin peroxidase complex (ABC) immunocytochemical technique was further developed for use in laboratory animals. Seventy-nine different antisera, including several monoclonal antibodies, were used to localize a variety of antigens including cell surface glycoproteins, oncogene-associated protein products, hormones, viruses, fetal antigens, enzymes and lysosomal proteins. Several of these antisera were used for the first time on tissue sections in an effort to evaluate their specificity for oncogenesis. Problems in fixation and interpretation were solved for specific antibodies including oncogene protein products and fetal antigens allowing us to localize these antigens in tissue sections in the various stages of carcinogenesis. The utilization of antibodies

to lysosomal granules of the large granular lymphocyte, the effector cell of natural killer activity, has provided a diagnostic tool for LGL leukemia and related diseases.

Pulmonary neoplasms in rats and mice were characterized by histogenesis and immunocytochemical studies. Antibodies to surfactant apoprotein and Clara cell antigens were utilized to show that the vast majority of naturally occurring pulmonary tumors of rats and mice and tumors induced by DEN and ENU in mice and nitrosomethylurea (NMU) in rats were of alveolar Type II cell origin. In these systems, no tumors of Clara cell origin could be found in contrast with conclusions published by others using morphologic techniques alone.

A new papovavirus was found in athymic nude rats. The virus caused a wasting disease characterized by salivary gland infection and pneumonia with intranuclear inclusion bodies. The disease was diagnosed by the localization of group-specific antigens in lesions with the use of the ABC immunocytochemical technique. Although no tumors were associated with viral infection, others have reported naturally occurring salivary gland tumors in nude rats. Infected material was given to three investigators for virus isolation, which has been unsuccessful to date.

The pathogenesis and promotion of tumors were studied using liver initiation-promotion systems in mice and rats; an NMU-induced thyroid tumor system in rats; skin painting studies in mice; and an aged F344 rat liver model system developed in this Section. From these efforts and a review of those of other investigators, we conclude that tumor promotion can be an irreversible biological process which may require only a short period of exposure to the promoter for effective tumor promotion. For example, in the skin of Sencar mice, we showed that after only two exposures to TPA, effective skin tumor promotion was seen. In addition, the tumors promoted after only short-term exposure to TPA, grew progressively and did not regress after exposure to TPA was terminated. In the thyroid gland, removal of the goitrogenic iodinedeficient diet at various time periods after exposure to NMU, allowed some of the promoted proliferative lesions to progress to large tumors. In mouse liver, the tumor promoter di(2-ethylhexyl)phthalate (DEHP) was effective as a tumor promoter after only 28 days of exposure while phenobarbital (PB) was only effective after continuous exposure.

The possible mechanisms for tumor promotion in rodent liver were investigated. DEHP was found to promote the development of liver tumors in mice but not in rats. DEHP causes liver enlargement, hyperplasia, and peroxisomal proliferation in both species; these effects are therefore not sufficient mechanisms for tumor promotion in this system, although DEHP is a complete carcinogen in both species. Aged F344 rats, which have naturally occurring gamma-glutamyl transpeptidase (GGT) negative focal proliferative basophilic hepatocellular lesions, were given phenobarbital in drinking water to determine the role of this promoter on these naturally occurring foci. Phenobarbital was found to induce focal eosinophilic GGT-positive, hepatocellular foci de novo and not to promote the growth or increase the incidence of the basophilic foci.

The TPPS provides guidance in pathology and evaluation of carcinogenesis investigations to other scientists at the NCI, U.S. regulatory agencies, and to the National Toxicology Program. Section members have participated in Government reviews of nitrite carcinogenicity and carcinogenic drugs, and as expert witnesses in Government proceedings.

The Ultrastructural Studies Section uses techniques of light, transmission and scanning electron microscopy as well as immunological techniques 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.

During the past two years, the Section has established a research program in the framework of the Laboratory and, on a collaborative basis, support is provided to members of the Laboratory and other institutions with regard to various aspects of the biology of neoplastic transformation. Within the context of understanding the biology of cell transformation, the Section has concentrated, during the last year, on the following research projects: (1) evaluation of morphological and functional changes following chemical transformation of epithelial cells, (2) ultrastructural identification of natural killer cells, (3) characterization of the thymic environment during T-cell lymphoma development, and (4) evaluation of the role of retinoids in embryonal development.

The availability in this Laboratory of nontransformed, initiated and chemically transformed epithelial cell cultures provides a basis for the investigation of functional changes during transformation of epithelial cells. The Ultrastructural Studies Section has initiated and is continuing a program directing special emphasis towards the investigation of morphologically definable transformation features, i.e., phenotypic changes, as these are as yet not well understood in epithelial cells. However, knowledge of these characteristics and the ways they change following tumorigenic transformation is important generally to the study of the mechanisms of carcinogenesis and particularly to study the mechanisms by which an epigenetic chemical carcinogen induces transformation. In one series of experiments several cell lines originating from rat liver tissue, some untransformed and some transformed by DL-ethionine, maintained in the Nutrition and Metabolism Section of the Laboratory, serve as models for the study of different parameters in the course of transformation. The characterization of these control and ethionine-transformed, liver-derived

cell lines has been achieved and, by using cytological and histochemical methods, evidence has been presented for (1) the epithelial nature of these cells, (2) their origin from liver epithelium, and (3) the identification of cellular alterations in nucleus and cytoplasm that resulted specifically from ethionine treatment and subsequent transformation. For the understanding of genetically stable modifications in morphology due to transformation, cytoskeletal arrangements and adhesion characteristics of the transformed cells have been compared to those of the nontransformed controls using immunochemistry, reflection contrast and phase microscopy, and electron microscopy. As recent studies have stressed the importance of the cytoskeleton (microtubules, actin cables, intermediate filaments) and extracellular attachment points (laminin, fibronectin, focal contacts) for the preservation and regulation of cellular configuration in interphase cells and during mitosis, changes in phenotype, as exemplified in our model system, are used as one criterion for cell transformation. Our electron microscopic studies reveal that major factors for phenotypic differences between tumorigenic and control cells are in the area of cell-cell and cell-substrate adhesion. The results of our studies give evidence, in cultures of transformed cells, for an increase in cell-substrate adhesion due to increases in the number of focal contacts and in the expression of fibronectin; concomitantly, a loss of cell-cell adhesion via intermediate junctions is indicated. The changed adhesion



patterns in the transformed cell cultures may be defined as progressive deficiencies in cell contact interactions. Although decreased cell-cell and cell-substrate adhesion is commonly associated with tumorigenic transformation, our observation of decreased cell-cell contact associated with increased cell-substrate adhesion in the transformed liver epithelium makes this system a unique and valuable model for the study of the transformation process independent of a reduction in cell-substrate adhesion.

In a second series of experiments, the promotable mouse epidermal cell line, JB6, is subjected to a similar protocol for the study of adhesion characteristics with the goal of characterizing specifically promotion-specific events.

Work continues, in collaboration with the Tumor Pathology and Pathogenesis Section of this Laboratory, on the development of a marker system in intact tissue at the ultrastructural level for identifying natural killer cells. The use of cryostat sections followed by a preembedding staining technique has given encouraging results, and, therefore, should allow the establishment of a protocol which produces specific antibody staining and acceptable ultrastructural preservation. This protocol could then be applied to other model systems in which identification at high resolution of antigenic markers in intact tissue would be beneficial.

The Section Head has continued a joint research project with the Institute of Pathology at the University of Cologne, Germany. The research project encompasses studies of the thymic microenvironment, and is especially concerned with regulatory mechanisms provided by the thymic epithelium. These mechanisms are necessary for the maturation and proliferation of prethymic T-progenitor cells to mature lymphocytes of the T-cell type. The aim of the ongoing study is to elucidate the mechanisms of the intrathymic differentiation block of prethymic lymphoid stem cells that gives rise to systemic malignant lymphoma of the Th-cell type. In vivo experiments, using the Moloney virus-induced lymphoma in the BALB/c mouse as a model, have been performed and the phenotype and distribution of the major thymic cell populations have been characterized at different stages of tumorigenesis by light and electron microscopy. Immunofluorescence studies for the presence of thymopoietin II and serum thymus factor were carried out to determine the functional state of the epithelial cells. The results show that the reticular epithelial cells of the thymus, which provide a microenvironment necessary for the differentiation of prethymic stem cells to lymphocytes of the T-lineage, are a prime target for retrovirus infection; these cells are infected by the lymphoma-producing virus, undergo phenotypic changes and are rendered functionally defective prior to lymphoma development. It is concluded from the results obtained that functionally incompetent epithelial cells fail to stimulate the maturation of T-progenitors, causing the progressive accumulation of T-cell precursors, and initiating a dysregulative lymphoma. These studies have been expanded to include the evaluation of the thymic microenvironment in mice of different strains, such as AKR, C3H, C57Bl, and BALB/c, expressing a varying capability for the induction of lymphoma. Our data show that microenvironmental changes in the thymus during the AKR lymphoma development are comparable to those obtained in the MMLV-induced lymphoma system. The data appear to be specific for lymphoma development per se as thymi of mice expressing a low capability of lymphoma development do not show shifts in the thymic epithelial cell population during the life cycle of the animal.

The Section Head has been invited by the Laboratory of Chemoprevention to join a collaborative study investigating the regulatory role of retinoids on the development of the avian embryo. The growth and differentiation of retinoid-deficient and control embryos of 5 to 15 somites have been examined in vivo by light microscopy and transmission and scanning electron microscopy. Fertile retinoid-deficient eggs were obtained from flocks of quail maintained on a retinoid- and carotenoid-deficient diet, supplemented only with small amounts of retinoic acid methyl ester as described by J.N. Thompson et al. (Brit. J. Nutr. 23: 471, 1969). Retinoid deprivation during embryonal development causes abnormalities in organs of epithelial and mesenchymal origin, most dramatically preventing the formation of the extra-embryonal circulatory system in the avian embryo. Our studies show that in the deficient embryo the following sequences of events lead to abnormalities: (1) the heart forms a cardia bifida at the 7-9 somite stage characterized by the formation of two individual tubes each closed at the area of the sinus venosus. Further normal development of the heart is arrested. The closed heart chambers dilate due to the accumulation of plasma. (2) Vitelline arteries and veins normally connecting the embryonal with the extra-embryonal circulatory system do not develop at the sinus venosus. (3) The extra-embryonal vascular system of blood islands and small vessels develops only partially throughout the area opaca and area pellucida adjacent to the embryo proper. Similar defects in the development of the circulatory system can be demonstrated by culture of normal 24-hr embryos on retinoid-deficient agar medium; conversely, normal development is observed upon culture of retinoid-deficient embryos on retinoid-containing agar medium. Studies examining the ability of retinoids to control the expression of oncogenes and peptide growth factors in this embryonic system are in the planning stage.

The Developmental Biology and Biochemistry Section (1) isolates, identifies and characterizes bioregulators of cellular growth and differentiation; (2) develops and applies model systems to evaluate and compare the functional activities and interactions of cellular growth factors during embryogenesis and experimental carcinogenesis; and (3) explores the use of growth factors as markers of tumor development and control.

With the resignation of the former Section Chief, Dr. Arnold Fowler, to accept a position in the Food and Drug Administration, the program of the Section has undergone a significant change. The original program of the Section involved the identification, isolation and characterization of placentally derived growth regulators. The placenta, which harbors a small population of potentially malignant cells, shares numerous properties with cancers and provides a unique model system for investigating the intricate balance between normal and neoplastic growth control. To date, studies have demonstrated the presence of mitogenic stimulating factors, colony stimulating factors and interferon-like activity in mouse placental extracts taken after 13 days of gestation. These investigations have led to the isolation and characterization of a new class of interferon (MuIFN-P1) which is distinguishable from classical mouse interferon alpha, beta and gamma by serological differences and unusual decay kinetics. Furthermore, a series of comparative studies has verified the presence of this atypical interferon in placental extracts from all mouse strains examined and, thus, provisionally support the notion of its general presence in all pregnant mice. MuIFN-P1 is classified as an interferon on the basis of its transcriptionally dependent, species specific antiviral activity. Like classical interferons, MuIFN-P1 also induces the enzyme 2-5A synthetase-considered an interferon specific

event--and inhibits growth factor mediated mitogenesis. MuIFN-P1 activity is highest in the placenta at term and is composed of two distinct species that function synergistically in their induction of the antiviral state. Both species are biologically and antigenically distinct from classical interferons. Though the function of MuIFN-P1 remains unclear, it does not appear to be antiviral *in situ* and all data collected are consistent with its classification as a feto-maternal cellular biological response modifier. The detection of an antiviral activity in extracts from rat, hamster, monkey and human placentas also supports this hypothesis. This project has been concluded and the terminal report is presented this year.

A new research program that potentially can make optimum use of the experience and capabilities of the personnel of the Section, notably in the areas of growth factor biochemistry and immune reagent development and application, concerns the systematic study of the expression of selected oncogenes in chemically induced animal tumors. This project, in collaboration with Dr. Mariano Barbacid, Litton Bionetics, Inc., Frederick, Maryland, will initially explore the time-course of expression of mutant ras family oncogenes during the development of chemically induced neoplasms in rats. By means of monoclonal or polyclonal antibodies to oncogene products, demonstrable in tissue sections at the cellular level by immunoperoxidase methods, evidence for or against a role of such genes in the induction of neoplasia will be systematically pursued. Transfecting Ki-ras genes have been detected in serially passaged renal mesenchymal tumors. In collaboration with the Tumor Pathology and Pathogenesis Section a concerted effort will be made to distinguish between appearance of oncogene products in tumor cells at the earliest identifiable stages of their evolution (consistent with a role in initial transformation to the neoplastic phenotype) or the gradual appearance of such expression during progression of an established primary neoplasm (see *Nature* 309: 518, 1984). As the Section has fewer personnel at the present time than the minimum necessary to maintain a self-sustaining level of effort, the Section as such will be disestablished and its personnel, together with the new oncogene project, will be transferred to form a Development Biology Working Group in the Office of the Chief.

The Chemistry Section plans and conducts laboratory research on the chemistry of organic and inorganic carcinogens. This includes (1) investigations on mechanisms of carcinogen formation, with the aim of understanding and ultimately preventing formation of such compounds *in vitro* and in the environment; (2) studies on 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 (3) comparative investigations of molecular interactions between chemical carcinogens and the cells of different organs and species, to identify factors potentially contributing to organ specificity and species differences in chemical carcinogenesis.

The activities of the Chemistry Section for the past year have focused on the chemistry of N-nitroso compounds. Specific emphases have included (1) development of new or improved synthetic methods useful in carcinogen chemistry, and preparation of specific nitrosamines and related compounds for chemical and biological studies; (2) the biological chemistry of nitrosamines, with special attention to the properties of suspected metabolic intermediates as well as to the rate modifying effects of deuterium substitution; (3) mechanistic studies

on N-nitrosation reactions of environmental interest, particularly those accelerated by electrophilic species, including transition metal complexes; and (4) chemical reactivity investigations, especially those aimed at developing decontamination and disposal methods useful for controlling the hazards associated with carcinogenesis research. Possible implications of this work with respect to the overall goal of human cancer prevention are sought.

The Section has undertaken a major project, in collaboration with the Perinatal Carcinogenesis Section and the Tumor Pathology and Pathogenesis Section, on structure/activity relationships in tumor promotion by barbiturates (see Project Number Z01CE05299-03 LCC). Barbiturate derivatives will be prepared in kilogram quantities in order to test the hypothesis that tumor-promoting activity is associated with long-acting sedative/anticonvulsive properties and that organotropism in tumor promotion is determined by the pattern of substitution at C-5.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04542-12 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Chemistry of N-Nitroso Compounds

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

PI: L. K. Keefer Chief, Chemistry Section LCC NCI

Others: T. Anjo Visiting Associate LCC NCI

H. Hu Staff Fellow LCC NCI

## COOPERATING UNITS (if any)

IARC, Lyon, France (A. J. Likhachev and M. Castegnaro); Program Resources, Inc., Frederick, MD (G. Lunn, E. B. Sansone, and A. W. Andrews); Smith Kline &amp; French Laboratories, Philadelphia, PA (B. Mico); Hoffmann-La Roche, Inc., Nutley, NJ (W. Garland); Clemson University, Clemson, SC (J. C. Fanning)

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

Chemistry Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

## TOTAL MAN-YEARS:

2.8

## PROFESSIONAL:

2.8

## OTHER:

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.)

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

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

L. K. Keefer	Chief, Chemistry Section	LCC	NCI
T. Anjo	Visiting Associate	LCC	NCI
H. Hu	Staff Fellow	LCC	NCI

Objectives:

(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 as a means of contributing to the general fund of knowledge about such materials.

Methods Employed:

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

Major Findings:

Important information regarding the organ specificity and other mechanistic aspects of dimethylnitrosamine (DMN) carcinogenesis has been obtained by deuterium isotope effect studies performed in collaboration with Drs. Bruce Mico of Smith Kline & French Laboratories and William A. Garland of Hoffmann-La Roche. Six-week-old male Fischer rats were given DMN or its fully deuterated analog (DMN-d6) either orally or by tail-vein injection, and the blood concentrations of nitrosamine were determined by gas chromatography-mass spectrometry as a function of time after administration. The existence of a first-pass effect on the metabolism of DMN was directly confirmed, and values for the bioavailability of both DMN and DMN-d6 at doses similar to those which produce liver cancer in these animals could be estimated. A clear isotope effect was observed on the disappearance of the nitrosamine from blood after intravenous administration; its magnitude indicated that the greater apparent strength of the carbon-deuterium (vs. C-H) bond roughly halves the rate at which the rat can metabolize DMN. A similar inhibition of hepatic first-pass metabolism of DMN was observed. The associated lower exposure of that organ to the metabolically activated carcinogen presumably accounts for the diminished hepatocarcinogenicity of DMN-d6 Z01CE04542-12 LCC compared to DMN after administration in drinking water to rats. A dramatically inhibitory effect of ether on DMN metabolism was discovered during the preliminary stages of this investigation; as a consequence, the studies described above were all performed on unanesthetized animals. The overall results of the above bolus investigations

were also observed in constant infusion studies, confirming the value of the latter, more economical approach in pharmacokinetic studies of this kind.

A synthetic chemical transformation of possible commercial significance was developed in collaboration with Drs. G. Lunn and E.B. Sansone of Program Resources, Inc. In these studies, unsymmetrical hydrazines useful as propellants, herbicides, drugs, and in cancer research laboratories were prepared conveniently and inexpensively by treatment of the corresponding nitrosamines with aqueous titanium trichloride solution. In a related investigation, a variety of chemical compound types containing nitrogen-nitrogen or nitrogenoxygen bonds were shown to be quantitatively cleaved to the corresponding amines by simply stirring them with aluminum-nickel alloy powder in aqueous or partially aqueous alkaline media at room temperature; pyridine and certain other aza aromatic compounds were also hydrogenated under these conditions. Since the starting materials for the aluminum-nickel reaction can be powerfully carcinogenic while the product amines are presumably innocuous, the method has been investigated as an approach to hazard control in the cancer research laboratory; we have found that chemical wastes produced during laboratory operations with carcinogens containing N-N or N-O bonds can generally be decontaminated using Al-Ni alloy and aqueous base in preparing them for safe disposal. However, nitrosamides such as nitrosomethylurea were found to produce some diazoalkane under these conditions; other destruction methods for nitrosamides have been investigated in collaboration with Drs. Lunn and Sansone as well as Dr. M. Castegnaro and his colleagues at the International Agency for Research on Cancer, with mutagenicity testing of the chemically treated nitrosamide solutions being performed by Dr. A. W. Andrews of Program Resources, Inc. In a biological study conducted in collaboration with Dr. A. J. Likhachev and his colleagues in Lyon, Leningrad, and Menlo Park, the influence of aging on DNA alkylation and other effects of methyl(acetoxymethyl)nitrosamine in vivo in rats was studied.

Extensive studies of alkanediazotate chemistry have been conducted, as these species are thought to be critical intermediates in the toxicological activation of numerous carcinogens, including nitrosamines, hydrazines, and azoxy compounds. In particular, the E conformer of thallium(I) methanediazotate has been prepared and characterized physicochemically. In contrast to previously reported methanediazotates, the thallium(I) compound is a crystalline material whose properties suggest considerable tendency toward covalency: it is readily soluble in certain non-polar solvents; it melts reversibly at a low temperature; it is monomeric and non-conducting in chloroform solution; a molecular ion was found in its electron impact mass spectrum; and X-ray crystallography revealed a degree of association between the thallium center and several nearby oxygen atoms in the lattice. The compound's excellent stability and solubility properties have proven most advantageous, for example in overcoming the problems of purification and analysis usually encountered with other diazotates. The Z stereoisomer has also been identified and partially characterized by trapping it in situ immediately after it was synthesized, although it has thus far defied isolation. While the direct applicability of the thallium compounds to biological studies will probably be limited by the metal's notorious toxicity, they have been shown to serve as excellent synthons for some novel N-nitroso compounds of potential interest in carcinogenesis research. Among these, for example, are the first reported quaternary ammonium diazotates, as well as two

Lunn, G., Sansone, E. B., Andrews, A. W., Castegnaro, M., Malaveille, C., Michelon, J., Brouet, I. and Keefer, L. K.: Destruction of carcinogenic and mutagenic N-nitrosamides in laboratory wastes. In O'Neill, I. K., Miller, C. T., von Borstel, R. C., Long, J. E. and Bartsch, H. (Eds.): N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer. IARC Scientific Publications No. 57., Lyon, France, International Agency for Research on Cancer. (In Press)

Lunn, G., Sansone, E. B. and Keefer, L. K.: Reduction of nitrosamines with aqueous titanium trichloride: Convenient preparation of aliphatic hydrazines. J. Org. Chem. (In Press)

Swann, P. F., Mace, R., Angeles, R. M. and Keefer, L. K.: Deuterium isotope effect on metabolism of N-nitrosodimethylamine in vivo in rat. Carcinogenesis 4: 821-825, 1983.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04580-10 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

The Role of Lipotropes in Carcinogenesis

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

PI:	L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
Others:	M. J. Wilson	Chemist	LCC	NCI
	N. M. Shivapurkar	Visiting Associate	LCC	NCI
	K. L. Hoover	Staff Fellow	LCC	NCI
	Z. He	Visiting Fellow	LCC	NCI

## COOPERATING UNITS (if any)

Litton Bionetics, Inc., Rockville, MD (Dr. Allen Manus); Hotel-Dieu de Quebec, Quebec, Canada (Dr. Luc Belanger)

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

Nutrition and Metabolism Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

4.4

## PROFESSIONAL:

3.4

## 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.)

The mechanisms responsible for the alteration of chemical carcinogenesis by the dietary lipotropes, choline, methionine, folic acid and vitamin B-12 have been studied. The metabolism and carcinogenic activity of ethionine in different species is being compared. Correlations between the tissue levels of S-adenosyl-methionine, S-adenosylhomocysteine, and 5-methylcytosine in animals treated with carcinogens, liver tumor promoters and methyl-deficient diets are being determined. The effects of methionine antagonists, and of dietary methionine and choline on carcinogenesis in liver and other organs are under investigation using standard bioassays. The effects of methylase inhibitors on the methylation of macromolecules and on carcinogenesis in vivo are determined.

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

L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
M. J. Wilson	Chemist	LCC	NCI
N. M. Shivapurkar	Visiting Associate	LCC	NCI
K. L. Hoover	Staff Fellow	LCC	NCI
Z. He	Visiting Fellow	LCC	NCI

Objectives:

To determine the mechanism(s) by which physiological methyl deprivation produces liver carcinomas. To determine the extent to which methyl deprivation contributes to carcinogenesis in extrahepatic tissues.

Methods Employed:

The effects on carcinogenesis in rodents of dietary regimes altering the in vivo bioavailability of the chief physiological methyl donor, S-adenosylmethionine are investigated. The dietary components varied include the methyl group-providing compounds, methionine and choline; the vitamins responsible for methyl group biosynthesis, folic acid and vitamin B<sub>12</sub>; and the methionine antagonist ethionine.

Both complete and two-stage carcinogenesis studies are employed. The early histological events associated with hepatocarcinogenesis are monitored by light microscopy using specialized stains. Tissue levels of S-adenosylmethionine, S-adenosylethionine, S-adenosylhomocysteine, 5-methyldeoxycytidine in DNA, and polyamines are determined using appropriate combinations of HPLC and chromatographic systems developed in this laboratory, as well as of standard spectrophotometric and radioisotopic techniques.

The cellular activities of the enzymes, DNA methylase and ornithine decarboxylase are determined by published methods. Serum levels of alpha-fetoprotein in rats undergoing carcinogenesis by methyl deprivation and the transfecting activity of DNA from tumors arising in methyl-deficient rats are determined by collaborative studies with other groups.

Major Findings:

The pursuit of this project has led to the major observation that dietary methyl deprivation alone causes liver cancer in rats. The chronic administration of methionine- and choline-deficient diets produced a high incidence of liver cancer in uninitiated rats as well as in diethylnitrosamine-initiated rats. Chronic feeding of the methyl-deficient diets also leads to the dedifferentiation of pancreatic acinar cells to hepatocyte-like cells. Methyl deprivation, even in uninitiated rats, leads to the formation of preneoplastic lesions, such as enzyme-altered foci and elevated levels of serum alpha-fetoprotein commonly seen during hepatocarcinogenesis by chemicals. To date dietary methyl deprivation has not

not altered tumor formation in the livers of C3H mice or in the extrahepatic tissues of rats or mice.

However, an elevated incidence of hepatocellular carcinomas in C3H, BALB/c and Swiss mice was observed upon long-term feeding of the methionine antagonist, ethionine. Further, the hepatocarcinogenic and liver tumor-promoting activities of phenobarbital in C3H mice are markedly inhibited by high dietary levels of methionine and choline.

In general the biological effects of methyl deprivation and ethionine administration can be correlated with their biochemical effects in different tissues. For example, of all rat organs studied, the liver suffered the greatest decline in the ratio of *S*-adenosylmethionine to *S*-adenosylethionine (in ethionine-fed rats) or to the physiological methylase inhibitor, *S*-adenosylhomocysteine (in choline- and methionine-deficient animals). In both cases such decreases were accompanied by a significant decline in the 5-methyldeoxycytidine content in hepatic DNA. The chronic administration of a methionine- and choline-devoid diet to C3H mice which are resistant to the hepatocarcinogenic effects of methyl deprivation, produced only slight alterations in the ratios of hepatic *S*-adenosylmethionine to *S*-adenosylhomocysteine and no significant change in the proportion of 5-methyldeoxycytidine in hepatic DNA. These results provide good evidence that dietary methyl insufficiency results in hypomethylated DNA and, at least under some conditions, plays a major role in hepatocarcinogenesis.

#### Significance to Biomedical Research and the Program of the Institute:

One of the basic aims of the National Cancer Institute is the prevention of cancer by a delineation of the mechanism by which cancers are induced. The reasons for studying the role of physiological methyl donors in carcinogenesis are both practical and theoretical. In practice, several physiological conditions associated with an elevated risk of cancer formation in humans are also accompanied by an abnormal stress on the body's pool of methyl donors. These include: 1) High fat intake. High fat diets increase the metabolic requirements for methionine and choline (hence the term lipotropes). 2) Familial polyposis. Biologically normal fibroblasts from colon cancer patients with this disease have an increased demand for methionine compared to the fibroblasts from their disease-free relatives. 3) Tyrosinemia. Patients born with this genetic disease have high serum levels of alpha-fetoprotein, develop a high incidence of liver carcinoma, and have a defective biosynthesis of *S*-adenosylmethionine. 4) Liver cancer in certain African populations. This disease has been associated with an elevated aflatoxin intake, hepatitis and a low protein, and thus low methionine, intake. The theoretical reasons for studying methyl deprivation in carcinogenesis are centered on indirect evidence implicating hypomethylation, particularly of DNA, in cancer causation. This includes the observations that 1) the chronic administration of several hepatocarcinogens and liver tumor promoters decreases the hepatic levels of *S*-adenosylmethionine in rats; 2) specific genes from several human and experimental tumors are hypomethylated compared to the same genes in the corresponding normal tissues; 3) azacytidine, an inhibitor of DNA methylation, is tumorigenic in several rodent tissues; 4) *S*-adenosylethionine, a major metabolite of the hepatocarcinogen, ethionine, and an effective inhibitor of DNA methylation, is a cell transformant. The present studies provide good evidence that physiological

methyl insufficiency under specific conditions plays a major role in hepatocarcinogenesis in rodents. Successful generalization of the hypothesis that methyl insufficiency or hypomethylation is a major contributing factor to carcinogenesis would provide 1) screening methods for populations at risk for specific types of cancer, 2) the prospect of early intervention to minimize such risks, and 3) an alternate mechanism to the common model systems of chemical carcinogenesis based upon the direct alkylation of DNA by an electrophilic metabolite of an exogenous compound.

#### Proposed Course:

This project is an integrated approach to an understanding of the mechanism by which and the extent to which physiological methyl deprivation causes cancer. Future studies will test the hypothesis that systems or agents which favor hypomethylation in vivo will enhance carcinogenesis. The effects of physiological methyl donors on carcinogenesis will be extended to include other dietary modifications known to alter the availability of S-adenosylmethionine in vivo, other carcinogens and antagonists of methionine, and other tissues and species. In particular, more direct evidence for the involvement of S-adenosylmethionine and of 5-methylcytosine in carcinogenesis will be sought. Dose-response studies on the formation of enzyme-altered foci and of liver tumors in rats as a function of methionine and choline content in the diet will be performed. Collaborative studies will be undertaken to determine the extent of methylation of specific genes and the transfecting activity of DNA from the liver tumors obtained in methyl-deficient rats. Alternate hypotheses to DNA hypomethylation as the mechanism of carcinogenesis by methyl deprivation will be tested by the long-term feeding of a diet deficient in another amino acid and by seeking abnormally alkylated bases in the hepatic DNA of methyl-deficient animals.

#### Publications:

Hoover, K. L., Lynch, P. H. and Poirier, L. A.: Profound influence of short-term severe methionine, choline, vitamin B<sub>12</sub>, and folate deficiency and hepatocarcinogenesis in rats injected with a single low dose of diethylnitrosamine. JNCI (In Press)

Mikol, Y. B., Hoover, K. L., Creasia, D. and Poirier, L. A.: Hepatocarcinogenesis in rats fed methyl-deficient, amino acid-defined diets. Carcinogenesis 4: 1619-1629, 1983.

Shivapurkar, N. and Poirier, L. A.: Levels of S-adenosylmethionine and S-adenosylethionine in four different tissues of male weanling rats during subchronic feeding of DL-ethionine. Biochem. Pharmacol. (In Press)

Shivapurkar, N. and Poirier, L. A.: Tissue levels of S-adenosylmethionine and S-adenosylhomocysteine in rats fed methyl-deficient, amino acid-defined diets for one to five weeks. Carcinogenesis 4: 1051-1057, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE04582-09 LCC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Metal Interactions in Carcinogenesis

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

PI: L. A. Poirier Chief, Nutrition and Metabolism Section LCC NCI

Others: K. S. Kasprzak Visiting Scientist LCC NCI  
 K. L. Hoover Staff Fellow LCC NCI  
 M. P. Waalkes Staff Fellow LCC NCI

COOPERATING UNITS (if any)

Litton Bionetics, Inc., Rockville, Maryland (Dr. Allen Manus)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Nutrition and Metabolism Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

3.1

PROFESSIONAL:

2.6

OTHER:

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 antagonism between the essential divalent metals, calcium, magnesium, and zinc and the divalent metal carcinogens, lead, nickel and cadmium are under investigation in metabolic, chemical and carcinogenicity studies. Magnesium has been shown to inhibit lung adenoma formation in lead- and nickel-treated strain A mice, as well as cadmium- and nickel-induced sarcomas in rats. Part of the protection afforded by magnesium against the tumorigenic activity of cadmium and nickel is due to the diminished accumulation of the carcinogenic metal at the target site. In chemical studies cadmium binding to DNA was found to be inhibited by zinc, magnesium and calcium. Extension of the possible antagonistic effects of the physiologically essential divalent metals against tumor formation by nickel, lead, and cadmium in other target sites will be explored.

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

L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
K. S. Kasprzak	Visiting Scientist	LCC	NCI
K. L. Hoover	Staff Fellow	LCC	NCI
M. P. Waalkes	Staff Fellow	LCC	NCI

Objectives:

The accumulation of evidence indicates that the activated form of most organic carcinogens consists of a reactive electrophile. Possible mechanisms by which the metal carcinogens exert their activity remain relatively unexplored. The hypothesis that they act via an antagonism of the physiologically essential metals, calcium, magnesium and zinc is being tested.

Methods Employed:

The carcinogenic and toxic activities of lead, nickel and cadmium in the presence of various amounts of calcium, magnesium or zinc are studied *in vivo* and *in vitro* using standard protocols. These include long-term feeding and/or injection of the suspect compound alone, or combined with the physiological metal salt, into rats and mice followed by examination at necropsy for tumors and other pathological changes. Metabolism of the carcinogenic metals in rats and mice and in tissue culture, their effects upon thymidine incorporation into DNA, and the effects of calcium, magnesium and zinc upon the metabolism and toxicity of the carcinogenic metals are determined by radioisotopic (radiometric and autoradiographic), and standard analytical methods (chemical analysis, chromatography, ultracentrifugation). Techniques for the isolation of viable interstitial cells of rat testes, target cells of cadmium carcinogenesis, have been developed.

Major Findings:

The major contribution constituted by these studies is that the physiological effects of the carcinogenic divalent metals are very often inhibited by the physiologically essential divalent metals, calcium, magnesium and zinc. Magnesium administered *in vivo* along with the carcinogenic metal has been shown to inhibit lung adenoma formation in strain A mice by lead and nickel and sarcoma production in rats by nickel and cadmium. The inhibition by magnesium of nickel carcinogenesis was partially the consequence of reduced nickel uptake in the target tissue, and in the lungs of strain A mice was accompanied by a suppression of the stimulating effects of nickel upon DNA synthesis. Zinc, which previous studies had shown to be a strong inhibitor of cadmium carcinogenesis, decreased nickel toxicity in rats by inhibiting nickel-induced hyperglycemia. The effects of calcium on metal carcinogenesis are more complex. Calcium injection inhibited lung adenoma formation in mice by lead and nickel, but, when administered alone, produced an elevation in such tumors. Neither the feeding nor the injection of calcium altered the yield of testicular tumors or injection site sarcomas produced by a single dose of cadmium.

Finally, calcium feeding enhanced the formation of renal carcinomas while suppressing the accumulation of the carcinogenic metal in the kidneys of rats fed lead for eighteen months.

In vitro, calcium, magnesium and zinc competitively antagonized the binding of cadmium to DNA, with a relative potency similar to that found in vivo for tumor prevention. Studies of the interactions of cadmium with isolated interstitial cells of testes indicate that cadmium is actively taken up by such cells and that zinc will antagonize this uptake. To date the most consistent antagonism to the physiological effects of the carcinogenic divalent metals has been provided by magnesium and zinc.

#### Significance to Biomedical Research and the Program of the Institute:

The aim of these studies is to increase the base of theoretical knowledge by which the potential carcinogenic hazards to man of carcinogenic metals can be diminished. Metals constitute one of the largest and broadest categories of chemical carcinogens to which humans are exposed. Metals are often among the most active carcinogens known. As a class of carcinogens they are relatively underinvestigated. The biochemical similarity of their mode of action, if any, to the organic carcinogens remains obscure. The evidence accumulated to date indicates that an antagonism to the divalent cations, calcium or magnesium, may constitute part of the mechanism by which the divalent metal carcinogens exert their activity. Successful demonstration of the molecular locus of antagonism between the physiological metals and the divalent carcinogens could help to identify the intracellular targets of metal carcinogens.

#### Proposed Course:

Fundamental studies on the dose-response curves, species sensitivity and route of administration in cadmium carcinogenesis will be conducted. Attempts will be made to extend the antagonism between carcinogenic and physiological divalent metals by determining the effects of dietary magnesium on renal carcinogenesis by lead and of zinc on sarcoma production by nickel. The possible potentiation of metal carcinogenesis by dietary deficiency of essential metals will be explored using zinc deficiency and cadmium tumorigenicity as a model. The intracellular targets of cadmium and nickel will be examined by determining their binding sites, functional effects and genetic damage in vivo and in vitro. Initial studies on cell transformation by nickel and cadmium will be undertaken both within the Laboratory and in collaboration with other groups.

#### Publications:

Kasprzak, K. S. and Poirier, L. A.: Effects of calcium and magnesium acetates on tissue distribution of cadmium in Wistar rats. In Savory, J. and Brown, S. S. (Eds.): Chemical Toxicology and Clinical Chemistry of Metals. London, Academic Press Ltd., 1983, pp. 377-380.

Kasprzak, K. S. and Poirier, L. A.: Effects of calcium, magnesium and sodium acetates on tissue distribution of Ni(II) in strain A mice. In Savory, J. and Brown, S. S. (Eds.): Chemical Toxicology and Clinical Chemistry of Metals. London, Academic Press Ltd., 1983, pp. 373-376.

Poirier, L. A., Kasprzak, K. S., Hoover, K. L. and Wenk, M. L.: Effects of calcium and magnesium acetates on the carcinogenicity of cadmium chloride in Wistar rats. Cancer Res. 43: 4575-4581, 1983.

Poirier, L. A., Theiss, J. C., Arnold, L. J. and Shimkin, M. B.: The inhibition by magnesium and calcium acetates of lead subacetate- and nickel acetate-induced lung tumors in strain A mice. Cancer Res. 44: 1520-1522, 1984.

Waalkes, M. P. and Poirier, L. A.: In vitro cadmium-DNA interactions: Cooperativity of binding and competitive antagonism by calcium, magnesium, and zinc. Toxicol. Appl. Pharmacol. (In Press)



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04680-14 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Development and Application of In Vitro Systems Involving Epithelial Cells

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

PI: M. J. Wilson Chemist LCC NCI

Others: L. A. Poirier Chief, Nutrition and Metabolism Section LCC NCI  
 U. I. Heine Chief, Ultrastructural Studies Section LCC NCI  
 K. L. Hoover Staff Fellow LCC NCI  
 J. L. Junker Staff Fellow LCC NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

Nutrition and Metabolism Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

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

Epithelial cells derived from the livers of 10-day-old Fischer 344 rats are used as a model system for studying the mechanism of carcinogenesis resulting from an insufficiency of methyl donors. Liver cells transformed by ethionine, a hepatocarcinogenic methionine antagonist, and the corresponding control cells have been examined histochemically, immunochemically and morphologically. The data obtained provide evidence for the epithelial nature of the cells and their derivation from liver epithelium, as well as indicate cellular alterations which accompany transformation. Transformation of liver cells, as manifested by growth in soft agar, has also been achieved following treatment with 3-deazaadenosine (DAA). This compound is metabolized to 3-deazaadenosylhomocysteine, a potent inhibitor of S-adenosylhomocystein (AdoHcy) hydrolase, and results in an accumulation of AdoHcy, a competitive inhibitor of most physiological methylation reactions.

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

M. J. Wilson	Chemist	LCC	NCI
L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
K. L. Hoover	Staff Fellow	LCC	NCI
J. L. Junker	Staff Fellow	LCC	NCI

Objectives:

Studies involving the successful culture of epithelial cells derived from the livers of 8- to 10-day-old Fischer strain 344 rats and malignant transformation of such cultured cells by a variety of carcinogens continue in this laboratory. Our primary aims in current investigations have been to 1) characterize the cultures morphologically and cytochemically, and 2) establish liver cells in culture as a model system for studying the effects, including carcinogenic effects, of compounds known or thought to interfere with normal cellular methylation reactions. Aberrations in DNA methylation may produce alterations in gene expression resulting in neoplastic transformation.

Methods Employed:

Methods for culturing rat liver cells continued to be developed in this laboratory. In addition, histochemical and morphological methods were used to characterize the cells. Cells were assayed histochemically for glycogen accumulation, acid and alkaline phosphatase, adenosine triphosphatase and gamma glutamyl transpeptidase activities. The chromosome numbers were determined following exposure of the cultures to colchicine. Nucleolar and nuclear area determinations were made using an automated image analyzer. Morphologically, the cell cultures were examined by TEM and SEM. Immunofluorescence was used to examine keratin, actin, fibronectin and tubulin and reflection contrast microscopy to examine adhesion plaques. Markers of transformation were tumor production following injection of the cells into a syngeneic host and growth in soft agar.

Major Findings:

Studies in this laboratory revealed that liver cells in culture, like hepatocytes in vivo, were susceptible to transformation by ethionine. The liver cell line transformed by ethionine and the corresponding nontumorigenic control cell line were examined histochemically and morphologically in order to confirm the epithelial nature of the cells and to identify specific cellular alterations induced by ethionine treatment and subsequent transformation. Histochemically, the control and ethionine-transformed cells were shown to store glycogen and possess detectable glucose-6-phosphatase activity, both indicative of their origin from liver epithelium. Morphologically, analysis with an automated image analyzer revealed an increase in the number of nucleoli/nucleus as well as in the nucleolar/nuclear ratio in ethionine-transformed cells. Ultrastructural studies revealed

a decrease in the length and frequency of cellular tight junctions and hyperplasia of the inner nuclear membrane in the transformed cells. Nucleolar perichromatin granules, apparently indicative of defective rRNA processing, were also observed in ethionine-transformed cells. Immunofluorescent localization of cytoskeletal proteins indicated cells transformed with ethionine contained prominent actin stress fibers and produced more cellular fibronectin than corresponding low passage control cultures. Unlike most transformed cultures, liver cells transformed with ethionine have a higher number of adhesion plaques per unit cell area than corresponding low passage control cells.

Liver epithelial cells have also been used to assess the carcinogenic potential of 3-deazaadenosine (DAA). Cells exposed to DAA for 12 weeks were capable of anchorage-independent growth. Preliminary evidence indicates DAA treatment *in vitro* also results in hypomethylation of nuclear DNA. The tumorigenic potential of the cultures is being assessed. Transformation with DAA provides further evidence for a causative role of physiological methyl deprivation in carcinogenesis since the administration of DAA *in vivo* results in the accumulation of AdoHcy and subsequent inhibition of enzymatic methylations.

#### Significance to Biomedical Research and the Program of the Institute:

The availability of liver cells in culture susceptible to transformation by ethionine and other inhibitors of cellular methylation reactions provides a unique model system for studying the role of aberrant methyl metabolism in carcinogenesis.

#### Proposed Course:

The neoplastic potential of DAA as well as its effects on DNA methylation, will continue to be examined. The ability of DNA isolated from ethionine- and DAA-transformed liver cells to produce foci in a standard and transfection assay will also be determined. The metabolism of *S*-adenosylmethionine and its analogues, including their abilities to cross cell membranes, will be determined under conditions known to modify transformation.

#### Publications:

Heine, U. I., Wilson, M. J. and Munoz, E.: Characterization of rat liver cells transformed by DL-ethionine. In Vitro 20: 291-301, 1984.

Junker, J. L., Wilson, M. J., Munoz, E. F. and Heine, U. I.: Examination of a liver cell line tumorigenically transformed by DL-ethionine. In Bailey, G. W. (Ed.): Proceedings of the Electron Microscopy Society of America. San Francisco, San Francisco Press Inc., 1983, pp. 782-783.

Wilson, M. J. and Hatfield, D. L.: Incorporation of modified amino acids into proteins *in vivo*. Biochim. Biophys. Acta 781: 205-215, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04812-16 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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: J. L. Junker Staff Fellow LCC NCI

M. J. Wilson Chemist LCC NCI

K. Takahashi Visiting Associate LCC NCI

C. H. Fox Sr. Scientist LB NCI

## COOPERATING UNITS (if any)

Biological Products Laboratory, 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

## TOTAL MAN-YEARS:

3.2

## PROFESSIONAL:

2.3

## OTHER:

0.9

## CHECK APPROPRIATE BOX(ES)

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

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

Rat liver cells and mouse epidermal cells are used for studying the mechanism of chemical carcinogenesis in epithelial cells. A cell line, derived from rat liver and tumorigenically transformed by exposure of the cells in culture to DL-ethionine, serves as a model to determine genetically stable cell modifications due to transformation, particularly those relating to cell-substrate and cell-cell adhesion. The characterization of the ethionine-transformed cell line and its nontransformed control has been achieved and evidence has been presented for 1) the epithelial nature of these cells, 2) their origin from liver epithelium, and 3) the identification of cellular alterations that resulted specifically from ethionine treatment and subsequent transformation. Cytoskeletal arrangements and adhesion characteristics of the transformed liver cells have been compared to those of the nontransformed controls using immunochemistry, reflection contrast and phase microscopy, and electron microscopy. Our results give evidence, in cultures of transformed cells, for an increase in cell-substrate adhesion due to increases in the number of focal contacts and in the expression of fibronectin; concomitantly, a loss of cell-cell adhesion via intermediate junctions is indicated. The changed growth patterns in the transformed cell cultures are thus defined as progressive deficiencies in cell contact interactions. The promotable mouse epidermal cell line, JB6, is subjected to a similar protocol with the goal of characterizing promotion specific events.

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

Ursula I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
James L. Junker	Staff Fellow	LCC	NCI
Mary J. Wilson	Chemist	LCC	NCI
K. Takahashi	Visiting Associate	LCC	NCI
Cecil H. Fox	Sr. Scientist	LB	NCI

Objectives:

To determine the morphological correlates of transformation, particularly those relating to cell-substrate and cell-cell adhesion, in cultured rat liver epithelial cells by comparing nontumorigenic controls with cells which have become tumorigenic following exposure to DL-ethionine. To determine, by expanding the study to include other cell lines, markers of tumorigenic transformation as well as of tumor promotion among epithelial cell lines in general. To compare results obtained in vitro with tumors in the animal to ascertain that the results of in vitro studies are relevant to tumor development in vivo.

Methods Employed:

Two cell lines, TRL 1215 (Idoine, J. B. et al. *In Vitro* 12: 541-553, 1976) and JB6 (N. Colburn, Project Number Z01CE05383-01 LVC) and their appropriate controls, were the model systems used in our studies. Routine culture methods were used for propagation of the cell lines. Tumor promotion was accomplished by exposure of initiated cells to the tumor promoters, 12-O-tetradecanoylphorbol13-acetate (TPA) and Di(2-ethylhexyl)phthalate (DEHP). Progression of cells to the tumor cell phenotype was measured by colony promotion in soft agar or tumor production in vivo. Immunofluorescence was used to determine the localization and distribution of cytoskeletal proteins such as keratin, actin, fibronectin, and tubulin. Morphometry was used to determine projected cell areas and nuclear/cytoplasmic ratios. Reflection contrast microscopy was used to examine adhesion plaques. Scanning, transmission and immunoelectron microscopy were performed to examine cell structures and cell-cell adhesions at high resolution.

Major Findings:

The characterization of nontransformed and DL-ethionine transformed rat liver-derived cell lines of the same passage level by morphological and histochemical means to ascertain their epithelial nature and to establish their origin from liver epithelium has been achieved. The presence of intermediate junctions, bile canaliculi-like structures, prekeratin, glycogen accumulation, and glucose-6-phosphatase activity confirm their origin from epithelial parenchymal cells of the liver. Persistent modifications of the phenotype resulting from ethionine transformation are variations in cell shape and size, focal multilayered growth, increase in the nucleolar/nuclear ratio and reduction in the number of cells displaying a primary cilium. Hyperplasia of the inner nuclear membrane, observed

in approximately 40 percent of the cells, elongation and branching of mitochondria, and a reduction in length and frequency of cell junctions are also characteristic of the transformed cells.

Recent studies have stressed the importance of the cytoskeleton (microtubules, actin cables, intermediate filaments) and extracellular attachment points (fibronectin, focal contacts) for the preservation and regulation of cellular configuration in interphase cells and during mitosis. Changes in phenotype, as exemplified in our model system, are used as one criterion for cell transformation. Our studies reveal that major factors for phenotypic differences between tumorigenic and control cells are in the area of cell-cell and cell-substrate adhesion. Decreased levels of intermediate junctions and an increase in intercellular spaces were observed only in ethionine-transformed cultures. These cells also showed increased cell spreading, compared to low passage controls. This increased spreading was accompanied by prominent actin stress fibers and increased expression of cellular fibronectin. Fibronectin was shown to be a component of the abundant extracellular matrix which is produced by the ethionine-treated cells between cell layers. Ethionine-treated cells also showed a higher number of adhesion plaques (focal contacts) per unit of cell area than did low passage controls. This finding of increased adhesion accompanying transformation is in contrast to the frequent observations in both fibroblast and epithelial cell cultures that tumorigenic cells are less adherent than controls.

Conversely, initiated JB6 cells were found to be rich in cellular fibronectin and actin stress fibers relative to their permanently transformed counterparts. The ultrastructural changes in JB6 cells that accompany the response to promoters of skin carcinogenesis in mice are of special interest since this cell line predicted complete or second stage promotion capacity for mouse skin by DEHP, previously known only as a promoter for mouse liver (see Project Number Z01CE05303-03 LCC).

#### Significance to Biomedical Research and the Program of the Institute:

Most malignant tumors of man are carcinomas; yet, most of the experimental models developed for the study of transformation and malignancy consist of cells derived from mesenchyme. The availability in this Laboratory of nontransformed, initiated and chemically transformed epithelial cell cultures provides a basis for the investigation of functional changes during transformation of epithelial cells. Cell growth unbounded by normal control mechanisms is the hallmark of tumor formation. A loss of contact inhibition of movement, growth, and cell division has long been associated with transformation. This implies that the way in which a cell reacts with its surroundings whether through cell-cell or cell-substrate contacts is an important aspect of tumorigenic and metastatic potential, as well as of normal differentiation, growth and repair. Thus far, previous studies in this Laboratory have shown that ethionine-treated cells, in addition to forming tumors when injected into syngeneic rats, are capable of anchorage independent growth and in culture form multilayered foci. Such indications of abnormal growth control could be expected from alterations in the interactions of the cells with their environment. Although decreased cell-cell and cell-substrate adhesion are commonly associated with tumorigenic transformation, our observation of decreased cell-cell contact associated with increased cell-substrate adhesion in the transformed liver epithelium makes this system a unique and valuable model for the study

of the transformation process independent of a reduction in cell-substrate adhesion.

Proposed Course:

To further the understanding of cell-cell and cell-substrate interactions during tumorigenesis two lines of research will be pursued: 1) Cell-matrix interactions will be studied by investigating the role of two major matrix proteins, laminin and type IV collagen. 2) Cell-cell interactions will be investigated using freeze-fracture to examine tight junctions, using freeze-fracture and immunocytochemistry to examine the nature and distribution of different types of cell junctions.

Publications:

Heine, U. I., Wilson, M. J. and Munoz, E.: Characterization of rat liver cells transformed in culture by DL-ethionine. In Vitro 20: 291-301, 1984.

Junker, J. L., Cottler-Fox, M., Wilson, M. J., Munoz, E. F. and Heine, U. I.: Transformation-associated increase of adhesion, fibronectin, and stress fiber development in a liver epithelial cell line. JNCI (In Press)

Junker, J. L., Wilson, M. J., Munoz, E. F. and Heine, U. I.: Examination of a liver cell line tumorigenically transformed by DL-ethionine. In: Bailey, G. W. (Ed.): Proceedings of the Electron Microscopy Society of America. San Francisco, San Francisco Press, Inc., 1983, pp. 782-783.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE04967-09 LCC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Role of Genetic and Physiological Factors in Ontogeny and Carcinogenesis

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

PI:	P. T. Allen	Microbiologist	LCC	NCI
Other:	A. O. Perantoni	Microbiologist	LCC	NCI

COOPERATING UNITS (If any)

Program Resources, Inc., Frederick, MD (O.S. Weislow); Genentech, Inc., San Francisco, CA (C.W. Czarniecki); Uniformed Services Univ. of Health Sciences, Bethesda, MD (R.H. Silverman); Food and Drug Admin., Rockville, MD (A.K. Fowler)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Developmental Biology and Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The expression and control of bioregulatory macromolecules in fetal development and the effects of their interactions on cellular proliferation, differentiation and immunocompetence compared to related factors of tumor cell origin are under investigation. The interferon-like activity found in placental tissues of the mouse (MuIFN-P1) was examined in depth because of the well-established anti-proliferative properties which the interferons exhibit in several neoplastic diseases and tissue culture systems, and their known immunomodulatory effects. MuIFN-P1 was found, through the use of several antisera, to be serologically distinct from the classical families of mouse interferons. The antiviral state induced by MuIFN-P1 was more stable than that induced by the classical interferons in kinetic studies examining the persistence of cytoprotective and yield inhibitory functions against both positive and negative strains RNA viruses (MM and vesicular stomatitis viruses). MuIFN-P1 was nonetheless found to possess the characteristics of a bona fide interferon in that it is a soluble protein requiring a time-dependent cellular transcriptional event, it is highly specific for mouse cells, it primes for the increased production of type-1 interferon at optimal concentrations similar to the optima for the classical interferons, and it induces the production of 2-5 A synthetase specifically in mouse cells and in a time-dependent manner. Further, MuIFN-P1 treatment of target cells suppresses their vulnerability to natural killer cells. Placental interferon is not unique to the mouse, but occurs in other rodents which have been examined (rat and hamster), as well as in man and the patas monkey.



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

P. T. Allen	Microbiologist	LCC	NCI
A. O. Perantoni	Microbiologist	LCC	NCI

Objectives:

To develop and examine model systems for identifying and characterizing fetal/maternal and tumor-associated bioregulatory macromolecules, and to assess and compare their interactions in cellular recognition and growth regulatory processes. To develop immunological reagents to examine and contrast markers of growth regulation during prenatal ontogeny or other normal proliferative processes and in tumorigenesis.

To establish in vitro bioassay systems to examine the interactions of growth regulators in normal and pathological growth and/or differentiation.

Methods Employed:

Interferons were assayed by titration of their antiviral activity on appropriate target cells in microtray cultures, assessing the protection against virus-induced cytopathogenicity via a computer-assisted ELISA reader. Interferon neutralization was quantitated by the comparative assay of interferon preparations in the presence and absence of neutralizing antisera. 2-5 A synthetase was assayed by determination of radiolabeled ATP incorporation into 2',5'-(Ap)nA oligonucleotide by poly rI-poly rC affinity purified cell extract. Infectious virus concentration was determined by a plaque assay procedure on monolayer cultures of appropriate cell lines using a semisolid overlay of medium containing methyl cellulose as the stabilizing agent. Protein concentration was determined by a semimicro Coomassie blue spectrophotometric procedure. Purity of chromatographically fractionated materials was assessed by SDS polyacrylamide gel electrophoresis.

Major Findings:

Placenta-associated macromolecules with growth modulating functions have been under investigation within this project over the past 3 years. The homogenate of mouse placenta contains one or more soluble protein growth factors (PIGF) which induce mitogenesis and anchorage independent growth. Also present is an unusual interferon (IFN) distinct from the previously known molecular classes of IFNs. Characterization of this bioreactive agent has been essentially completed. By the 12th day of gestation the normal murine placenta contains an acid-labile substance with antiviral activity which has been shown to have many of the characteristics essential for its classification as an IFN, including nonparticulate response upon ultracentrifugation, loss of activity upon incubation at 56° C or in the presence of trypsin, and a lack of antiviral activity in actinomycin-treated cells. This activity (designated MuIFN-P1) was found to be labile at pH 2.0, a characteristic of both IFN-gamma and IFN-alpha of patients with immune disorders. The titer of

MuIFN-P1 increases throughout gestation. Crude preparations of MuIFN-P1, prepared by homogenization of near-term placenta in a high-salt buffer, typically exhibit antiviral activity in the range of 500 to 1,000 International reference units (RU)/ml. The bioreactivity of MuIFN-P1 was resolved by exclusion chromatography on Bio-Gel P100 into two components, a major peak of 27 kd (MuIFN-P1/27) and a minor peak of 70 kd (MuIFN-P1/70). Concentrated preparations of these semipurified components typically have had titers of 20 to 40 RU/ml and 250 to 500 RU/ml for the 70 kd and 27 kd moieties, respectively. Characterization of the MuIFN-P1/27 has been straightforward, but the low activity level obtained for MuIFN-P1/70 has restricted its characterization. Although partially neutralized by high concentrations of a high-titered antiserum prepared against mouse (L-cell) IFN, further serological study has demonstrated that MuIFN-P1/27 is antigenically distinct from the classical mouse IFNs. In view of this distinctiveness, it became essential to examine additional parameters to determine that MuIFN-P1 was in fact a bona fide IFN.

IFNs characteristically exhibit a well-defined pattern of species specificity, commonly being most active in cells of the species of origin, and either inactive or less active in cells of other species (although particular special case exceptions are well documented). In a study utilizing cell lines representing six mammalian species (mouse, cow, human, hamster, rat and dog), three preparations of MuIFN-P1 (crude MuIFN-P1, MuIFN-P1/27 and MuIFN-P1/70), and appropriate classical IFN controls, the three MuIFN-P1 preparations showed strongly expressed specificity for murine cells, while the control IFNs exhibited the expected species specificities and cross reactivities as reported by other laboratories.

Another common property of the IFNs is their induction of an enzyme that synthesizes from an ATP substrate a series of adenosine oligomers with a 2'-5' linkage, collectively referred to as 2-5 A. The 2-5 A synthetase is universally associated with the IFNs and is not known to be induced except through an IFN-mediated pathway. The ability to induce the production of 2-5 A synthetase is thus an additional criterion in determining that a particular substance with antiviral activity is in fact an IFN. To examine this characteristic, L cell cultures were exposed to MuIFN-P1 and assayed for 2-5 A synthetase at intervals of up to 48 hr. A low level of constitutive 2-5 A synthetase was seen in untreated cultures, but by 24 hr of IFN exposure the activity had increased by a factor of 200. The specificity of 2-5 A synthetase induction was examined in comparison to other IFNs by the use of a second cell line, MBDK, of bovine origin in which MuIFN-P1 has no antiviral activity, but HuIFN-alpha does. Cultures of both cells were exposed to a battery of placental and classical mouse and human IFNs, then assayed for 2-5 A synthetase levels. As anticipated all of the murine IFNs, as well as the recombinant HuIFN-alpha AD, induced high titers of 2-5 A synthetase in L cells. In MBDK cells, only HuIFN-alpha AD and HuIFN-leukocyte induced 2-5 A synthetase levels significantly higher than the assay's lower limit of detection. These data demonstrate that MuIFN-P1, like other previously characterized IFNs, induces the production of 2-5 A synthetase in the cells which it protects against virus infection.

Another identifying characteristic of the IFNs is their ability to "prime" cultures for increased production of type 1 IFN. MuIFN-P1 was examined for priming activity in comparison to MuIFN-alpha/beta and MuIFN-gamma. Following pre-exposure to serial dilutions of the priming IFNs, MuIFN type 1 was induced by inoculation with MM virus, and the IFN yield was determined. All IFN preparations had marked priming activity and exhibited a clear dose-response effect.

The optimal priming concentration for all of the IFNs was 0.5 to 1.25 RU/ml. The priming ability of MuIFN-P1 was thus quite similar to that of type 1 and type 2 IFNs, further confirming the placental activity as a bona fide IFN.

A synergistic response between specific IFN classes, i.e., combinations of gamma IFNs and either alpha or beta IFNs, has been reported; mixtures of alpha IFN and beta IFN are not synergistic. These effects are thought to result from differences in the mechanism of action between MuIFN type 1 and MuIFN type 2, possibly at the level of IFN:receptor interaction. In experiments utilizing cytoprotection against MM virus as the measure of IFN activity, the observation of synergy between MuIFN-gamma and MuIFN-alpha/beta was readily confirmed. Combinations of these two IFNs gave up to fourfold more antiviral activity than expected on the basis of individual titers observed in parallel cultures. In contrast, mixtures of MuIFN-P1/27 and either MuIFN-gamma or MuIFN-alpha/beta exhibited significantly lower levels of potentiation (1.3- to 1.7- fold). MuIFN-P1 was thus seen to be distinguishable from both of the other IFN classes compared in these synergy studies.

IFNs exert their effects through the induction of secondary responses in the affected cells. The development of the IFN-induced antiviral state is thus time dependent, requiring 12 to 18 hours for full expression. In a comparative study MuIFN-P1 and MuIFN-alpha/beta exhibited very similar kinetics of antiviral development.

IFN-induced antiviral activity can be maintained indefinitely in the presence of IFN. However, on removal of IFN from the supernatant culture fluid, the protected state begins to decay. The cytoprotection induced by MuIFN-P1 against MM virus began to decline 2 to 3 days after removal of the MuIFN-P1 from L cell cultures, as expected for a bona fide IFN. In a comparative study the activity of MuIFN-P1 decayed significantly more slowly than either MuIFN-alpha/beta or MuIFN-gamma. This parameter was examined in depth to determine its validity, through the use of an additional challenging virus, vesicular stomatitis (VS) virus, and through the use of an additional mode of assay for IFN activity, the inhibition of infectious virus production. The antiviral state induced by MuIFN-P1 was more stable than MuIFN-alpha/ beta or MuIFN-gamma by three out of four assay methods. These results demonstrate that MuIFN-P1 can be distinguished from both MuIFN-alpha/beta and MuIFN-gamma by the greater persistence of the antiviral state induced. This study supports and extends earlier findings that MuIFN-P1 represents a class of interferons not previously identified.

Investigations on the effect of MuIFN-P1 on natural killer (NK) cell activity are in progress. Pretreatment of target cells with MuIFN-P1 suppresses their sensitivity to NK cell-mediated cytotoxicity. The effects of pretreating NK cells with MuIFN-P1 are less well characterized, but suppression rather than enhancement has been most consistently observed.

The placenta is a complex organ, consisting of a variety of fetal and maternal tissues. Existing data do not clearly indicate if MuIFN-P1 originates in fetal or maternal tissues, nor do they precisely reveal the nature of the cells involved in the elaboration of MuIFN-P1. Short-term culture of Percoll gradient-fractionated placental cells indicates that specific subpopulations of cells are associated with MuIFN-P1 production. Furthermore, the MuIFN-P1 appears tightly bound

to the cells with which it is associated, in that highest yields are obtained from those cultures which are frozen and thawed compared to those that release the IFN into the culture fluid during incubation under standard culture conditions.

In addition to studies in the mouse, placenta-associated IFN has been detected in several other mammalian species, including man, patas monkey, hamster and rat. Titers in the rat and hamster are comparable to those in the mouse, while those in primates are lower by 1.5 to 2 orders of magnitude. Interestingly, rat IFN-P1 is fully active in mouse cells, although mouse MuIFN-P1 is inactive in rat cells.

#### Significance to Biomedical Research and the Program of the Institute:

The interaction between the developing fetus and the mother has a number of obvious analogies to neoplastic growth in a host animal. Fetal growth and development, for example, serve as a normal control model for the perturbed or unregulated growth and differentiation in the neoplastic state. The placenta in particular is a useful model for examining the interaction of host factors controlling the proliferation, differentiation, expansion and invasiveness of potentially malignant cells. Furthermore, this organ is a rich source of hormones, mitogens, colony stimulating factors and at least one inhibitor of a type known for suppression of cell proliferation and immune modulation--interferon. The placenta also harbors a plethora of potentially malignant cells (e.g., cytotrophoblast) and a variety of poorly defined factors which permit these and other cells of fetal origin to escape maternal immune detection and elimination. These factors function to effectively maintain a stable fetomaternal relationship on one hand, but mimic the tumor-host relationship on the other. More importantly, the identification of new biological response modifiers associated with the placenta may permit their application in chemotherapeutic regimens should a clearer understanding of these phenomena emerge.

#### Proposed Course:

This report concludes studies on interferons, including MuIFN-P1, within the LCC, and terminates this project. Suggestions for continuation of this work elsewhere include the following. Future efforts regarding MuIFN-P1 should be directed toward generating monoclonal and/or polyclonal immune reagents for detection of this macromolecule in situ in tissue sections and cell cultures in order to extend the understanding of its normal function. Further efforts to identify and purify growth stimulatory macromolecules in embryonal, fetal and neoplastic tissues in adequate quantity to permit biochemical, biophysical and biological characterization are needed. Efforts should be made to develop in vitro model systems to assess and compare growth factors and their interactions in cellular proliferation, differentiation and immunocompetence.

#### Publications:

Czarniecki, C. W. and Allen, P. T.: Disparate response of encephalomyocarditis virus and MM virus to interferon in JLS-V9R cells. Antiviral Res. (In Press)

Weislow, O. S., Kiser, R., Allen, P. T. and Fowler, A. K.: Partial purification of a placental interferon with atypical characteristics. J. Interferon Res. 3: 291-298, 1983.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05092-06 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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:	A. E. Palmer	Research Veterinarian	LCC	NCI
Others:	J. M. Rice	Chief	LCC	NCI
	J. M. Ward	Chief, Tumor Pathol. & Pathogen. Section	LCC	NCI
	L. M. Anderson	Expert	LCC	NCI
	P. J. Donovan	Chemist	LCC	NCI

## COOPERATING UNITS (if any)

Melo Laboratory, Inc., Rockville, MD (Dr. J. Cecmanic and S. Harbaugh)

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

3.2

## PROFESSIONAL:

2.0

## OTHER:

1.2

## CHECK APPROPRIATE BOXES)

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

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

Nonhuman primates of the species Erythrocebus patas (patas), Macaca mulatta (rhesus), Macaca fascicularis (cynomologus) and Cebus apella (cebus), are subjected to direct-acting and metabolism-dependent chemical carcinogens by transplacental or direct exposure. In some cases the carcinogen-treated animals are subsequently exposed to chemicals that promote the development of neoplasms in rodents. Mechanisms of organ and species differences in the effects of chemical carcinogens and tumor promoters among rodent and nonhuman primate species are investigated. DNA repair capacity and its inducibility in different tissues are being surveyed in this context. 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. Phenobarbital has been shown to promote development of hepatocellular neoplasms in patas monkeys after initiation by transplacental or direct exposure to nitrosodiethylamine even when as long as 4 years--the duration of infancy and adolescence in this species--elapsed between initiation and inception of promotion. This is the first demonstration of tumor promotion in a nonhuman primate species.

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

A. E. Palmer	Research Veterinarian	LCC	NCI
J. M. Rice	Chief	LCC	NCI
J. M. Ward	Chief, Tumor Pathol. & Pathogen. Section	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
P. J. Donovan	Chemist	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 precisely characterize neoplastic and selected nonneoplastic lesions from treated animals by light and electron microscopy, histochemistry, explantation to cell or organ culture, transplantation and by other procedures which may be appropriate. To attempt to demonstrate the phenomenon of tumor promotion in nonhuman primates and to determine whether cell and tissue specificities of tumor-promoting chemicals demonstrated in rodents are similar in nonhuman primate models.

Methods Employed:

Carcinogenic chemicals specifically selected or designed for a given study are purified after purchase, or synthesized de novo and characterized thoroughly by chromatographic and spectroscopic procedures. Radiolabeled compounds are similarly prepared as required. The carcinogens are administered in precise doses to non-pregnant or exactly timed-pregnant nonhuman primates (patas, rhesus, cebus or cynomolgus monkeys) and the treated animals and their offspring are followed carefully for the development of the tumor. Agents which previously have been demonstrated to promote tumors in rodents are similarly purchased, purified or synthesized de novo and administered to primates after completion of an initiating regimen of exposure to organ-specific carcinogens.

Tumor-bearing nonhuman primates are intensively monitored to study tumor growth, body weight and clinical pathological changes. Selected animals are evaluated for tumor markers such as alphafetoprotein. Selected tumors which may cause suffering are carefully evaluated on an individual basis, and may be surgically removed to reduce suffering and to prolong life. When tumors are judged to be inoperable, and to be causing suffering or threatening life, animals are killed by euthanasia. Thorough gross postmortem examinations are performed and all gross lesions plus major organs are evaluated by light microscopy. Selected neoplasms are evaluated by electron microscopy and/or are cultured by cell or explant culture in vitro, then transplanted to athymic (nu/nu) mice. Tumors are evaluated by histochemistry as appropriate to conclusively define histogenesis.

Major Findings:

Studies on carcinogenesis by ethylnitrosourea (ENU) and diethylnitrosamine (DEN) in the Old World monkey, Erythrocebus patas, have been continued and

in recipients at approximately those necessary for therapeutic efficacy (suppression of convulsions) in man. After 1.5 years of phenobarbital exposure, one male offspring exhibited rising levels of alpha-fetoprotein and at laparotomy was found to have multiple nodular masses in the liver, which on biopsy were identified as hepatocellular neoplasms. This observation has been confirmed in other animals, and indicates that (1) DEN is an effective transplacental carcinogen in nonhuman primates; (2) latent neoplastic cells may persist for years in primate liver without proliferating; and (3) these cells can be stimulated by phenobarbital to proliferate to form a tumor, indicating that at therapeutic doses this barbiturate is a tumor promoter in the patas monkey and that its promoting effects are comparable in regard to target organ and target cell in both rodents and this primate species.

The tumor-promoting potential of phenobarbital after the direct (intraperitoneal) exposure of juvenile male patas monkeys to DEN was studied. Nine animals were given mildly toxic doses of DEN (0.4 mmoles/kg at 14-day intervals) for 20 doses (cumulative dose of 7.3 mmoles/kg). Two deaths occurred, after 18 and 20 doses of DEN due to hepatotoxicity, and these animals had preneoplastic hepatic lesions. Sodium phenobarbital (NaPB) was administered in the drinking water at 15 mg/kg/day, beginning 14 days after the last dose of DEN. After 275 days of NaPB treatment there were significantly more adenomas and carcinomas in these animals than in those that received DEN only, which confirms the conclusion reached from the transplacental study described above.

Systemic strongyloidiasis which previously caused fatal disease among colony-maintained patas monkeys has not occurred within the colony within the past year. This is attributed to more rigid measures taken to exclude any possibility that infectious/parasitic diseases may enter the colony.

#### Significance to Biomedical Research and the Program of the Institute:

Research on animal models of human childhood neoplasms should provide an insight into the types of causative agents and modes of exposures responsible for childhood cancer. It is to be expected that natural selection would tend to eliminate genotypes in the human population which predispose individuals to the development of fetal neoplasms before attaining reproductive age, yet the incidence of embryonal neoplasia in childhood is relatively constant. Epidemiological studies have pointed to the occurrence of childhood neoplasms in association with certain types of congenital malformations which are not inherited and suggest that environmental agents, alone or in combination, may play a role in the induction of such neoplasms. The inducibility of tumors very similar to the pediatric tumors of man by chemical carcinogens in laboratory rodents and primates further supports this view. Most tumors induced transplacentally in rodents are of adult types and appear during adult life in individuals exposed in utero, resembling the human experience with diethylstilbestrol. The ENU studies in monkeys have provided experimental data indicating that both adult and pediatric tumor types develop in at least one species of primates in response to carcinogenic exposure in utero and suggest that chemical carcinogens may be involved in the prenatal genesis of pediatric and possibly certain adult types of tumors in man. The demonstration of the inducibility of uterine choriocarcinoma by chemical carcinogens, at low exposure levels, further illustrates the importance of preventing human exposure to carcinogenic chemicals during pregnancy in either the workplace or environment.

The phenomenon of tumor promotion, while well established in rodents, is based on very limited data from which to extrapolate to man. The generality of the phenomenon and the extent to which organ-specific effects can be predicted in one species on the basis of bioassays conducted in another remain to be established. There is for tumor promotion, as yet, no unifying conceptual hypothesis exploitable for interspecies comparison, comparable to the role of primary damage to DNA in mutagenesis and probably in neoplastic transformation by genotoxic chemicals. It appears from experiments in rodents that promotion, unlike tumor initiation, is not persistent and that the underlying toxic effects thus are not cumulative. If, as seems likely, tumor promotion plays a significant role in the development of human cancer, the requirement for continual exposure to the promoting agent may provide prevention strategies that are much more readily applicable than in the case of persistent and cumulative genetic toxicity.

#### Proposed Course:

Monkeys exposed to ENU or DEN either transplacentally or directly (after weaning) will continue to be observed for the development of tumors. In vivo studies will emphasize (1) further refinement of definition of periods of maximal prenatal susceptibility to direct-acting versus enzyme-activated transplacental carcinogens; and (2) the extent to which the enzymes, which activate different classes of metabolism-dependent carcinogens, can be induced by either the carcinogens or other agents in fetal, maternal and placental tissues at different stages of prenatal development. The possibility that the phenomenon of tumor promotion can be demonstrated in primates prenatally exposed to ENU, either by increasing the incidence of hepatocellular tumors by postnatal exposure to phenobarbital, chlorinated hydrocarbons, etc., will be further explored. DNA repair phenomena, especially alkyl-acceptor protein-mediated repair and its possible induction by exposure to alkylating carcinogens, will be studied in organs and separated cell types at different stages of prenatal and postnatal development to evaluate the possible roles of DNA repair phenomena in cell- and organ-specific carcinogenesis, especially as these vary among primate and available rodent species in response to metabolism-independent alkylating agents. Induced tumor DNA will be studied by transfection and hybridization to attempt to detect the expression of oncogenes. The possible role of aflatoxins, environmental carcinogens prevalent in the regions of the world notable for a high incidence of gestational choriocarcinoma, as causative agents for human choriocarcinoma will be explored by direct testing in pregnant patas monkeys.

#### Publications:

Binkerd, P. S., Hendrickx, A. G., Rice, J. M. and Palmer, A. E.: Embryonic development in Erythrocebus patas. Am. J. Primatol. 6: 15-29, 1984.

Rice, J. M.: Exposure to chemical carcinogens during pregnancy: Consequences for mother and conceptus. In: Proc. First World Conf. Trophoblast Neoplasms. New York, Plenum Press. (In Press)

Winterer, J., Palmer, A. E., Cicmanec, J., Davis, E., Harbaugh, S. and Loriaux, D. L.: Endocrine profile of pregnancy in the patas monkey (Erythrocebus patas). Endocrinology (In Press)



## CONTRACT IN SUPPORT OF THIS PROJECT

MELOY LABORATORIES, INC. (N01-CP-41016)Title: Resources for Transplacental Carcinogenesis in PrimatesCurrent Annual Level: \$274,866Man Years: 3.2Objectives:

This project is designed to demonstrate and characterize transplacental carcinogenesis in nonhuman primates, especially the Erythrocebus patas, an Old World monkey. Additionally, related phenomena are studied, including the increased risk of carcinogenesis in adult females exposed to chemicals during pregnancy, tumor promotion, and mechanisms of cell and organ specificities and of species differences in the effects of both chemical carcinogens and tumor promoters.

Major Contributions:

Ethylnitrosourea (ENU) has been shown to be a potent carcinogen in the rhesus (Macaca mulatta) and patas monkeys. In both species the fetus is more susceptible than is the adult, and this susceptibility is more pronounced during the first and early second 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 four 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.

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. 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 tumor incidence. The demonstration of tumor promotion in nonhuman primates provides significant evidence of the importance of this phenomenon to man.

Proposed Course:

Animals previously exposed to carcinogens will continue to be closely monitored for tumor development, and all tumors will be intensively studied. In addition, studies to demonstrate more precisely the varying sensitivity of the fetus during gestation are under way. Limited numbers of animals will be treated with agents known to be promotive in rodents, after limited transplacental exposure to carcinogens. The transplacental effects of chemicals other than ENU and DEN will be explored. The direct and transplacental carcinogenic effects of aflatoxin B<sub>1</sub> will be studied.

CONTRACT IN SUPPORT OF THIS PROJECT

MELOY LABORATORIES, INC. (N01 CP 25613)

Title: Tumor Promotion in Cynomolgus Monkeys (Macaca fascicularis)

Current Annual Level: \$116,793

Man Years: 1.5

Objectives:

This project is intended to demonstrate the phenomenon of tumor promotion in cynomolgus monkeys and to explore the promotive activity in this species of several chemicals known to promote tumors in rodents. The liver model was chosen because diethylnitrosamine (DEN) has been studied extensively and shown to be a predictable hepatocarcinogen in this species. Preliminary findings suggest that DEN initiates patas monkey liver when given intravenously or transplacentally. The effect of promoter compounds in vivo on liver metabolism, morphology and enzyme induction will be studied.

Major Contributions:

The contract is in its second year and preliminary studies have shown major differences in the in vivo response of cynomolgus liver as compared to Fischer rat liver to several chemicals known to promote liver tumors in rats.

A major portion of the colony is under study for DEN initiation followed by promotion with promoter compounds. Animals are being closely monitored for tumor development, both by gross examinations and serum alphafetoprotein levels. To date no tumors have been identified.

Proposed Course:

Study animals will be closely monitored for evidence of tumor development. Tumors may be surgically excised in selected cases to determine the effect of this procedure on developing tumors and to study the pathogenesis of the process. Whenever tumor-load is determined to be causing suffering or is life threatening, the animal will be killed by euthanasia. Tumors will be studied histologically and histochemically by light and electron microscopy, by cell or organ culture for growth characteristics and by transplantation into athymic mice. Selected tumors will be studied by DNA transfection and hybridization to determine the presence of and/or expression of oncogenes. Studies to determine the impact of chemical liver tumor promoters on the induction of liver enzymes in the cynomolgus monkey liver and to compare this behavior both to that of nonpromoters and to the effect of these chemicals on rodent liver are planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05093-06 LCC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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. J. Donovan	Chemist	LCC	NCI
	A. O. Perantonì	Microbiologist	LCC	NCI
	O. Barbieri	Guest Researcher	LCC	NCI
	L. F. Dove	Bio. Lab. Technician (Animal)	LCC	NCI

COOPERATING UNITS (if any)

Microbiological Associates, Inc., Bethesda, MD (M. L. Wenk)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701

TOTAL MAN-YEARS:

3.2

PROFESSIONAL:

2.2

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.)

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 defined medium for growth of rat and mouse ureteric bud epithelium in monolayer culture has been developed in which epiderman growth factor and selenium have proved essential and insulin, hydrocortisone, and transferrin have proved highly beneficial. 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 mutations at two to three loci (resistance to ouabain and 6-thioguanine, and to diphtheria toxin in the hamster) were assayed in vitro with simultaneous determination of survival ability. Organ specificity of induced gene mutation is being determined in embryonal cells isolated from organs of various species exposed in utero at comparable stages of gestation. Quantitative dose curves for transplacentally induced mutations were also obtained for selected carcinogens, including the polycyclic hydrocarbons, 7,12-dimethylbenz[a]anthracene, 3-methylcholanthrene, and benzo[a]pyrene, the nitroso compounds, N-nitrosoethyl (and methyl) urea and N-nitrosodiethylamine, ethyl carbamate, and methyl (and ethyl) methane sulfonate.

PROJECT DESCRIPTIONNames, 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	Microbiologist	LCC	NCI
O. Barbieri	Guest Researcher	LCC	NCI
L. F. Dove	Bio. Lab. Technician (Animal)	LCC	NCI

Objectives:

To identify and characterize those aspects of morphogenetic differentiation which modify the consequences of prenatal exposure to chemical carcinogens, especially in the nervous and genitourinary systems. The ultimate objective is to elucidate the control of expression of the neoplastic phenotype in transformed cells. To devise and apply improved quantitative selective mutation systems to embryonal and fetal primary cells in culture, 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 treated transplacentally at different stages of gestation with ENU; also to simultaneously determine survival ability. 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 determine the organ specificity in various species of gene mutation 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 in vitro quantitatively determined parameters with transplacental tumorigenesis data.

Methods Employed:

Gravid animals are injected with different doses of agents at precise periods of gestation. Primaries are made from either the whole carcass or selected organs of different species. After 24 hours fetal cells are isolated and then frozen over liquid nitrogen for later assay. Mutation frequencies to ouabain resistance, 6-thioguanine resistance, and where applicable, resistance to diphtheria toxin are determined by thawing the cells and growing them for an optimum expression time, then seeding for colony formation for survival studies or seeding them into the selective media for mutation determination. Cells utilized for transformation studies are grown for various periods after isolation from the fetus and then seeded in agar with and without promoter and simultaneously in wet media for survival determination.

Short- and long-term cell and organ culture techniques are developed and the features of tissue rudiments maintained therein characterized by histochemical, light microscopic, and ultrastructural techniques. Cultures of both normal fetal organ rudiments and selected tumors are utilized to explore the effects of morphogenetic differentiation and its induction on the behavior of tumors of

undifferentiated cell type, including nephroblastic tumors of the kidney. Tumors are induced transplacentally or by direct treatment in experimental animals to provide suitable material for study and are transplanted serially in appropriate recipients to develop standard, manipulable models for studies in vitro. Surgical procedures relating to tissue transplantation are adapted as necessary to study the capacity of various recipient sites to modify differentiation of selected transplantable tumors. Substances and tissues known to influence differentiation are combined with tumors and normal undifferentiated inducing tissues, and interactive events in the differentiation of normal and neoplastic tissues are characterized. All studies are performed in more than one species, selecting species (such as the rat and mouse) which can be readily manipulated in the laboratory and in which responses of selected organ systems (such as the kidney and brain) to chemical carcinogens during fetal life vary, extremely, both in quantitative responses to chemical carcinogens and in the nature of tumors induced.

#### Major Findings:

The methodology of mutation selection has been improved resulting in increased recovery of induced mutants. It was, therefore, possible to obtain quantitative dose response curves of transplacentally induced mutation by various chemical carcinogens, as reported previously. The sensitivity to transplacentally induced mutation at different stages of gestation has been determined for the Syrian hamster. In vivo studies are currently under way to determine dose responses and age-dependence of transplacental carcinogenesis in this species. The time of maximum sensitivity to transplacental mutation induction is presently being determined in the mouse and rat.

The relative potency of induced mutation seemed to parallel the transplacental efficacy of almost all compounds. The polycyclic hydrocarbon, 7,12-dimethylbenz[a]anthracene at 0.1 mmole/kg gave the largest transplacental effect, 1300-fold over the control. The direct acting carcinogen N-nitrosoethylurea at 1 mmole/kg induced mutation 500 times that of the control. Among other compounds for which an effect was determined were the following with the dosage given in parenthesis : N-nitrosodiethylamine (0.5 mmole/kg, 7X), ethylmethanesulfonate (0.5 mmole/kg, 120X), methylmethanesulfonate (0.5 mmole/kg, 45X), ethyl carbamate (5.0 mmole/kg, 7X), benzo[a]pyrene (0.1 mmole/kg, 17X), 3-methylcholanthrene (0.1 mmole/kg, 70X), and N-nitrosomethylurea (1.0 mmole/ kg, 330X).

The sensitivity of induced mutation during different stages of gestation was determined and does not follow the same periods of susceptibility as transplacental carcinogenesis. It seems to coincide more with the period of maximum susceptibility to embryo lethality and teratogenesis. At this time the cells have several distinguishing characteristics: they have extremely rapid division rates, the DNA content of the embryo increasing about 1000 times during this period; and they are participating in a series of morphogenetic events while undergoing a series of successive steps of individual differentiation. It would appear that other determinants for the origin of cancer might be appropriately sought as a simple mutagenic initiation is probably not sufficient. Efforts to understand the differences in the spectrum of renal tumors observed in the rat versus the mouse following transplacental treatment with carcinogens have been expanded. The observation that mouse kidneys develop only adult type tumors despite their age at exposure while

rats often manifest embryonal renal tumors supports the idea that differences in the developmental processes in these two animals may determine the pattern of neoplastic expression. Our approach to this problem has required expertise in microdissection of fetal metanephric rudiments. We now can routinely reproduce the classic separations of ureteric bud from metanephrogenic mesenchyme performed by Grobstein. In addition, we have found the appearance of gamma-glutamyl transpeptidase (GGT) activity in mouse and rat fetal kidneys to be an extremely useful marker for primitive renal proximal tubular epithelia.

The acquisition of these techniques now permits us to study those factors important in the induction and normal differentiation of fetal metanephric rudiments and also to investigate those factors which prevent completion of the normal process, resulting in expression of the nephroblastoma. Preliminary attempts to promote differentiation in rat nephroblastomas using homotypic or heterotypic inducers have been unsuccessful, but media conditions in these organ culture studies have not yet been optimized. The separated ureteric bud, for example, deteriorates rapidly in our organ culture media; however, supplementation of the media with several hormones stabilizes cellular integrity. Further experimentation will therefore be necessary to determine if the failure to induce nephroblastoma differentiation is simply a problem of culture conditions or instead an inability of tumor tissue to respond to the normal signals of differentiation. We have therefore devoted considerable effort to the development of a defined medium that would allow for the survival and growth of the homotypic inducer, the ureteric bud, in monolayer culture without loss of the bud cells' inductive capacity and which would also show no toxicity toward the tumor cells. We have accomplished this with both rat and mouse buds and found the cultured cells to require selenium and epidermal growth factor for survival and growth and to benefit from the presence of insulin, hydrocortisone, and transferrin for growth. Triiodothyronine and prostaglandin E<sub>1</sub> have little effect in our system. Despite their serial propagation in monolayer, these bud cells have not lost their ability to induce differentiation since cocultivation of bud cells with normal metanephrogenic mesenchyme is characterized by tubulogenesis in the mesenchyme. We are presently evaluating this process with markers for different segments of differentiated nephron. Because of their serial propagation, considerable biological material necessary for biochemical studies of cell surface factors important to differentiation is now available.

In the mouse, efforts to establish a renal embryonal tumor model continue still with limited success. Entire kidney rudiments or separated metanephrogenic mesenchyme from animals treated transplacentally with nitrosoethylurea have been implanted in adult syngeneic hosts but as yet without the appearance of tumors. Survivability of these microscopic implants is in question, however, leading us to initiate studies in which transplacentally treated mesenchyme is amplified through growth as a primary monolayer culture and then injected into syngeneic hosts.

#### Significance to Biomedical Research and the Program of the Institute:

Studies in rodents have shown that a fetus may be as much as two orders of magnitude more susceptible to carcinogens than an adult of the same species, strain, and sex. The precise reasons for this enhanced vulnerability are not clearly understood, and the fact remains unexplained that, in rodents, carcinogens acting on differentiating fetal tissues principally induce tumors of adult epithelial morphology. Many tumors

which develop as a consequence of transplacental exposure to carcinogens are morphologically identical to those inducible in adults. In the mouse kidney, for example, only adenomas and a few carcinomas originating from proximal convoluted tubules develop after prenatal exposure to carcinogens when the kidney is mostly undifferentiated. This suggests that the fundamental genetic damage inflicted on undifferentiated fetal cells does not preclude subsequent programmed differentiation. The fact that differentiation overrides expression of neoplastic transformation in a given organ system (e.g., the kidney) of certain species such as the mouse, but does not do so in others such as the rat, provides a route to exploration of the basic nature of cellular differentiation to the control of neoplastic growth in the context of prenatal susceptibility to carcinogens.

The ability to determine experimentally *in vitro*, the relative potency of transplacental carcinogens to induce mutation in the somatic cells of the fetus is an important methodological advance. Since susceptibility to carcinogens during this time period is greatly increased relative to adults, prenatal testing of putative carcinogens has sometimes been advocated. However, the cost and difficulties of transplacental carcinogenesis experiments would be prohibitive except in some cases. This *in vivo/in vitro* method would partially fulfill this need.

Second, there is at present no clear explanation of the vast differences in susceptibility to transplacental carcinogens among different organs of different species. Also, vulnerability is specifically time-dependent, being nil in periods before organogenesis and rising to a maximum just before birth. The fundamental question posed by both observations is whether the resulting transplacental tumor incidence is proportional to genetic damage as measured by mutation frequency. One of the alternative explanations is that the genetic damage inflicted by the mutagen initiates, but the controlling factor in tumorigenesis is the process of differentiation and anything that influences that process.

#### Proposed Course:

Rat renal "blastema-cell" tumors will continue to be studied in transplantation and in cell and organ cultures to determine whether the morphologically undifferentiated tumor can be induced to form characteristic epithelial structures resembling renal tubules and whether the enzymes characteristic of renal epithelium will develop as markers of morphologically demonstrable differentiation. In organ culture, both natural (ureteric bud) and heterotopic (fetal spinal cord) inductive tissues will be used as potential inducers as well as chemical agents which are known to affect other *in vitro* systems in which morphogenetic differentiation occurs (cAMP, IUDR, DMSO). Initially, the goal of this program is to determine whether the lesser tendency of fetal rat kidney (in comparison with that of the mouse) to form differentiated epithelial tumors after exposure to transplacental carcinogens is due to interspecies differences in cellular responsiveness to mediators of morphogenetic differentiation.

A major characteristic of the response of rodents to chemical carcinogens during intrauterine development is that susceptibility to neoplastic transformation generally is not demonstrable prior to completion of definitive organogenesis, which marks the beginning of the fetal period of development. True embryos, in which undifferentiated tissues are only beginning to form identifiable organ



rudiments, are subject to teratogenic damage but are not, in general, at risk for subsequent tumor development as a consequence of exposure to carcinogens during this stage of development. The question arises whether this indicates that potential neoplastic transformants are generated, but are prevented from expressing their neoplastic genotypes phenotypically by proliferation to generate a tumor. The latter might be accomplished through cell-cell interactions or other mediators of programmed normal differentiation to which cells altered by carcinogens are still responsive.

Mutagenesis in fetal hamster, rat, mouse, and eventually nonhuman primate tissues will be investigated systematically to establish whether genotoxic damage, demonstrable immediately by the mutation assays, correlates with organ-specific and age-dependent transplacental carcinogenesis by various agents in these species. A future project is planned using the fetal hamster in cell cultures from which morphologic transformation of mesenchymal cells is demonstrable. Prenatal hamsters will be subjected transplacentally to a carcinogenic dose of metabolism-independent carcinogen at different stages of development, from implantation of the blastocyst through late fetal life. Cultures prepared from the conceptuses thus exposed will be studied for the presence of transformed cells in an effort to demonstrate the presence of latent transformed cells in fetal tissues that appear refractory to carcinogenesis during early development. The fact that transformation is readily demonstrated in fetal hamster fibroblasts that originate from the soft connective tissues in which tumors are not seen following transplacental exposure to carcinogens strongly suggests that such an approach will be fruitful.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05157-05 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Biochemical Mechanisms of Organ Specificity in Chemical Carcinogenesis

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

PI: J. M. Rice Chief LCC NCI

Others: B. P. Chen Research Chemist LCC NCI

L. M. Anderson Expert LCC NCI

A. E. Palmer Research Veterinarian LCC NCI

R. G. Blasberg Medical Officer DCHPH NCI

## COOPERATING UNITS (if any)

Univ. of North Carolina, Chapel Hill, NC (D. Kaufman); Microbiological Assoc., Inc., Bethesda, MD (M. Wenk); Chemical Industry Inst. of Toxicology, Research Triangle Park, NC (J. Swenberg); Albert-Ludwigs-Universitat, Freiburg, Germany (P. Kleihues); St. Jude's Children's Hosp., Memphis, TN (T. Brent)

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

Perinatal Carcinogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

0.

## CHECK APPROPRIATE BOX(ES)

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

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

Factors that modify tissue responses to chemical carcinogens in different organ systems are studied to evaluate their contribution to changing susceptibilities to carcinogenesis in certain tissues during prenatal and postnatal development. Tissues and organ systems currently under study include the nervous system, kidneys, and liver. Modifying processes now being studied include excision repair of DNA, DNA repair mediated by alkyl acceptor protein (AAP), the changing cellular susceptibility to carcinogens during different stages of the cell cycle, and maternal vs. fetal metabolism in the bioactivation of transplacental carcinogens. An assay for functional repair of DNA damaged by mutagenic and carcinogenic agents or rendered partially apurinic by mild acid hydrolysis has been improved. In vitro repair of lac operon-containing bacteriophage DNA, using 30,000 x g supernatants from wild type *E. coli* plus all four deoxyribonucleoside triphosphates has been accomplished and is being extended to the use of comparable homogenates of fetal tissues and dissociated specific cell types to explore ontogeny of DNA capacity during prenatal development. Formation of O6 and N7 methylguanine adducts in DNA of fetal and maternal tissues from gravid rats given the transplacental carcinogen, procarbazine, but not in neonatal rats injected directly, provides evidence that maternal metabolic activation of the carcinogen is crucial for transplacental carcinogenesis by this agent.

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

J. M. Rice	Chief	LCC	NCI
B. P. Chen	Research Chemist	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
A. E. Palmer	Research Veterinarian	LCC	NCI
R. G. Blasberg	Medical Officer	LCHPH	NCI

Objectives:

To define the roles of biochemical processes that significantly modify the susceptibility of fetal and selected adult organs to chemical carcinogens. Current specific objectives include the following: to investigate the development of the capacity for excision repair and alkyl acceptor protein (AAP) mediated repair of DNA in fetal tissues, including brain and liver, during the course of intrauterine development and to evaluate the role of repair capacity or its absence in high prenatal susceptibility to tumorigenesis in these organ systems in different species; to evaluate the extent to which susceptibility of a specific target cell, the hepatocyte, varies in susceptibility to chemical carcinogens during different stages of the cell cycle, a factor of major importance in understanding the intrinsically high susceptibility to chemical carcinogens of fetal tissues which have high rates of cell division; and to investigate the mechanism of action of the antitumor agent and transplacental carcinogen, procarbazine.

Methods Employed:

Nucleic acid repair in organs taken directly from fetal tissues is accomplished by direct measurement of enzyme activities, such as apurinic endonuclease, which are required for DNA repair. Pregnant animals including both rodents and nonhuman primates are treated with various doses of carcinogens at precisely defined times during gestation; and at selected intervals after treatment, fetal organs are dissected under a microscope and dissociated for study. The cell cycle kinetics of the regenerating rat liver are determined, after subjecting F344 rats to a two-thirds partial hepatectomy, by pulse labeling the regenerating liver with thymidine, quantitating the incorporation of this DNA precursor by liquid scintillation counting of isolated DNA, and by evaluating the proportions of cells undergoing DNA synthesis and in mitosis through high resolution autoradiographic techniques. Rats are injected via the portal vein with selected doses of direct-acting carcinogens at precisely defined times after partial hepatectomy and the carcinogenic response, as well as the evolution of preneoplastic lesions, are determined as a function of time elapsed since partial hepatectomy and as a function of the stage of the cell cycle at which carcinogen treatment took place. Increased synchrony in regenerating liver is achieved by post-hepatectomy injection with hydrocortisone hemisuccinate at 5-hour intervals for a total of three injections. Procarbazinemethyl-<sup>14</sup>C was injected i.v. into timed pregnant and i.p. into newborn BD IX strain rats, in which procarbazine is known to be a transplacental carcinogen for the developing nervous system; DNA is isolated from target and nontarget maternal, neonatal and

fetal tissues, hydrolyzed, and analyzed quantitatively for the formation of alkylated purines by HPLC.

#### Major Findings:

The cytostatic drug, procarbazine, has previously been shown to be a potent trans-placental neurotropic carcinogen in rats. Following a single i.p. administration of (<sup>14</sup>C-methyl)procarbazine (110 mg/kg) on the 22nd day of gestation, methylation products in cellular DNA were determined in fetal and maternal rat organs. The concentration of the major adduct, N7-methyl-guanine, was highest in the maternal liver (224 mol/mol guanine). Fetal and nonhepatic maternal tissues exhibited significantly lower levels but differed little from each other. In brain, lung, intestines and placenta the O<sup>6</sup>-methylguanine/N7-methylguanine ratio was close to 0.11, indicating that procarbazine like other methylating carcinogens initiates malignant transformation via methyl diazonium hydroxide as the ultimate reactant. Following a single dose of (<sup>14</sup>C-methyl)procarbazine to newborn animals, methyl-purine values were 30 to 60 times lower than after prenatal administration. This suggests that DNA alkylation in nonhepatic tissues occurs by systemic distribution of a proximate carcinogen formed in the adult rat liver.

The DNA functional assay for apurinic/apyrimidinic endonuclease and for other enzymes essential for DNA repair in specific tissues has been improved, and in vitro repair of lac operon-containing apurinic bacteriophage DNA probes has been accomplished using 30,000 x g supernatants of wild type E. coli. Repair requires the presence of all four deoxyribonucleoside triphosphates. An effort is in progress to extend this methodology to extracts of fetal rodent and nonhuman primate tissues as a quantitative assay for DNA repair capacity in fetal tissues during prenatal development.

Studies on the effects of chemical carcinogens on the regenerating rat liver have shown that in synchronized hepatocyte populations in regenerating livers, susceptibility to neoplastic transformation by the alkylating agent, methyl(acetoxymethyl)-nitrosamine (DMN-OAc), is greatest in cells that are actively synthesizing DNA. Thus, the S phase of the cell cycle, especially its earlier portion, is a period during which there is markedly enhanced intrinsic cellular vulnerability to neoplastic transformation. DMN-OAc, an ester of the presumed reactive metabolite of dimethylnitrosamine, which is dependent on esterase for its activation rather than on any oxidative enzyme system, resulted in a high incidence of hepatocellular tumors, nodules, and foci, and caused practically no tumors in other organ systems with the single exception of the thyroid when injected into partially hepatectomized rats via the portal vein. Our experiments indicate that this is the ideal agent to use for cell cycle studies since levels of esterases do not change over the period of hepatectomy and regeneration in liver and labeling of DNA by DMN-OAc-C<sup>14</sup> is essentially constant, irrespective of the stage of the cell cycle in regenerating liver when the label carcinogen was given. Data from this study, previously reported as in progress, are being prepared for publication. An additional finding of great interest is apparent enhancement, by a promoting mechanism, of carcinogenesis in the thyroid by phenobarbital (see Project Number Z01CE05303-03 LCC), confirming findings recently reported in Japan of this second tissue in which the barbiturate is capable of systemic tumor promotion in rodents.

A preliminary survey, in collaboration with Dr. J. Swenberg, CIIT, Research Triangle Park, NC, of the interorgan and intercellular distribution and inducibility of AAP in rodents and nonhuman primates has continued. This modality of DNA repair is present at significantly higher levels in patas monkeys (see Project Number Z01CE05092 LCC) than in corresponding tissues of rats. This representative nonhuman primate species closely resembles man with regard to AAP levels in those tissues that have been investigated in both species.

In an application of transplacental carcinogenesis methods and results to other programs of cancer research within the NCI, specifically the pharmacokinetics of antitumor agents in the brain in collaboration with the Division of Cancer Treatment, transplacental exposure of Sprague-Dawley rats to nitrosoethylurea was used to generate primary cerebral gliomas for the study of regional blood flow and regional blood-to-tissue transport in these tumors.

#### Significance to Biomedical Research and the Program of the Institute:

Organ specificity in carcinogenesis by different classes of agents is of obvious importance to the problem of extrapolating between species and in understanding the spectrum of tumors ascribed to environmental causes in human beings. Most studies in this area have concentrated on the capacity of target cells to metabolize carcinogens to ultimate reactive forms. Other possible mechanisms have received little attention in comparison and deserve exploration. The biochemical basis of the well-established high fetal susceptibility to chemical carcinogens remains poorly understood. The fetus is clearly at greatly elevated risk from exposure to chemical carcinogens, a fact that must be considered in estimations of relative human risk from environmental exposure to such agents. The factors that contribute to this enhanced susceptibility remain to be adequately evaluated. The role of DNA repair processes, best shown by enhanced susceptibility to UV carcinogenesis in individuals deficient in such repair as a result of the hereditary condition, xeroderma pigmentosum, indicates that repair is significant in controlling the consequences of damage to cells inflicted by at least some carcinogenic agents, but the development of this capacity during prenatal life has been studied very little. It could easily contribute, in part, to the susceptibility of fetal tissues which may be deficient in activity or fidelity of DNA repair.

It has long been known that dividing cells are more intrinsically susceptible to chemical carcinogens than post-mitotic cells or cells that are not cycling. The exact reasons for this are not clear; a reasonable hypothesis is that DNA may be more vulnerable to damage in certain phases of the cell cycle, and that in cycling cells, there is a greater probability that DNA repair will not be completed before programmed DNA synthesis encounters a defective portion of the genome bearing a carcinogen-induced lesion. Whether, in fact, cells are especially vulnerable to carcinogens at a particular stage of the cell cycle is thus of importance not only for perinatal carcinogenesis, but for carcinogenesis in adult tissues where mitotic activity is high, including various lining epithelia such as intestinal mucosa which constitute major sites of important neoplasms of man.

#### Proposed Course:

The prenatal development of the capacity for excision repair and for alkyl acceptor protein-mediated repair of DNA in liver, brain, and other tissues will continue to

be studied in order to allow meaningful comparison of species of experimental animals that differ greatly in prenatal susceptibility to carcinogens in various organ systems. Apurinic/aprimidinic endonuclease activity in various fetal rat tissues at different stages of prenatal development will be assayed in collaboration with Dr. Thomas Brent, St. Jude Children's Hospital, Memphis, TN.

Studies on the role of the cell cycle in susceptibility to carcinogenesis, and possibly other forms of genotoxic damage including mutation, will be continued. Techniques developed in the course of these studies, including characterization of the biology and morphology of liver cell tumors in rats resulting from a single transient exposure to an alkylating agent, will be applied elsewhere in the program of this Section. We plan to attempt to resolve apparent discrepancies between capacities for DNA repair in fetal liver and brain in rats and mice and the distinct differences in susceptibility to oncogenesis in these species.

Publications:

Blasberg, R. G., Kobayashi, T., Horowitz, M., Rice, J. M., Groothuis, D. R., Molnar, P. and Fenstermacher, J.: Regional blood flow in ethylnitrosourea-induced brain tumors. Ann. Neurol. 14: 189-201, 1983.

Blasberg, R. G., Kobayashi, T., Horowitz, M., Rice, J. M., Groothuis, D. R., Molnar, P. and Fenstermacher, J.: Regional blood-to-tissue transport in ethylnitrosourea(ENU)-induced brain tumors. Ann. Neurol. 14: 202-215, 1983.

Wiestler, O. D., Kleihues, P., Rice, J. M. and Ivankovic, S.: DNA methylation in maternal and fetal rat tissues following prenatal administration of procarbazine. J. Cancer Res. Clin. Oncol. (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05299-03 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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:	B. A. Diwan	Expert	LCC	NCI
Others:	J. M. Rice	Chief	LCC	NCI
	J. M. Ward	Chief, Tumor Pathol. & Pathogen. Section	LCC	NCI
	L. M. Anderson	Expert	LCC	NCI
	A. E. Palmer	Research Veterinarian	LCC	NCI
	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
	P. Blumberg	Chief, Molec. Mech. of Tumor Promotion Section	LCCTP	NCI

## COOPERATING UNITS (if any)

Microbiological Associates, Inc., Bethesda, MD (M. L. Wenk)

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

Perinatal Carcinogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

2.2

## PROFESSIONAL:

1.6

## OTHER:

0.6

## CHECK APPROPRIATE BOX(ES)

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

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

The phenomenon of tumor promotion in two-stage carcinogenesis was systematically explored in various rodent species in conjunction with transplacental carcinogenesis. Tumor-promoting activities of various pharmacologically defined classes of compounds (e.g., barbiturates, benzodiazepine tranquilizers) and environmental pollutants (e.g., the plasticizer, di-2-ethylhexylphthalate, and its major metabolites) are determined by sequential administration to animals of a transient, low level exposure to a genotoxic carcinogen followed by the test agent under study. The appearance of preneoplastic and neoplastic lesions is quantitated in target animal tissues. Organ specificities and interspecies correlations in tumor promotion are investigated for clues to the mechanism(s) of action of tumor promoters. Barbital has been found to promote carcinogenesis in the rat renal cortical epithelium, as well as in the liver and thyroid follicular parenchyma. Long-acting anticonvulsant/sedative activity correlates with tumor promotion by barbiturates, and organ specificity varies with the pattern of substitution at C-5. Tumor-initiating capabilities of transplacentally active agents in various fetal tissues are determined following postnatal exposure of prenatally-treated offspring to various topical and systemic promoters. Thyroid follicular neoplasms and preneoplastic hepatocellular foci appeared in rats exposed prenatally to nitrosomethylurea and given phenobarbital postnatally, demonstrating the persistence in these "non-target" tissues of latent neoplastic cells resulting from transplacental exposure to the carcinogen. Premalignant JB-6 mouse epidermal cells are used to investigate the mechanisms of tumor promotion by various drugs and environmental pollutants.

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

B. A. Diwan	Expert	LCC	NCI
J. M. Rice	Chief	LCC	NCI
J. M. Ward	Chief, Tumor Pathol. & Pathogen. Section	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
A. E. Palmer	Research Veterinarian	LCC	NCI
U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
P. Blumberg	Chief, Molec. Mech. of Tumor Promotion Section	LCCTP	NCI

Objectives:

1) To determine the cell- and organ-specific tumor-promoting effects of barbiturates, benzodiazepine tranquilizers and di-2-ethylhexylphthalate and its metabolites in various rodent species, 2) to investigate the relationship between the structure and promoting activity of barbiturates and other classes of tumor-promoting compounds, 3) to characterize and define the limits of their organ, strain and species specificities, 4) to demonstrate the occurrence of potentially neoplastic cells (initiated cells) in organs and tissues which appear refractory to tumor development by transient transplacental or systemic exposure to directly acting or pulse carcinogens alone, and 5) to employ promotable JB-6 mouse epidermal cells to investigate the mechanisms of the promoting action of these agents.

Methods Employed:

In transplacental initiation studies, precisely timed, pregnant mice, rats, and Syrian hamsters are exposed to chemical carcinogens at defined periods during gestation. Offspring derived from these mothers and young animals exposed to carcinogens by conventional routes (postnatal initiation) are subsequently exposed to nongenotoxic agents known or suspected to promote tumorigenesis in one or more organs. Preneoplastic proliferative lesions and neoplasms resulting from such treatments are identified and classified by histological, histochemical and ultrastructural parameters. For routine quantitative evaluations of preneoplastic focal proliferative lesions in the liver, an automated image analyzer (Videoplan, Carl Zeiss, Inc., N.Y.) and Zeiss Stereology Software are used. Premalignant JB-6 mouse epidermal cells are exposed to suspected tumor promoters and the progression of these cells to tumor cell phenotypes is measured by colony formation in soft agar at 14 days. Ultrastructural changes accompanying promoter-mediated phenotypic transformation are characterized by scanning and transmission electron microscopy (see Project Number Z01CE04812-16 LCC).

Major Findings:

The results of a completed study on the tumor-promoting abilities of four barbiturates in rat liver clearly showed that both phenobarbital and barbital are strong liver tumor promoters. No such promoting effects were observed with amobarbital or barbituric acid. In addition to the enhancement of liver tumorigenesis, barbital



promoted the development of renal epithelial tumors while phenobarbital promoted the development of thyroid follicular cell neoplasms in N-nitrosodiethylamine (DEN)-treated rats. It therefore appears that 1) barbiturates selectively promote carcinogenesis in certain epithelia, 2) their selectivity depends on molecular structure, 3) not all barbiturates promote, and 4) only long-acting agents are effective promoters. Substitution of both hydrogen atoms at the C5 position of the pyrimidine ring by alkyl or aryl groups appears to be essential but not sufficient for the tumor-promoting activity of barbiturates.

Studies performed in juvenile male mice (B6C3F1) showed that phenobarbital given during adolescence markedly inhibits the development of hepatocarcinogenesis (both preneoplastic and neoplastic lesions) induced by DEN exposure at birth. No such effects were seen in mice exposed similarly to amobarbital which neither inhibited nor promoted the development of focal hepatic lesions in DEN-pretreated mice. The hypothesis that the inhibitory effect on hepatocarcinogenesis is a consequence of androgen deprivation (feminization) resulting from enzyme induction and metabolic derangement by phenobarbital administration during the critical period of sexual maturation is being systematically tested.

The results of prenatal initiation and postnatal promotion experiments in rats proved that N-nitrosomethylurea (NMU), in addition to being a complete transplacental carcinogen for the rat kidney and nervous system, is a strong initiator of thyroid tumors in male offspring and also a relatively weak initiator of preneoplastic liver lesions in female offspring. Thyroid tumors and liver lesions were observed only in offspring that received NMU prenatally and phenobarbital postnatally. Thus prenatal administration of chemical carcinogens followed by postnatal application of tumor promoters can result in tumor formation at sites where no tumor would occur in the absence of promotion. Experiments are in progress using other topical and systemic promoters (i.e., 12-O-tetradecanolyphorbol-13-acetate [TPA], saccharin, barbital and clofibrate) to test whether NMU and other directly acting genotoxic agents administered transplacentally can initiate carcinogenesis in skin and other internal organs of rats.

Preliminary results from studies in adult mice suggest that different inbred strains significantly differ in their responses to two-stage liver carcinogenesis initiated by DEN and promoted by phenobarbital. Thus, the DBA/2 strain of mice was the most susceptible while NFS/N and C57BL/6 were the most resistant; the strain, C3H/He, showed intermediate responsiveness. Studies are in progress to investigate the biochemical mechanisms responsible for these strain differences in mice.

The plasticizer, di-2-ethylhexylphthalate (DEHP), a confirmed promoter of hepatocellular carcinogenesis in mice, was found to transform the promotable mouse epidermal JB-6 clonal lines, C141, C121, and R219. However, DEHP did not initiate or promote skin carcinogenesis in female CD1 mice. DEHP was also inactive as a complete promoter of skin carcinogenesis initiated by 7,12-dimethylbenz[a]anthracene (DMBA) in SENCAR mice. Like the plant-derived natural product, mezelein, however, DEHP significantly enhanced skin carcinogenesis in SENCAR mice when initiation by DMBA was followed by short-term applications (2x/week, 2 weeks) of TPA prior to application of DEHP. Thus DEHP promotes transformation in JB-6 cells and acts as a second-stage promoter in mouse skin. DEHP therefore has promoting capability for at least two distinct kinds of epithelium in mice. The possible mechanism

of DEHP promotion is being explored. Unlike other skin tumor promoters, however, DEHP does not compete for TPA receptors and may act through different mechanisms. Of the two major metabolites of DEHP tested, mono-2-ethylhexylphthalate promoted transformation of JB-6 cells but 2-ethylhexanol was ineffective.

#### Significance to Biomedical Research and the Program of the Institute:

Barbiturates are widely used as hypnotic, anticonvulsant and sedative drugs while benzodiazepine (oxazepam, diazepam) tranquilizers are routinely used therapeutically in the treatment of anxiety, tension and other minor emotional disorders. Concern over possible adverse effects of benzodiazepine tranquilizers arises because of reports that oxazepam produced liver neoplasms in mice and diazepam had neoplasm-promoting activity. The demonstration of tumor-promoting activity of therapeutic drugs at therapeutic dosage levels is of obvious significance to public health if such promoting action is not limited to rodent species only. Rigorous analyses of the relationship between the structure and promoting activity of various barbiturates and determination of their organ- and species-specificity may provide clues as to their mechanisms of action as tumor promoters. If organ-specificity and dosage requirements of these drugs prove to be consistent in all of the rodent species and many of the nonhuman primate species, it may be possible to predict the effects of such agents in man.

#### Proposed Course:

Systemic analyses of the relationship between structure and promoting activity of different barbiturates will be extended. To gain further insight into the relationship between their structure and promoting activity, barbiturates with appropriate molecular structures have been synthesized in the Chemistry Section of our laboratory and are being tested for their tumor-promoting activities. These experiments are designed to answer two specific questions: 1) Is long-acting/anticonvulsant activity characteristic of promoting compounds? 2) Do diastereomers differ in their promoting activity since they are known to differ in their pharmacological properties? Tumor-promoting activity and organ-specificity of each barbiturate will be investigated in at least three rodent species--mouse, rat and hamster. If organ-specificity of barbiturates varies with the structure and if it is consistent in all rodent species, These these studies will be extended to non-human primates. Biochemical mechanisms for genetic (strain) differences in tumor promotion will be determined. Studies employing promotable JB-6 cell models will be extended to the investigations of the mechanisms of tumor promotion by suspect drugs and environmental pollutants. These studies will be carried out in consultation and collaboration with other Sections in the LCC which are working on similar projects in tumor promotion: Office of the Chief, Tumor Pathology and Pathogenesis Section, and Ultrastructural Studies Section.

#### Publications:

Diwan, B. A., Rice, J. M., Ward, J. M., Ohshima, M. and Lynch, P. H.: Inhibition by phenobarbital and lack of effect of amobarbital on the development of liver tumors induced by N-nitrosodiethylamine in juvenile B6C3F1 mice. Cancer Lett. 23: 223-243, 1984

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01CE05301-03 LCC
PERIOD COVERED October 1, 1983 to September 30, 1984		
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:	M. Ohshima Visiting Scientist	LCC NCI
	C. Reynolds Senior Staff Fellow	BRMP NCI
	A. Palmer Research Veterinarian	LCC NCI
	J. Rice Chief	LCC NCI
	U. Rapp Chief, Viral Pathology Section	LVC NCI
COOPERATING UNITS (if any) Veterans Administration Hosp., Pittsburgh, PA (G. Singh); Dept. of Pathology, Harvard Univ. Medical School, Cambridge, MA (C. David); Pathology/Histotechnology Laboratory, Program Resources, Inc., Frederick, MD (R. Kovatch, F. Argilan)		
LAB/BRANCH Laboratory of Comparative Carcinogenesis		
SECTION Tumor Pathology and Pathogenesis Section		
INSTITUTE AND LOCATION NCI, NIH, Frederick, Maryland 21701		
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER: 0.
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The pathology and biology of experimentally induced and naturally occurring neoplasms of rodents are characterized and compared using serial sacrifice studies, immunocytochemistry, automated image analysis with stereology, conventional light microscopy, ultrastructure and histochemistry. Detailed histogenesis investigations were performed for mouse, rat, hamster and monkey liver, rat thyroid gland, mouse and rat lung, and rat pituitary gland. Hepatocellular carcinomas were found to originate in all species from initial focal proliferative hepatocellular lesions. Antibodies to surfactant apoprotein and Clara cell antigens allowed us to show that the vast majority of naturally occurring pulmonary tumors of rats and mice, and tumors induced by N-nitrosodiethylamine and N-nitrosoethylurea in mice and N-nitrosomethylurea in rats were of alveolar type II cell origin; no tumors of Clara cell origin were found. The avidin-biotin peroxidase complex (ABC) immunocytochemical technique was further developed for use in laboratory animals. Seventy-nine different antisera, including several monoclonal antibodies, were used to localize in tissue sections a variety of antigens including cell surface glycoproteins, oncogene-associated protein products, hormones, viruses, fetal antigens, enzymes and lysosomal proteins of large granular lymphocytes (LGL; natural killer cells). The latter has provided a diagnostic tool for LGL leukemia and a powerful method for studying disease processes that involve LGL. A new papovavirus was found in athymic nude rats. The virus caused a wasting disease characterized by salivary gland infection and pneumonia with intranuclear inclusion bodies. The disease was diagnosed by the localization of group-specific antigens in lesions with the use of the ABC immunocytochemical technique. Although no tumors were associated with viral infection, others have reported naturally occurring salivary gland tumors in nude rats. Infected material was given to three investigators for virus isolation, which has been unsuccessful to date.		

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

J. M. Ward	Chief, Tumor Pathol. and Pathogen. Section	LCC	NCI
M. Ohshima	Visiting Scientist	LCC	NCI
C. Reynolds	Sr. Staff Fellow	BRMP	NCI
A. Palmer	Research Veterinarian	LCC	NCI
J. M. Rice	Chief	LCC	NCI
U. Rapp	Chief, Viral Pathology Section	LVC	NCI

Objectives:

To characterize the biology and pathology of naturally occurring and experimentally induced tumors of laboratory animals.

To identify differences and significance of differences between naturally occurring and induced tumors.

Methods Employed:

Induced and naturally occurring tumors of laboratory animals, primarily rats, mice, hamsters and nonhuman primates, were characterized by conventional histological methods, histochemistry, immunocytochemistry, ultrastructural studies, serial sacrifice experiments and transplantation. During the past year the project concentrated on selected model systems to study the histopathogenesis of tumors. Particular emphasis was placed on application and utilization of the avidin-biotin peroxidase (ABC) immunocytochemical method for localization of marker antigens of tissues in various stages of tumor development. The ABC technique had to be modified to allow specific antigens to be detected immunologically in fixed tissue sections. In particular, oncogene-associated proteins (src, myc), intermediate filaments (vimentin, keratin), and alphafetoprotein required special fixatives or tissue handling for successful identification of these antigens in fixed tissue specimens.

Lung tumors potentially of alveolar or bronchiolar origin were studied in rats given a single injection of nitrosomethylurea (NMU). Rats were sacrificed from 16 to 52 weeks later and lungs were perfused with formalin. ABC immunocytochemistry was used with polyclonal antisera to rat surfactant apoprotein or Clara cell antigens, on fixed tissue sections from rats in this experiment. Lung tumors potentially of alveolar or bronchiolar origin were studied in mice injected with nitrosoethylurea or N-nitrosodiethylamine (DEN) or in control B6C3F1 or A strain mice.

Hepatocellular neoplasms were investigated using serial sacrifice techniques, autoradiography, histochemistry and immunocytochemistry. Quantitated image analysis and stereologic techniques allowed us to study the sequential histological changes in specific focal proliferative lesions. Mice or rats were injected once with DEN or N-ethyl-N-hydroxyethylnitrosamine (EHEN) and sacrificed at various times thereafter. Some groups of animals were also placed on diets with tumor promoters.

Representative portions of the liver were prepared and stained for histological features, autoradiography, histochemical localization of gamma-glutamyl transpeptidase, other enzymes and for immunoreactive alpha-fetoprotein. Livers from monkeys injected with DEN were also studied in a similar fashion.

Large granular lymphocyte (LGL) leukemia, a common natural disease of the F344 rat, is under intensive investigation. The cause, histopathogenesis and nature of the neoplastic cell was studied by a variety of techniques. Sodium nitrite has been found to inhibit the development of the natural disease. F344 rats were fed nitrite in the diet at 2000 ppm, a dose which inhibited the natural development of leukemia in old rats. Rats were sacrificed at various time intervals to study in vitro natural killer cell activity, and pathology of the lymphoid system. A polyclonal rabbit antiserum was prepared to rat LGL granules and used for localizing cells containing granules in various tissues, with ABC immunocytochemistry. The histopathogenesis of the natural disease was studied by immunocytochemical localization of OX-8 antigen with mouse monoclonal antibodies.

Nude rats developed a wasting disease. The cause of the disease was investigated by histological, immunocytochemical, ultrastructural and viral isolation techniques.

#### Major Findings:

Lung tumors and focal hyperplastic lesions induced by carcinogens in rats and mice invariably arose from alveolar type II cells as shown in morphological, ultrastructural and immunocytochemical studies. Surfactant apoprotein was always found in early (small) focal proliferative lesions and in the majority of neoplasms in both species. Clara cell antigen was never found in mouse lung tumors but was found in a few large lung tumors of rats. It was concluded that the majority of naturally occurring and chemically induced tumors of the lung of rats and mice are of alveolar type II cell origin.

Quantitative and histochemical analyses of proliferative focal hepatocellular lesions provided convincing evidence that hepatocellular carcinomas arise commonly within adenomas of rodent and monkey liver. However, specific markers of preneoplasia and neoplasia varied significantly among the species. Alpha-fetoprotein was found in adenomas and carcinomas of mice and patas monkeys but only in carcinomas of rats.

LGL leukemia was identified by immunocytochemical localization of granule antigens in fixed tissue sections, thus providing a valuable diagnostic tool. Similar antigens were also localized to other tissues including intestine, liver and kidney, probably representing lysosomal proteins in these tissues. The early stage of LGL leukemia was found to originate in the marginal zone of the spleen. Sodium nitrite had no effect on natural killer (NK) activity in blood or spleen during periods of exposure ranging from 5 days to 6 months. This suggests that nitrite may inhibit the development of LGL leukemia by preventing neoplastic progression, rather than by destroying LGL stem cells. A papovavirus was found to be associated with wasting disease in nude rats. The virus caused severe parotid salivary gland infection, intranuclear inclusion bodies and pneumonia. The virus was thought to be a member of the miopapovavirus subgroup of the papovaviridae family based on ultrastructure and immunocytochemical reaction with antibodies to disrupted SV40 virus. Efforts

at viral isolation and viral DNA characterization by three independent collaborating investigators were unsuccessful. *Pneumocystis carinii* infection was also found in three rats injected with NMU.

Significance to Biomedical Research and the Program of the Institute:

Rodent tumors are used by investigators for assessment of the carcinogenic effects of chemicals, studies on the mechanisms of carcinogenicity or the role of modifiers of carcinogenesis, and in safety tests for chemicals required by U.S. regulatory agencies. Knowledge of the nature, morphology and natural history of both natural and induced tumors is necessary for careful and accurate evaluations of, and regulatory decisions based on, these animal experiments and their uses.

Proposed Course:

Particular emphasis will concern the localization and expression of oncogene-associated protein products in fixed tissue sections at the various stages of carcinogenesis, in part by collaboration with other investigators within the LCC (see Project Number Z01CE05399-01 LCC). The ABC immunocytochemical technique will be extended to demonstrate specific antigens at different stages in the carcinogenic process with the aim of providing markers of neoplasia. Immunocytochemistry will also be utilized to characterize more accurately the induced and naturally occurring neoplasms of rodents.

Publications:

Goodman, D. G., Ward, J. M., and Reichardt, W. D.: Splenic fibrosis and sarcoma in F344 rats fed diets containing aniline, P-chloroaniline, azobenzene, o-toluidine, 4,4'-sulfonyldianiline or DaC red 9. JNCI (In Press)

Hall, W. C. and Ward, J. M.: A comparison of the avidin-biotin-peroxidase complex (ABC) and peroxidase-anti-peroxidase (PAP) immunocytochemical techniques for demonstrating Sendai virus infection in fixed tissue specimens. Lab. Anim. Sci. (In Press)

Losco, P. and Ward, J. M.: The early stage of large granular lymphocyte leukemia in the F344 rat. Vet. Pathol. 21: 286-291, 1984.

Ohshima, M., Ward, J. M., Brennan, L. M. and Creasia, D. A.: A sequential study of liver carcinogenesis in male F344 rats induced by methapyrilene hydrochloride. JNCI 72: 759-768, 1984.

Reynolds, C. W., Bere, E. W., Jr., and Ward, J. M.: Natural killer activity in the rat. III. Characterization of transplantable large granular lymphocyte (LGL) leukemias in the F344 rat. J. Immunol. 132: 534-540, 1984.

Reynolds, C. W. and Ward, J. M.: LGL lymphoproliferative diseases in man and experimental animals. A summary of these cells, their characteristics and potential experimental uses. In Lotzova, E. and Herberman, R. B. (Eds.): Immunobiology Of Natural Killer Cells. West Palm Beach, CRC Press (In Press)

Ward, J. M.: Morphology of potential preneoplastic hepatocyte lesions and liver tumors and a comparison with other species. In Popp, J. A. (Ed.): Current Perspectives in Mouse Liver Neoplasia. Washington, D.C., Hemisphere Press (In Press)

Ward, J. M.: Proliferative lesions of the glandular stomach and liver in F344 rats fed diets containing Aroclor 1254. Environ. Health Perspect. (In Press)

Ward, J. M., Argilan, F. and Reynolds, C. W.: Immunoperoxidase localization of large granular lymphocytes in normal tissues and lesions of athymic nude rats. J. Immunol. 131: 132-139, 1983.

Ward, J. M., Hamlin, M. H., II, Ackerman, L. J., Lattuada, C. P., Longfellow, D. G. and Cameron, T. P.: Age-related neoplastic and degenerative lesions in aging male ACI/SeghapBr rats. J. Gerontol. 38: 538-548, 1983.

Ward, J. M., Lock, A., Collins, M. J. Jr., Gonda, M. A. and Reynolds, C. W.: Papovaviral sialoadenitis in athymic nude rats. Lab. Anim. Sci. 18: 84-89, 1984.

Ward, J. M. and Lynch, P. H.: The transplantability of naturally occurring benign and malignant neoplasms and age-associated nonneoplastic lesions of the aging F344 rat as biologic evidence for the histologic diagnosis of neoplasms. Cancer Res. 44: 2608-2615, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05303-03 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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 LCC NCI  
Pathogenesis Section

Others: M. Ohshima Visiting Scientist LCC NCI  
B. A. Diwan Expert LCC NCI  
A. E. Palmer Research Veterinarian LCC NCI  
J. M. Rice Chief LCC NCI

## COOPERATING UNITS (if any)

Microbiological Associates, Inc., Bethesda, MD (Drs. M. Wenk and F. Spangler)

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

Tumor Pathology and Pathogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

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

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

The pathogenesis and promotion of tumors were studied using a mouse and rat liver initiation-promotion system, a nitrosomethylurea (NMU)-induced thyroid tumor system in rats, skin painting studies in mice, and an aged F344 rat liver model system. Tumor promotion can be an irreversible biological process which may require only a short period of exposure to the promoter for effective tumor promotion. For example, in the initiated skin of Sencar mice after only two exposures to 12-O-tetradecanoylphorbol-13-acetate (TPA), effective skin tumor promotion was seen. Tumors promoted after only short-term exposure to TPA grew progressively and did not regress after exposure to TPA was terminated. In the thyroid gland, withdrawal of a goitrogenic iodine-deficient diet at various time periods after exposure to NMU allowed some of the promoted proliferative lesions to progress to large tumors. In mouse liver, the tumor promoter di(2-ethylhexyl)phthalate (DEHP) was effective as a tumor promoter after only 28 days of exposure while phenobarbital (PB) was only effective after continuous exposure. DEHP was found to promote the development of liver tumors in mice but not in rats although it is a complete carcinogen for the liver in both species. DEHP caused liver enlargement, hyperplasia, and peroxisomal proliferation in both species; these processes thus cannot be sufficient as possible mechanisms for tumor promotion in this system. Aged F344 rats, which have naturally occurring gamma-glutamyl transpeptidase (GGT)-negative focal proliferative basophilic hepatocellular lesions in their livers, were given PB in their water to determine the role of the promoter on these naturally occurring foci. PB was found to induce focal eosinophilic GGT-positive, hepatocellular foci de novo and not to promote the growth or increase the incidence of the naturally occurring basophilic foci.



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

J. M. Ward	Chief, Tumor Pathol. and Pathogen. Section	LCC	NCI
M. Ohshima	Visiting Scientist	LCC	NCI
B. Diwan	Expert	LCC	NCI
A. E. Palmer	Research Veterinarian	LCC	NCI
J. M. Rice	Chief	LCC	NCI

Objectives:

To characterize the sequential morphologic and biologic steps in the development of cancer, and to systematically study the nature of cellular and organ specificities and species differences in response to tumor promoters.

Methods Employed:

The role of initiation and short-term irreversible or continuous tumor promotion in the development of neoplasia is under investigation using selected model systems including mouse and rat liver, rat thyroid and mouse skin. The mouse liver initiation-promotion system developed by us was utilized for study of the nature of tumor promotion by di(2-ethylhexyl)phthalate (DEHP) and phenobarbital (PB). B6C3F1 mice were injected at 4 weeks of age intraperitoneally with N-nitrosodiethylamine (DEN) in tricaprylin at a dose of 80 mg/kg. One week later the mice were placed on diets containing the phthalate or water with phenobarbital for 0, 7, 28, 84 or 168 days. After 24 weeks in the experiment, the animals were sacrificed, eight representative sections of liver were fixed, embedded in paraffin, sectioned and stained. Focal hepatocellular proliferative lesions were evaluated using an automated image analysis system. A similar experiment was performed with F344 rats. Since DEN also induces lung tumors in mice, and kidney, lung and nasal tumors in rats, these organs were also studied for a possible effect of the tumor promoters.

The role of dietary iodine deficiency and goiter is being studied in F344 rats. At six weeks of age, rats received nitrosomethylurea (NMU) intravenously at a dose of 41 mg/kg. Two weeks later, the rats were placed on the Remington iodinedeficient test diet for 8, 12, 16, 20, 24, 28, or 32 weeks and sacrificed at various time intervals up to 32 weeks. At necropsy, the thyroid glands were weighed and multiple histologic sections were prepared for computerized image analysis and histologic evaluation. Gross and microscopic findings in other organs were also studied since NMU induces tumors in numerous tissues. The role of PB in thyroid tumor promotion was studied by histologic, hormonal and biological studies.

The effectiveness of short-term exposure to tumor promoters and tissue sensitivity of Sencar mice to carcinogens is being studied in Sencar mouse skin. Sencar mice were painted with 7,12-dimethylbenz[a]anthracene (DMBA) once at 7 weeks of age. Two weeks later they were painted with 12-O-tetradecanoylphorbol-13-acetate (TPA) for 1, 2 or 4 exposures in 1 or 2 weeks. The skin tumors that developed were counted and measured for size weekly, to follow the progressive growth or clinical

regression of the tumors. At 24 weeks, all mice were sacrificed. In another experiment to compare the sensitivity of skin and internal organs to a carcinogen, BALB/c or Sencar mice were given one IP injection of DMBA followed by acetone or TPA on the skin. Mice were sacrificed at 52 weeks.

The origin of tumors induced in carcinogenesis experiments by classical tumor promoters is being investigated using an F344 model system we developed to study the effect of tumor promoters on spontaneous focal hepatocellular lesions. Aged male or female F344 rats 20-24 months of age were given PB in their drinking water for up to 10 months. Rats were sacrificed in groups or given tritiated thymidine, necropsied and liver lesions were quantified using image analysis and enzyme histochemistry.

#### Major Findings:

These studies provided evidence that tumor promotion in these systems and under certain conditions does not require continuous exposure to the tumor promoter for significant tumor promotion, i.e., promotion can be irreversible. DEHP was effective after only 30 days of exposure while phenobarbital required continuous exposure. While DEHP, however, was effective in mice after 28, 84 or 168 days of exposure, DEHP did not promote DEN-initiated foci after a continuous 112-day exposure in rats while PB was only effective after 112 days of exposure. Since DEHP induces liver enlargement, hepatocellular hyperplasia, increased hepatic DNA synthesis and peroxisomal proliferation in both species, it is suggested that these postulated mechanisms of tumor promotion are not sufficient.

Thyroid carcinogenicity, enhanced by dietary iodine deficiency for a continuous 32 weeks, was also enhanced significantly after only 16 or 20 weeks of dietary exposure, despite the fact that the goitrogenic thyroid hyperplasia and enlargement regressed soon after dietary supplementation with iodine. PB, a thyroid tumor promoter in rats, was found to have subtle but significant effects on thyroid hormone serum levels and histology.

Skin tumors in Sencar mice were promoted after only 2 or 4 exposures to TPA in 1 or 2 weeks. Papillomas developed in these mice and progressively grew in size despite lack of further exposure to the tumor promoter, TPA, after week 2. Although Sencar mice are uniquely sensitive to skin tumor promoters, the effectiveness of TPA after only 2 or 4 exposures suggests that some events responsible for promotion occur early and are irreversible in this system. Sencar mice were shown to be more sensitive to skin tumor development than were BALB/c mice after DMBA IP initiation and TPA promotion. BALB/c mice, however, were more responsive to internal tumor development than were Sencar mice.

PB induced a unique focal proliferative hepatocellular lesion in aged F344 male and female rats. The focal lesions were characterized by large hepatocytes with eosinophilic cytoplasm (hyperplasia of smooth endoplasmic reticulum) and phenotypically contained gamma-glutamyl transpeptidase. PB had no effect on the number or size of the spontaneous basophilic foci in rat liver or on DNA synthesis in the hepatocytes of these basophilic foci. These findings suggest that PB exerts its carcinogenic effect on rat liver by inducing de novo eosinophilic foci rather than by increase in growth and multiplicity of the spontaneous hepatocellular foci.

Significance to Biomedical Research and the Program of the Institute:

Tumor promotion is a unique phenomenon in biology. Its understanding and perhaps inhibition requires an understanding of the biological characteristics of tumor promoters. Since it is well known that some carcinogens may cause cancer in rodents after a single exposure, it is of utmost importance to define the nature and causes of the effectiveness of tumor promoters after short-term exposure. The mechanisms of induction of tumors by tumor promoters is also of utmost importance so that interspecies extrapolation to humans may be carried out for purposes of cancer prevention.

Proposed Course:

Using selected model systems, e.g., mouse and rat liver and rat thyroid models, we will more fully explore the role of tumor promoters in tumor promotion, especially after short-term exposures. We will also explore the biological and biochemical phenomena responsible for tumor promotion by DEHP in mouse liver but not in rat liver and the role of tumor promoters in tumor induction de novo or promotion of naturally occurring hepatocellular foci. This work is related to a complementary project (see Project Number Z01CE05299-03 LCC) in progress in the Perinatal Carcinogenesis Section, LCC, and to the study of tumor promotion in nonhuman primates in the Office of the Chief (see Project Number Z01CE05092-06 LCC).

Publications:

Ohshima, M. and Ward, J. M.: Promotion of N-methyl-N-nitrosourea-induced thyroid tumors by iodine deficiency in F344/NCr rats. JNCI (In Press)

Ward, J. M.: Increased susceptibility of livers of aged F344/NCr rat liver to the effects of phenobarbital on the incidence, morphology and histochemistry of hepatocellular foci and neoplasms. JNCI 71: 815-823, 1983.

Ward, J. M., Ohshima, M., Lynch, P. and Riggs, C.: Di(2-ethylhexyl)phthalate but not phenobarbital promotes N-nitrosodiethylamine-initiated hepatocellular proliferative lesions after short-term exposure in male B6C3F1 mice. Cancer Lett. (In Press)

Ward, J. M., Rice, J. M., Creasia, D., Lynch, P. and Riggs, C.: Dissimilar patterns of promotion by di(2-ethylhexyl)phthalate and phenobarbital of hepatocellular neoplasia initiated by diethylnitrosamine in B6C3F1 mice. Carcinogenesis 4: 1021-1029, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05350-02 LCC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

The Thymic Microenvironment During T-Cell Lymphoma Development

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

PI: U. I. Heine Chief, Ultrastructural Studies Section LCC NCI  
 Other: J. L. Junker Staff Fellow LCC NCI

COOPERATING UNITS (if any)

Pathology Institute, University of Cologne, Cologne, Germany (G.R.F. Krueger);  
 Biological Products Laboratory, Program Resources Inc., Frederick, MD  
 (E.F. Munoz)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Ultrastructural Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

0.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Maturation and proliferation of prethymic T-progenitor cells to mature lymphocytes depend on regulatory mechanisms in the thymus where the T-progenitors must interact with non-lymphoid, epithelial cells to be able to differentiate. The thymic epithelial cells provide a specific microenvironment capable of directing proliferation and maturation. It has been shown previously in mice that during the early phase of M-MuLV-induced lymphomagenesis, prethymic stem cells of the T-cell lineage derived from blood-forming tissues accumulate in the thymus where they encounter a differentiation block; subsequently, uncontrolled proliferation of the stem cells will lead to generalized lymphoma. The aim of this study is to elucidate the mechanism of the intrathymic differentiation block of prethymic lymphoid stem cells that gives rise to systemic malignant lymphoma of the Thy<sup>c</sup>-cell type. In vivo experiments, using the Moloney virus-induced lymphoma in the BALB/c mouse as a model, have been performed, and the phenotype and distribution of the major thymic cell populations have been characterized at different stages of tumorigenesis by light and electron microscopy. Immunofluorescence studies for the presence of thymopoietin II and serum thymus factor were carried out to determine the functional state of the epithelial cells. The results of our experiments show that the reticular epithelial cells of the thymus, which provide a microenvironment necessary for the differentiation of prethymic stem cells to lymphocytes of the T-lineage, are a prime target for transformation, as these cells undergo phenotypic changes, and are rendered functionally defective prior to lymphoma development. It is suggested that incompetent epithelial cells cause the progressive accumulation of non-differentiating T-cell precursors producing a dysregulative lymphoma. Evaluation of the thymic microenvironment in mice of different strains (AKR, C3H, C57B1, BALB/c) expressing varying capabilities for the induction of lymphoma indicates that the changes described above may be of wider significance for lymphomagenesis.

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

Ursula I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
James L. Junker	Staff Fellow	LCC	NCI

Objectives:

Our principal aim is to identify the role of the thymic microenvironment in T-cell lymphoma development. Special emphasis is directed towards the study of the thymic epithelial cells, as the latter may represent a critical target cell that by dysfunction initiates leukemogenesis.

Methods Employed:

Malignant lymphomas were induced by infecting newborn BALB/c HAN mice with 0.2 ml of Moloney murine leukemia virus suspension, 6.8 log FFU/ml. Infectivity studies were carried out at the University of Cologne, whereas the cell biology studies were pursued in this Laboratory. Thymic tissue, obtained from control and infected animals at biweekly intervals, was subjected to histological and cytological examinations using standard procedures for light, transmission and scanning electron microscopy. The quantitation of the cell population was performed on semithin sections in weekly intervals until tumor development. The functional state of the thymic epithelial cells was tested by identifying thymic hormone production by immunofluorescence using antisera against thymopoietin II and serum thymus factor. Comparative studies were carried out using mice of different origins, such as AKR, C3H, C57B1, and BALB/c.

Major Findings:

Current evidence shows that the thymic reticular epithelial cells have a major role in the maturation of prethymic stem cells of the T-cell lineage to functionally mature T-cells. Characterization of the reticular epithelial cells in the thymic cortex revealed in control and infected animals the presence of two differentiation stages of these cells classifiable as an immature type, resembling the epithelial cell at the periphery of the thymus primordium, and a mature cell type. Quantitation of the different cells during lymphoma development indicates a shift in the cell population as the number of mature reticular epithelial cells declines early during leukemogenesis, coinciding with a progressive degeneration of these cells. These events coincide with an elevation in the number of immature epithelial cells and the prevalence of a new epithelial cell type of hyperchromatic appearance. Concomitantly, immunofluorescence studies for thymopoietin II and serum thymus factor show a marked decrease of these hormones only in epithelial cells of infected mice. The accumulation of lymphoblasts in the thymic cortex of infected animals, denoting lymphoma development, was found to be secondary to the shift in the population of the reticular epithelial cells. Our data, concerning the chronological sequence of increase in as well as depletion of the different cell types in the thymus during leukemogenesis, which coincides with the appearance of deficiencies in the hormonal competence of the reticular epithelial cells, suggest that a

prime target for viral infection is the thymic reticular epithelial cell. The data are in agreement for both the Moloney murine leukemia virus-induced lymphoma and the AKR lymphoma. They appear to be specific for lymphoma development in general as thymi of mice expressing a low capability of lymphoma development do not show such shifts in cell population during the life cycle of the animal.

Significance to Biomedical Research and the Program of the Institute:

One of the missions of the National Cancer Institute is the elucidation of the mechanisms leading to tumor production and, consequently, the establishment of protocols for treatment. Research of animal models of human lymphomas provides insight into such mechanisms. The aim of this study is to investigate changes in the thymic microenvironment that may be causative for the development of T-cell lymphomas. Results of this study may serve as a basis for further investigations pertaining to mechanisms of cell-cell interactions during tumor promotion in general, which is a research area of major importance in this Laboratory.

Proposed Course:

Work is in progress to culture thymic epithelium and raise monospecific antibodies against a variety of epithelial cell proteins that may serve as markers in the characterization of functionally defective thymic epithelium. The availability of such markers will be of importance for the general research community.

Publications:

Heine, U. I., Krueger, G. R. F., Karpinski, A., Munoz, E. and Krueger, M. B.: Quantitative light and electron microscopic changes of thymic reticular epithelial cells during Moloney virus induced lymphoma development. J. Cancer Res. Clin. Oncol. 106: 102-111, 1983.

Heine, U. I., Krueger, G. R. F., Munoz, E. and Karpinski, A.: Altered thymic epithelial cells may be decisive for Moloney virus-induced lymphoma development. In Bailey, G. W. (Ed.): Proceedings of the Electron Microscopy Society of America. San Francisco, San Francisco Press, Inc., 1983, pp. 784-785.

Krueger, G. R. F., Karpinski, A., Heine, U. I. and Koch, B.: Differentiation block of prethymic lymphocytes during Moloney virus induced lymphoma development secondary to a thymic epithelial defect. J. Cancer Res. Clin. Oncol. 106: 153-157, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05351-02 LCC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

October 1, 1983 to September 30, 1984

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

PI: J. L. Junker Staff Fellow LCC NCI

Other: J. M. Ward Chief, Tumor Pathology and 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

TOTAL MAN-YEARS:

0.3

PROFESSIONAL:

0.2

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

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

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

Studies involve collaboration within the Laboratory on the characterization of neoplastic cells and the study of tumor histogenesis. The aim of the present investigation is to define by high resolution and immunoelectron microscopy changes in natural killer cells and targeted tumor cells which correlate with natural killer cell activity. Large granular lymphocytes (LGL) have been identified with natural killer cell activity. OX-8, a monoclonal antibody which reacts with a cell surfact antigen of LGLs and cytotoxic/suppressor T-lymphocytes, has been used in the immunoelectron microscopy of lymph nodes from nude rats which lack the T-cells. A postembedding staining procedure gave moderate preservation of lymphoid cell structure. Cells whose membranes were specifically stained by the antibody displayed characteristics consistent with their being LGLs (kidney-shaped nucleus, cytoplasmic granules). Nonlymphoid cells were, however, poorly preserved. Current investigations involve the establishment of a staining protocol which gives acceptable ultrastructural preservation of all cell types as well as specific antibody staining.

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

J. Junker	Staff Fellow	LCC	NCI
J.M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI

Objectives:

To identify at the ultrastructural level those cells which react with OX-8 antibody, a monoclonal antibody which is specific for a cell surface antigen on natural killer cells and cytotoxic/suppressor T-lymphocytes, so that this antibody can be used as a marker with which to study natural killer cell activity in nude rats. To establish in this Laboratory procedures for immunoelectron microscopy of intact tissue.

Methods Employed:

Immunoperoxidase staining was performed, using the avidin-biotin-peroxidase complex (ABC) system, on formaldehyde-fixed lymph nodes from nude rats. Sections 10  $\mu$ m were cut on a cryostat, thawed on a saline solution, and, following antibody staining, were prepared for transmission electron microscopy.

Major Findings:

The preembedding staining protocol produced adequate ultrastructural preservation. Nonspecific staining of membranes, however, was high, making definitive identification of specific staining difficult.

Significance to Biomedical Research and the Program of the Institute:

Development of a marker system for identifying natural killer cells in intact tissue at the ultrastructural level would be useful to the study of interactions between natural killer cells and tumor cells, and would, thereby, help in the understanding of natural defense mechanisms against cancer.

Proposed Course:

The results from preembedding staining of cryostat sections have been promising. Variations in the protocol will now be tested in order to optimize specific staining while minimizing nonspecific background.

This project serves to focus major collaborative undertakings between the Ultrastructural Studies Section and other Sections within the LCC, especially in the areas of ultrastructural pathology. Additional undertakings are anticipated as the need arises.

Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05352-02 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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:	O. Barbieri	Guest Researcher	LCC	NCI
	J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI

## COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (A. Dipple); Sloan-Kettering Institute, Rye, NY (I. Haller); University of South Florida, Tampa, FL (A. Giner-Sorolla); Lawrence Hospital, Bronxville, NY (J. Budinger)

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

Perinatal Carcinogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

1.8

## PROFESSIONAL:

1.0

## OTHER:

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.)

A multifaceted analysis of sensitivity factors in perinatal carcinogenesis is in progress. In a pharmacogenetic and pharmacokinetic study of transplacental carcinogenesis by methylcholanthrene (MC), genetic backcrosses were made to obtain, in the same mother, fetuses which were either inducible or noninducible for the enzymes which metabolize MC. The inducible fetuses from two dose groups exhibited a significantly higher incidence of lung tumors (two- to three-fold greater) than did those of the noninducible phenotype. This is the first direct demonstration of a determining role of enzyme inducibility in fetal susceptibility to a carcinogen. This finding will be confirmed and extended to other carcinogens and mouse strains and to noncarcinogenic enzyme inducers. Radioactive MC administered to pregnant mice attained high concentrations rapidly in the amniotic fluid and fetal blood and remained at high levels there, suggesting sequestration of the chemical in the fetal compartment. In an assay of the transplacental plus chronic lifetime exposure effects of the nitroso derivative of the drug cimetidine (nitrosocimetidine, NCM), no increase was seen in incidences of any neoplasm, but there was an apparent enhancement of lung tumor growth in females and lung tumor metastasis in males. This effect could be suggestive of either transplacental causation of lung tumors by NCM or direct effects on development of spontaneously-arising tumors. These possibilities will be tested in further studies in which NCM will be applied to lung and skin tumors initiated by other known carcinogens. New studies just starting include determination of promotion in suckling mice by polychlorinated biphenyls, and assessment of the transplacental neurogenic effects of nitrosamines in C3H mice, in an attempt to obtain an animal model for perinatal causation of brain tumors by nitrosamines.

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

L. M. Anderson	Expert	LCC	NCI
O. Barbieri	Guest Researcher	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI

Objectives:

To identify and describe the dynamic cellular, molecular, and organismal factors that determine the susceptibility of fetal and immature animals to carcinogens. Specific objectives for current projects are (1) determination of the role of maternal and fetal genotypes with regard to inducibility of aryl hydrocarbon metabolism in susceptibility to transplacental carcinogenesis by methylcholanthrene (MC); (2) establishment of transplacental pharmacokinetics of MC, as related to metabolism and tumorigenesis; and (3) assay of the transplacental plus chronic lifetime exposure effects of nitrosocimetidine (NCM), a DNA-alkylating derivative of a widely-used pharmaceutical drug.

Methods Employed:

The mouse strains used for the transplacental pharmacogenetics experiment were C57BL/6 (inducible for aryl hydrocarbon metabolism) and DBA (noninducible); in crosses and backcrosses of these strains, inducibility is inherited as a Mendelian dominant trait. (C57 x DBA) F<sub>1</sub> females mated with DBA males and DBA females mated with F<sub>1</sub> males were treated i.p. on the 17th day of gestation with one of several doses of MC or oil. Metabolic phenotyping of the progeny of these crosses was carried out at nine months of age by treatment with MC 48 hours prior to use of the livers in an isotopic assay for conversion of MC to primary and water-phase products. The rest of each mouse was subjected to complete necropsy and examined for tumors.

In a parallel pharmacokinetic study, <sup>14</sup>C-MC was administered i.p. to F<sub>1</sub> mothers bred to DBA males. After various intervals of time individual fetuses were taken for measurement by scintillation counting of placenta, amniotic fluid, fetal blood, liver, lung, and carcass, and maternal blood and liver.

In the assay of NCM, (C57BL/6 x BALB/c)F<sub>1</sub> mice were exposed to this agent in the drinking water at the usual human dose and at a dose ten times greater, from two weeks preconception through fetal and neonatal development and the remainder of their lives. Other groups were exposed to the precursors of NCM, cimetidine and sodium nitrite, singly or in combination. The mice were killed when moribund and examined for tumors.

Major Findings:

In the pharmacogenetic study, analysis of tumor incidence is nearly complete for the progeny of F<sub>1</sub> mothers receiving the two highest doses of MC (100 and 135 mg/kg). There has been a significant correlation ( $p < 0.05$  or  $0.01$ ) between average number

of tumors per mouse and of inducibility of the enzyme metabolizing MC: inducible ( $Ah^b/Ah^d$ ) $F_2$  offspring have exhibited on the the average two- to three-fold more lung tumors than  $Ah^b/Ah^d$  (noninducible) littermates. For some groups there has also been a similar significant difference in the percent of tumor bearing mice. This is the first direct demonstration that fetal enzyme-inducibility genotype is a determining factor in transplacental carcinogenesis.

In the parallel pharmacokinetic investigation,  $^{14}C$ -MC was found to attain a high concentration in amniotic fluid and fetal body fluids, and to remain high for the duration of gestation. Amounts in other fetal tissues were lower and reached peak levels at 24 to 36 hours. There was considerable variation in amounts of MC between litters and among individuals within the same litter.

Chronic administration of nitrosocimetidine to mice from early development until natural death did not result in an increase in the incidence of any neoplasm; there was a significant reduction in numbers of mammary tumors. However, the higher dose of NCM and of its combined precursors were associated with an unusually high incidence of very large lung tumors in females and of metastatic lung tumors in males.

#### Significance to Biomedical Research and the Program of the Institute:

The finding that mouse fetuses that are inducible with regard to MC metabolism are at increased risk for transplacental MC carcinogenesis is of considerable interest in light of the early appearance and wide quantitative variability of such enzymes in human fetal tissues. These results confirm indirect evidence from other studies that fetal sensitivity to carcinogens commences with ontogenetic appearance of activating enzymes. Further studies will reveal whether this correlation may be considered to be a general principle, and applicable to other types of carcinogens.

In the pharmacokinetic analysis, it was of particular interest that large amounts of the MC accumulated rapidly in the amniotic fluid and fetal body fluids and were retained there for a long period, even in a mother with inducible liver MC metabolism. Additional experiments will show whether an actual sequestration in these fluids takes place; if so, then exposure conditions of the fetus are qualitatively different from those of adults given the same dose, in a way that may greatly influence risk.

The assay of NCM confirmed that this important chemical, which could easily form in the human stomach and is a demonstrated alkylating agent in vitro, is not a tumor initiator in vivo. However, the possible effects of lung tumor growth and metastasis were of interest. This phenomenon might reflect transplacental initiation of some of the tumors, since lung tumors started in fetal lungs four to five days before birth grow more rapidly and metastasize more readily than do those induced in adult mice. An alternative explanation is that the NCM had a promotive or mutational effect on spontaneously-arising lung tumors. Either of these actions, if confirmed, would be an example of an important, unique effect of an alkylating agent that does not initiate tumors in adults.

Proposed Course:

The analysis of the results of the pharmacogenetic and pharmacokinetic experiments will be completed for both types of genetic backcrosses. In the next phase of the tumorigenesis aspect, the effects of the inducer,  $\beta$ -naphthoflavone, on the transplacental carcinogenic action of MC in the two types of crosses will be determined. The question to be asked is whether prior induction of the enzyme in mother and/or fetus, before exposure to the MC, will protect against or potentiate the tumorigenic effects. In the pharmacokinetic study, particular attention will be paid to the possibility of sequestration of unchanged carcinogen in the fetal compartment.

A possible effect of NCM on tumor growth and progression will be assessed first in adult mice, using lung tumors and skin papillomas induced by known effective carcinogens as targets. If negative results are obtained, NCM will be studied as a possible transplacental carcinogen.

Two new studies are being started. In the first, an attempt is being made to confirm that a single dose of polychlorinated biphenyls (PCBs) given to suckling mice may promote liver tumors initiated by dimethylnitrosamine (DMN) on the fourth day of life. Both numbers and sizes of preneoplastic and neoplastic liver lesions, and liver and body burdens of PCBs are being determined. In the second new project, transplacental effects of DMN and diethylnitrosamine are being tested in C3H/He mice, a species and strain which is both susceptible to transplacental induction of neurogenic tumors and endowed with fetal enzymes for activating nitrosamines. Nitrosamines cause few neurogenic tumors transplacentally in rats, but rat fetuses lack capacity for metabolic activation of these agents. Nitrosoureas are highly effective transplacental neurogenic carcinogens in rats. Recent epidemiological evidence has implicated nitrosamines in perinatal causation of brain tumors in children. Positive results from the experiment with C3H mice may establish a model for integration of animal experimentation with epidemiological suggestions.

Publications:

Anderson, L. M., Donovan, P. J. and Rice, J. M.: Risk assessment for transplacental carcinogenesis. In Flaherty, D. K. (Ed.): New Approaches in Toxicity Testing and their Application to Human Risk Assessment. Springfield, IL, C. C. Thomas Co. (In Press)

Anderson, L. M., Van Havere, K. and Budinger, J. M.: Effects of polychlorinated biphenyls on lung and liver tumors initiated in suckling mice by dimethylnitrosamine. JNCI 71: 157-163, 1983.

Dipple, A., Piggott, M. A. and Anderson, L. M.: 1,12-Dimethylbenz[a]anthracene-DNA adducts in cultured cells from mouse fetuses of different gestational ages. Cancer Lett. 21: 285-292, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05353-02 LCC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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

Other: J. M. Rice Chief, Perinatal Carcinogenesis Section LCC NCI

## COOPERATING UNITS (if any)

Temple University, Philadelphia, PA (G. Harrington and P. Magee); Litton Bionetics, Rockville, MD (A. Manus)

## LAB/BRANCH

Laboratory of Comparative Carcinogenesis

## SECTION

Perinatal Carcinogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.5

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

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

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

Three animal tumor models have been established for the study of modulation of sensitivity to carcinogens. In the first, athymic nu/nu mice have been found to be sensitive to skin tumorigenesis by a variety of agents, including dimethylbenz[a]anthracene (DMBA), DMBA followed by repeated application of the promoter, TPA, and repeated application of methylnitrosourea (MNU). Distribution on the body, latency, and histology of the tumors differed with each of these treatments: a single dose of DMBA resulted mainly in sebaceous adenomas distant from the treatment site; DMBA followed by TPA yielded in addition papillomas at the site; and MNU caused rapidly-growing carcinomas at the site only. Five to ten times more tumors were found on the nu/nu compared to the nu/+ mice with all treatments. This system will be used to investigate physiological and cellular factors determining sensitivity to skin carcinogenesis. In the second model, the role of induction of aryl hydrocarbon metabolizing enzymes in susceptibility to systemic carcinogenesis by methylcholanthrene (MC) was investigated using six mouse strains of varying inducibility. Highly inducible strains (C57BL and BALB/c) experienced few deaths (10-20%) six to nine months after the start of oral dosing with MC, whereas 70-90% of noninducible mice (DBA and AKR) dies with tumor during this period. Strains of moderate inducibility (Swiss and C3H) exhibited an intermediate number of deaths (30-50%). Treatment of the mice with the enzyme inducer, beta-naphthoflavone, prior to the MC resulted in reduced death rates for all strains. This project will be extended to other compounds, doses, and modes of administration, and may be useful in the design of practical cancer prevention measures for human populations. Thirdly, the high sensitivity of strain A mouse lung to rapid induction of tumors was used to confirm the carcinogenic action of a low dose (500 ppb in the water) of the environmental agent dimethylnitrosamine in mice fed different diets. This model will be further exploited to study modulation of dose-response and preneoplastic cellular changes during exposure to environmental levels of carcinogen.

PROJECT DESCRIPTIONNames, 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

Objectives:

To investigate reasons for differing susceptibilities to carcinogenesis among individuals and between organs, by analytical application of animal tumorigenesis models with unique qualitative or quantitative sensitivity to chemical carcinogens. Three such models have been employed. (1) Experiments with athymic nude (nu/nu) mice have been directed at elucidation of the role of thymus-dependent immune system function and/or normal skin factors in skin carcinogenesis by a variety of chemical initiators and promoters and UV light. The specific objective of the current phase of the project has been establishment of protocols for direct induction of tumors in nude mouse skin. (2) The importance of inductive increases in carcinogen-metabolizing enzymes, with regard to modulation of tumorigenic effects, has been studied in six inbred mouse strains which differ widely in their responsiveness to enzyme inducers. The specific objective of the experiment in progress has been to ascertain whether systemic carcinogenesis by oral methylcholanthrene (MC), with or without prior treatment with the noncarcinogenic enzyme inducer,  $\beta$ -naphthoflavone (NF), varies as a direct or inverse function of inducibility of aryl hydrocarbon oxygenase. (3) The high sensitivity of the lungs of strain A mice to tumor initiation permits experimental study of the effects of very low doses of environmental carcinogens. Tumorigenic effects of dimethylnitrosamine (DMN) at a concentration of 500 ppb in the drinking water have been examined in mice fed a variety of diets, and attempts have been made to detect steady-state levels of DMN in the blood of these mice.

Methods Employed:

Athymic nu/nu and phenotypically normal nu/+ mice (BALB/c background) have been maintained under pathogen free conditions. Ten mice of each sex and genotype were subjected to each of the following treatments: ethylnitrosourea intraperitoneally (i.p.) followed by weekly applications to the ears of 12-0-tetradecanoylphorbol-13-acetate (TPA); dimethylbenz[a]anthracene (DMBA) applied once to each ear; DMBA applied once to the ears followed by repeated applications of TPA; methylnitrosourea (MNU) applied once to each ear, with or without subsequent repeated treatment with TPA; repeated application of MNU; repeated exposure to ultraviolet light; or TPA treatment only. Detailed gross and histopathological examination of each skin is in progress. The role of genotype-related inducibility of aryl hydrocarbon metabolism in tumorigenesis has been investigated in C57BL and BALB/c (highly inducible), C3H and NIH Swiss (moderately inducible), and DBA and AKR (noninducible) female mice. The mice received repeated oral doses of MC, with or without prior i.p. treatment with the inducer NF. Strain A mice were given 500 ppb DMN in their drinking water or no treatment for periods of 12 to 18 weeks. They were fed Purina Laboratory Chow, or a modified Purina Mouse Chow, which was expected to result in an increase in DMN-induced lung tumors and fewer tumors in the controls,

compared to the Purina Laboratory Chow, because of the reduced amounts of nitrosamine-forming precursors and of nitrosamine-absorbing fibers in the Mouse Chow. Lungs were examined for tumor grossly and in 1-mm slices. Blood samples were analyzed for content of DMN by gas chromatography interfaced with a Thermal Energy Analyzer.

#### Major Findings:

Tumors were initiated on nu/nu skin by all of the treatments and in each group occurred in five- to twelve-fold greater abundance on these mice compared to identically-treated nu/+ mice. These differences were true for both ears (the site of chemical treatment) and the entire skin. Three treatment protocols were particularly effective in causing skin tumors, a single high dose of DMBA, a lower dose of DMBA followed by repeated applications of TPA, and repeated treatment with MNU. The patterns of distribution, latency, and histological characteristics were distinct for each of these groups. Tumors caused by a single high dose of DMBA began to appear within two months, were distributed over the entire body surface and only occasionally on the ears, and were in general slowgrowing, benign sebaceous adenomas or mixed tumors containing both sebaceous and squamous epithelial elements. In mice treated repeatedly with TPA after DMBA initiation, many papillomas developed in addition to the sebaceous tumors, and occurred particularly on the ears after a latency of five months or more. By contrast, repeated MNU treatment yielded papillomas and squamous cell carcinomas almost exclusively. These appeared late, after seven to eight months of treatment, but developed so rapidly that every mouse had to be killed with a carcinoma within three weeks after appearance of the first lesion. Their occurrence was limited to the site of treatment.

Results to date from the second model system, in which mice of six genetic strains of differing aryl hydrocarbon metabolism inducibility were treated with MC with or without prior exposure to NF, are summarized in the table below. The experiment was started in two phases, three months apart.

Strain	Inducibility	Time after First Treatment (months)	Number of Mice Still Alive (%) after Treatment with:		
			MC	NF then MC	NF or oil
C57BL/6NCr	++	9	27/30 (80%)	27/30 (90%)	15/15 (100%)
Swiss, [Cr:NIH(S)]	+	9	15/30* (50%)	17/30* (90%)	15/15 (100%)
DBA/2NCr	0	9	4/30* (13%)	13/30* (43%)	13/15 (87%)
BALB/cAnNCr	++	6	30/30 (100%)	29/30 (97%)	15/15 (100%)
C3H/HeNCr MTV-	+	6	24/30 (80%)	28/30 (93%)	15/15 (100%)

Strain	Inducibility	Time after First Treatment (months)	Number of Mice Still Alive (%) after Treatment with:		
			MC	NF then MC	NF or oil
AKR/NCr	0	6	10/30 (30%)	15/30 (50%)	15/15 (100%)

\*P<0.05, MC vs NF then MC

All except five of the MC-treated mice that have died have been grossly tumor-bearing, with lymphomas and mammary carcinomas being the most numerous. It is apparent from these preliminary results that rate of appearance of lethal neoplasms caused by oral MC is inversely related to inducibility of aryl hydrocarbon metabolism, at least for these six mouse strains. The noncarcinogenic enzyme inducer, NF, afforded protection against MC tumorigenesis, even in noninducible strains. Further analysis of the tumors of these mice, including numbers of primary lung tumors and forestomach papillomas, now in progress, will give more information about relative contributions of first-pass liver clearance and target organ effects.

In the third model system, strain A mice were exposed to 500 ppb DMN in their drinking water for varying periods and with different diets. The results confirmed that tumorigenicity of this low dose of DMN may be reliably demonstrated after the short exposure period of 16-18 weeks and with use of diets of widely different composition. Use of shorter exposure periods did not improve the tumor-incidence differential between control and treated mice. Neither did the Mouse Chow diet result in an improvement of the model, even though it was low in nitrosamine precursors and nitrosamine-absorbing fibers. No DMN was detected in the blood samples taken from these mice, with a limit of detection of 1 ppb, including blood pooled from tumor-bearing mice exposed to DMN for 16 weeks. This finding indicates that even though a tumorigenic dose of nitrosamine was clearly being delivered to a peripheral target organ, steady-state levels of the chemical could not be detected in the blood.

#### Significance to Biomedical Research and the Program of the Institute:

Enhanced susceptibility of nude mouse skin to carcinogenesis by chemicals and UV light, especially to topical administration of DMBA, DMBA/TPA, and MNU, demonstrated by our current experiment in progress, confirms this system as an important new model for analysis of sensitivity factors in carcinogenesis. It is of particular interest that the distribution, latency, and histological type of the tumors were unique for each treatment protocol. Thus the model is now ready for systematic exploitation in investigations of interactions of tumor-causing chemical regimen (type of chemical, single-stage carcinogenesis vs. initiation-promotion) with physiological and cellular determinants (absence of thymus gland nude mouse skin anomalies). A clear correlation has been seen, in six mouse strains, between early death due to MC-caused tumors and reduced or absent inducibility of the enzymes which metabolize MC. The public health relevance of this finding is apparent, since people are in general exposed systemically to agents such as polycyclic aromatic hydrocarbons, and are thought to show genetic variation in mixed function oxygenase inducibility. The protective effect of enzyme induction



in systemic carcinogenesis has long been established as a general principle, but has often been overlooked in both epidemiological and experimental animal studies. Application of this principle could be useful both in identification of individuals at risk and in creation of non-interventionary cancer prevention plans, such as ones involving diet modification.

Since people are exposed chronically to low doses of carcinogens, in the ppb range in food, cigarette smoke, commercial products, etc., it is useful to have a practical animal model in which tumorigenic effects of such low levels can be reliably quantified. In the current phase of our ongoing program utilizing the sensitivity of strain A mouse lung for this purpose, an attempt was made to maximize the demonstrated difference between DMN-treated and control mice by manipulation of diet and by modification of time of exposure. The various maneuvers did not result in a significant amplification of this difference, but they did confirm that a tumorigenic effect of 500 ppb DMN in the drinking water can be reproducibly demonstrated. Such an effect has now been shown in two animal facilities, with strain A mice from two sources, and in mice fed four widely differing diets. This model can now be considered to be established, and used for further investigations of tumorigenesis at low doses. The absence of detectable DMN in the blood of DMN-treated tumor-bearing mice is of interest in light of the considerable efforts made by other laboratories in analysis of trace amounts of nitrosamines in human blood. Our results suggest that lack of detection of such agents in the blood does not preclude delivery of tumorigenic doses to distal targets.

#### Proposed Course:

In the nude mouse skin tumorigenesis project, DMBA will be administered orally to nu/nu and nu/+ mice, to determine whether tumors, especially those with a prominent sebaceous component, may be caused by this mode of administration, as our current results suggest. In the next phase, experimental manipulations will be carried out in an attempt to discover whether the special sensitivity of the nude skin to tumorigenesis is due to absence of thymus gland or anomalies of the skin, or both. Thymectomies, thymus transplants, and reciprocal skin transplants between nu/nu and nu/+ mice will be carried out, followed by topical or systemic DMBA, DMBA/TPA, or MNU.

In the project focusing on the role of enzyme inducibility in tumorigenesis, analysis of tumor incidence and type for all target organs in each of the six strains will be completed and further experiments based on the results planned. Amounts of MC metabolizing enzymes may be measured in target organs of these strains and correlated with numbers of tumors. In the next experimental series, variation in the dose of both carcinogen and inducer, and chronic administration in the diet will be pursued. Other carcinogens, including ones to which people are exposed, and other inducers, especially ones which occur naturally in human foods, will be tried.

As part of the study of low-dose carcinogenesis in strain A mice, males will be fed the AIN-76 semisynthetic diet, which should be free of contaminating nitrosamines and which has been found to give the greatest differential effect when DMN-treated mice were compared with controls. Groups of 100 males will be given doses of DMN ranging from 1-500 ppb in the drinking water for 18 weeks, to determine the no-detectable-effect dose level and the shape of the dose-response curve

at low doses. In a second series, various doses of ethanol will be administered simultaneously with 500 ppb DMN. Since ethanol blocks liver metabolism of DMN, its presence should result in increased delivery of DMN to the lungs and enhanced lung tumorigenesis. In parallel experiments, formation and persistence of O<sup>6</sup>-methylguanine in DNA of target cells in the lung will be followed as a function of DMN dose and other experimental variables, by use of a sensitive HPLC-fluorimetric technique.

Publications:

Anderson, L. M., Priest, L. J., Deschner, E. E. and Budinger, J. M.: Carcinogenic effects of intracolonic benzo[a]pyrene in alpha-naphthoflavone-induced mice. Cancer Lett. 20: 117-123, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05399-01 LCC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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	Microbiologist	LCC	NCI
	S. Sukumar	Visiting Associate	LCC	NCI
	E. M. A. Santos	Visiting Fellow	LCC	NCI
	C. D. Reed	Sr. Health Services Ofcr.	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI

COOPERATING UNITS (If any)

Microbiological Assoc., Inc., Bethesda, MD (M.L. Wenk); Basic Research Program, Litton Bionetics, Inc., Frederick, MD (M. Barbacid); Program Resources, Inc., Frederick, MD (O.S. Weislow)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Developmental Biology and Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.5

OTHER:

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 the relationship of oncogene expression to progression from the normal to the neoplastic phenotype are to be studied using 3T3 transfection and hybridization techniques and monoclonal antibodies directed against the specific oncogene products. Four types of tumors will be generated by single injection of F344 rats using direct-acting alkylating agents: renal mesenchymal tumors induced by methyl(methoxymethyl)nitrosamine (DMN-OME), intestinal adenocarcinomas induced by methyl(acetoxymethyl)nitrosamine (DMN-OAc), hepatocellular carcinomas induced by intra-portal injection of DMN-OAc followed by phenobarbital promotion, and gliomas induced by transplacental exposure to nitrosoethylurea (ENU). In collaboration with Dr. Mariano Barbacid, DNA purified from these tumors will be utilized for 3T3 transfection assays, which are particularly effective for the detection of the *ras* oncogene, and in Southern blot hybridizations with available oncogene probes. Tumor DNA positive for an activated oncogene will be evaluated to determine whether the oncogene is of the wild-type or a mutant by restriction mapping and sequencing. Depending upon availability of oncogene products, hybridoma technology will be applied for the production of monoclonal antibodies specific for the product, and generated monoclonals will be used to characterize oncogene expression in preneoplastic, early and late stages of tumor development in vivo by immunohistochemical procedures. The demonstration of transfecting *ras* (Kirsten) genes in serially passaged rat mesenchymal renal tumors has been accomplished.

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

J. M. Rice	Chief	LCC	NCI
A. Perantoni	Microbiologist	LCC	NCI
J. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
C. Reed	Sr. Health Services Officer	LCC	NCI
S. Sukumar	Visiting Associate	LCC	NCI
E.M.A. Santos	Visting Fellow	LCC	NCI

Objectives:

To identify activated oncogene sequences in four specific types of chemically induced neoplastic rat tissues and to compare these observations with oncogene expression in normal, non-neoplastic tissues. To isolate and characterize the oncogene sequences found for 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.

Methods Employed:

Tumor generation: Four particular types of neoplasms in F344 rats are being induced to supply DNA for transfection experiments. These tumors include renal mesenchymal tumors induced by single neonatal i.p. injections of methyl(methoxymethyl)nitrosamine (DMN-OMe), intestinal adenocarcinomas induced by single i.p. injections of methyl(acetoxymethyl)nitrosamine (DMN-OAc) of 5 week-old animals, hepatocellular carcinomas induced by single intraportal injections of DMN-OAc to 100-gram rats which were subjected to partial hepatectomies 21-24 hours previously and subsequently promoted with phenobarbital in the diet, and gliomas of the brain induced by single transplacental exposure to nitrosoethylurea (ENU) at 15 days gestation. Each of these alkylating agents has a short half-life. Predictable latency periods after the single instillation until the development of the desired tumor and documented histogenetic sequences are associated with the development of each of these experimental tumor types.

Transfection experiments: DNA is purified from the various tumors and appropriate control tissues by standard protocols, precipitated with calcium phosphate, and incubated with a clone of NIH/3T3 cells known to be readily transfectable. Oncogene-positive clones will be isolated and will provide a source of DNA for oncogene sequence analysis, to be carried out in collaboration with Dr. Mariano Barbacid, Litton Bionetics, Inc., Frederick, Maryland.

Oncogene screening: Available clonal oncogenes will be radiolabeled and hybridized with tumor DNAs that have been digested with an appropriate restriction enzyme, separated by gel electrophoresis, and transferred to nitrocellulose. Any activated oncogenes found will be sequenced to determine their similarity to the wild-type allele.

Oncogene expression: Depending upon oncogene product availability monoclonal antibodies will be developed specifically for that product and will be used in immunohistochemical procedures to characterize expression of the oncogene during different stages of histogenesis in these chemically induced tumors.

Major Findings:

At present our efforts are directed primarily towards acquisition of techniques necessary for the pursuit of this study. However, a 3T3 transfection of DNA from serially passaged chemically induced renal mesenchymal tumors yielded positive foci for the ras oncogene. These foci have been cloned and found to contain the K-ras form of this oncogene. We have yet to determine if this oncogene represents a mutant or the wild-type allele.

Significance to Biomedical Research and the Program of the Institute:

The increasingly numerous genetic determinants that confer a neoplastic transformed phenotype on cells in which they or transition mutants of these oncogenes are highly expressed, strongly suggests a connection between abnormal genetic expression, including perhaps chemically inducible mutation or other forms of genetic damage, and the activity of certain growth factors in the evolution of at least some form of neoplasia. It is of great importance to establish what role specific oncogenes play in the evolution of tumors caused by known etiologic agents of human cancer, including both chemicals and radiation.

Proposed Course:

Systematically to explore by means of highly specific monoclonal/polyclonal antibodies the expression in experimental neoplasms of selected oncogenes, especially the family of 12th-codon mutant ras oncogenes that have been demonstrated in both human and animal neoplasms. Emphasis will be placed on distinguishing between expression at the earliest morphologically detectable stages of neoplastic proliferation (in which case the oncogene may be inferred to participate in neoplastic transformation) and acquisition of expression at a later stage of evolution of a tumor, as during an adenoma to carcinoma in situ transition or in metastases rather than primary neoplasms.

Publications:

Sukumar, S., Santos, E., Martin-Zanca, D., Arthur, A.V., Long, L. K. and Barbacid, M.: Transforming ras genes in human neoplasia and in chemically induced animal tumor systems. In Bishop, J. M., Greaves, M. and Rowley, J. D. (Eds.): Genes and Cancer, UCLA Symposia on Molecular and Cellular Biology. New York, Alan Liss, Inc. (In Press)

CONTRACT IN SUPPORT OF PROJECT NUMBERS:

Z01CE04580-10 LCC  
Z01CE04582-09 LCC  
Z01CE05353-02 LCC

LITTON-BIONETICS, INC. (N01-CP-31016)

Title: Holding Facility for Small Laboratory Animals

Current Annual Level: \$186,000

Man Years: 2.5

Objectives: The purpose of the contract is to provide animal holding facilities for rats, mice and hamsters treated with a variety of organic and inorganic chemical carcinogens and fed several different diets, together with technical support for administration of chemicals and necropsy of experimental animals.

Methods Employed: Standard carcinogenesis feeding and injection studies with metals and organic compounds are conducted. Initiation and promotion studies in rat liver are done by administering short pulses of the hepatocarcinogens, 2-acetylaminofluorene and N-nitrosodiethylamine. Promotion is performed by feeding phenobarbital or methionine antagonists. Typically, carcinogenesis studies are for 0.5 to 2 years. Preliminary toxicity studies are conducted to determine the appropriate carcinogen levels and dietary regimens to be employed. Other services include the routine weighing, termination, and necropsies of carcinogentreated animals.

Major Contributions: The carcinogenic activity of ethionine has been extended to three strains of mice. The simultaneous injection of magnesium carbonate with nickel subsulfide inhibits the sarcomagenic action of the latter in rats. The chronic administration of calcium acetate in the diets of rats receiving the renal carcinogen, lead subacetate, markedly enhanced the activity of the latter compound. The chronic administration of high dietary levels of methionine and choline in rats appeared to exert no significant effects on the liver tumor-promoting activity of phenobarbital and DDT in rats. Dimethylnitrosamine (DMN), at a dose of only 500 ppb administered in the drinking water for 16 weeks, was tumorigenic towards the lungs of strain A mice fed different chow diets, despite the absence of detectable DMN in their blood.

Proposed Course: The ongoing metals studies will be transferred to another laboratory. The contract will be terminated in September, 1984.

CONTRACT IN SUPPORT OF PROJECT NUMBERS:

Z01CE05157-05 LCC  
Z01CE05093-06 LCC  
Z01CE05299-03 LCC  
Z01CE05303-03 LCC  
Z01CE05399-01 LCC

MICROBIOLOGICAL ASSOCIATES (N01-CP-41014)

Title: Non-SPF Rodent Holding Facility for the Laboratory of Comparative Carcinogenesis

Current Annual Level: 2349,884

Man Years: 4

Objectives: The purpose of this contract is to provide support services for the Laboratory of Comparative Carcinogenesis for long-term holding, treatment and observation of rodents in carcinogenesis investigations emphasizing lifetime tumor induction in rodents and related activities. The contract is specifically utilized for conducting experiments that require species or strains of rodents not available from the Frederick Cancer Research Facility (FCRF) animal production area, since such animals cannot be introduced into the LCC animal research facilities at the FCRF.

Protocols are developed in collaboration with LCC investigators and approved by an LCC project officer. Protocols involve the preparation, handling and administration of chemical solutions to animals according to NCI guidelines for the safety of personnel; specifications for holding, treatment, and data collection (including gross pathology data) for mice, hamsters, rats and related species; administration of chemical carcinogens to animals by skin painting, gavage, parenteral injection or other routes; storage of labile animal diets, reagents, tissues or other materials under controlled temperature conditions; qualitative or quantitative analysis of carcinogen preparations or of tissues of carcinogen treated animals and other necessary details are provided by LCC investigators. All fixed tissue specimens from carcinogenesis studies are sent to FCRF for histology and evaluation by NCI pathologists.

Major Contributions: This contract has made possible research involving administration of chemical carcinogens to laboratory animals that could not have been accomplished at FCRF, including studies on the effects of retinoids on naturally occurring tumors of ACI rats, ENU in congenic strains of mice varying in their expression of murine retroviruses (in collaboration with Dr. Janet Hartley, NIAID), and carcinogenesis in mongolian gerbils, which vary strikingly from other rodents in their response to chemical carcinogens. It has also provided a means for continuing rats on long-term carcinogenesis protocols that were begun in another facility, enabling the LCC to terminate another comparable support contract (Litton Bionetics, Inc., N01-CP-31016) a year earlier than originally planned.

Proposed Course: To continue for the duration of the current contract to provide support to the LCC for studies requiring long-term holding of rodent species and

strains that cannot be accommodated at FCRF because they are not free of detectable potential rodent pathogens. During that time provision for a similar facility at FCRF will be developed to supplant services now provided by this contract, which will not be recompleted.



ANNUAL REPORT OF  
THE LABORATORY OF EXPERIMENTAL CARCINOGENESIS  
NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

The major goals of the Laboratory of Experimental Carcinogenesis (LEC) are to elucidate mechanism(s) of malignant transformation in human and animal cells by chemical carcinogens and other cancer causing agents; to determine critical cellular and genetic factors involved in initiation, promotion and progression of these transformed cells; and to apply, whenever possible, the knowledge obtained from these studies towards effective prevention of cancer in man. In order to obtain these goals, LEC plans, develops and conducts a research program that includes (1) identification and characterization of exogenous and endogenous factors controlling initiation, promotion and progression of chemically-induced tumors; (2) studies on the regulation of gene expression and differentiation in both human and animal neoplasia; (3) definition of the mechanism by which modifiers of cellular differentiation may inhibit and/or promote the neoplastic process; and (4) characterization of the metabolic processing and mutagenic potential of both known and suspected carcinogenic aromatic amines.

The LEC is a new laboratory that seeks to accomplish the goals listed above by bringing together expertise in the diverse disciplines of cell biology, chemical and viral carcinogenesis, molecular biology, protein and nucleotide chemistry, and computer science. The central hypothesis of research conducted by this Laboratory states that the neoplastic process, whether caused by chemical or biological agents, will not be defined without an integrated multidisciplinary research effort.

The Laboratory is composed of four sections, each of which is charged with major responsibilities towards the goals set out for LEC. Due to the integrated and multidisciplinary approach towards the understanding of the neoplastic process, considerable interactions occur between the sections. These areas of interaction are listed in the individual project reports.

The Chemical Carcinogenesis Section plans and develops laboratory research aimed at elucidating the mechanism(s) of malignant transformation in human and animal cells by chemical carcinogens and other cancer causing agents. The major efforts of the Section are focused on (1) identification and characterization of exogenous and endogenous factors controlling initiation, promotion and progression in chemically-induced murine hepatomas and human B-cell lymphomas; (2) applying advanced quantitative two-dimensional gel electrophoresis techniques of total cellular proteins to study protein changes during oncogenesis and to identify gene product(s) that are associated with the malignant transformation; (3) studying the metabolic processing and mutagenic potential of both known and suspected carcinogenic aromatic amines; and (4) definition of the mechanism by which modifiers of cellular differentiation may inhibit and/or promote the neoplastic process.

Metabolism and Mutagenicity of Chemical Carcinogens. Our work in this area has continued to focus on model carcinogenic aromatic amines and amides as well as on carcinogenic heterocyclic amines from pyrolysates of amino acids, meat and fish. Results so far obtained include (1) dose-dependent DNA binding and formation of individual DNA adducts (Gua-C8-AAF, Gua-C8-AF and Gua-N<sup>2</sup>-AAF) were observed in rat and mouse primary hepatocytes following exposure to N-hydroxy-2-acetylaminofluorene (N-OH-AAF) and N-acetoxy-2-acetylaminofluorene (N-OAc-AAF). The patterns of DNA adducts formed in vitro for N-OH-AAF were similar to those found in vivo. A positive correlation was found between the extent of DNA strand breaks, and the formation of either Gua-C8-AF or Gua-C8-AAF. (2) The data from studies of the heterocyclic amines 3-amino-1,4-dimethyl-5H-pyrido [4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1), 2-aminodipyrido-[1,2-a:3',2'-d]imidazole (Glu-P-2), and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)] indicate that cytochrome P-450-dependent N-hydroxylation of the heterocyclic amines is an obligatory step in the metabolic activation of these compounds in both subcellular and in whole cell systems; genotoxicity of these compounds quantitatively differ when measured in intact hepatocytes versus the Salmonella tester strain; and agents modulating the activity and the composition of the cytochrome P-450 system may greatly influence both toxicity and carcinogenicity of this compound in vivo; and (3) the results from a study comparing the capacity of human liver microsomes of 28 individuals to metabolize debrisoquine, bufuralol, aldrin and AAF indicate that common cytochrome P-450 isoenzymes are involved in the formation of AAF metabolites while the metabolism of debrisoquine, bufuralol and aldrin is unrelated to the metabolism of this carcinogen in human liver.

Mechanism of Chemically-Induced Murine Hepatomas. Progress continues to be made in our studies on the mechanism of chemically-induced murine hepatomas. Our efforts have been focused on (1) isolation and characterization of preneoplastic liver cell populations; (2) characterization of transplantation and growth of normal, preneoplastic and neoplastic rat hepatocytes in the anterior chamber of the eye of the isogenic host; and (3) modulation of cell surface receptors during chemically-induced hepatoma formation in the rat. Results obtained thus far include: (1) immunohistochemical studies using antibodies against the asialoglycoprotein surface receptor of normal rat hepatocytes have confirmed the lack of this receptor in preneoplastic areas in rat liver. The areas lacking the asialoglycoprotein receptor are entirely superimposable with glucose-6-phosphatase-deficient areas and partially overlapped with the gamma-glutamyltranspeptidase-positive areas in serial liver sections. (2) Due to the lack of asialoglycoprotein receptor on the surface of preneoplastic hepatocytes, an efficient method for separation of these cells from normal hepatocytes was developed using tissue culture plates coated with asialofetuin. Only normal cells attach, whereas preneoplastic and neoplastic cells do not. (3) In contrast to the reduction of asialoglycoprotein receptors during early stages of hepatocarcinogenesis, the surface receptor protein for transferrin is present at an increased level. (4) The transplantation to the anterior chamber of the rat eye offers a promising model to study attachment and growth of normal, preneoplastic, neoplastic and in vitro carcinogen-treated liver cells.

Regulation of Gene Expression and Differentiation in Neoplasia. We have continued to employ the techniques of molecular biology to examine gene expression during neoplastic progression as well as during normal growth and differentiation. Our aim is to identify and characterize both cellular and genetic factors that

are of critical importance in the oncogenic process. The experimental systems presently under study are (a) the human promyelocytic leukemia cell line HL60, (b) a variety of undifferentiated human B cells and B-cell lymphomas, and (c) the rat hepatoma cell line and primary hepatocytes. The results obtained in the HL60 cell line study include (1) functional association of c-myc expression with the differentiated state of the cell, and (2) expression of the 2.2-kb N-ras specific transcript is associated to some extent with the proliferative capacity of the cells. The results obtained and the experiments in progress in the human B-cell study include (1) chemical transformation of lymphoblastoid cell lines, (2) transfection of these "high grade lymphomas" into NIH 3T3 cells, (3) analysis of the transfection specific gene, and (4) two-dimensional gel analysis of proteins associated with the chemically-induced transformation state. The results obtained and the experiments in progress in the rat hepatoma and primary hepatocyte study include (1) partial construction of cDNA libraries which correspond to discrete stages in the neoplastic progression of rat hepatomas, (2) isolation of cDNA recombinant clones which are upregulated in actively proliferating rat liver (i.e., following partial hepatectomy) as compared to normal adult liver, and (3) characterization of the change in expression of a differentiation specific gene.

Analysis of Carcinogenesis and Differentiation by Two-Dimensional Gel Electrophoresis. Considerable progress has been made in improving the computer-based quantitative two-dimensional gel electrophoresis analysis. This type of electrophoresis allows the simultaneous separation of hundreds of polypeptides in a single polyacrylamide gel. We have acquired, and have significantly revised, a computer-based system to automatically analyze autoradiograms produced from these gels. We have also been successful in analyzing silver-stained gels. This system automatically finds and measures the intensity of any polypeptide resolved by these electrophoretograms. Newly developed programs automatically match together the spot patterns found in different gels. Still other programs link together a series of gels which may constitute an experiment, allowing the investigator to quantitatively follow the synthesis of any resolvable protein through that experiment, or series of experiments. The investigator may specify various parameters and ask the computer to list those spots whose pattern of synthesis may lie within or without those parameters. Finally, several sophisticated computer-graphics programs allow the investigator to visually compare and follow various polypeptides which may be matched on a virtually unlimited number of electrophoretograms. The ultimate aim of this facility is to develop a gel analysis system that is as completely automatic as possible in analyzing the gels involved in an experiment.

Several projects are ongoing that employ the two-dimensional gel electrophoresis facility. These include (A) examination of early events in chemically-induced hepatocarcinogenesis. Results obtained to date include: (1) Using the Solt-Farber initiation promotion protocol, early preneoplastic hepatocytes were induced in male rats, isolated, and then separated into "small" and "large" cell populations using elutriation centrifugation. Following carcinogen treatment two major constitutive polypeptides (pI 5.7, MW 50 kDa and pI 6.6/49 kDa) were not detected in either "small" or "large" cells. In contrast three new polypeptides (pI 6.5/53 kDa; pI 6.6/51 kDa; and pI 6.8/52 kDa) were expressed in both "small" and "large" cells but not in untreated control cells. "Small" and "large" showed no qualitative polypeptide differences. In contrast 8-10% of the 600-800 readily detected proteins were undergoing quantitative changes of at least four-fold during chemical carcinogenesis. (2) Hyperplastic nodules

(preneoplastic stage) were chemically induced in rat liver, individual nodules were dissected out, and then classified histologically as being either early preneoplastic, preneoplastic, or neoplastic. Only one minor cytosolic polypeptide (pI 6.75/31 kDa) found in normal rat liver was not expressed in either preneoplastic or neoplastic nodules. However, four new cytosolic (pI 6.35/75 kDa; pI 6.8/57 kDa; pI 5.55/50 kDa; pI 6.15.7/57-60 kDa) and three membrane associated polypeptides (pI 6.25/48 kDa; pI 6.75/26 kDa; pI 6.05/24 kDa) were expressed in both neoplastic and preneoplastic nodules but not in normal liver. Numerous quantitative differences were also detected among the various cell types. (3) Investigations of the homogeneity/heterogeneity of gene expression among individual nodules isolated from separate animals have revealed marked similarities among the early preneoplastic nodules.

(B) Analysis of gene expression during chemical transformation in hamster embryo cells (cooperating unit: J. DiPaolo and J. Doniger, Laboratory of Biology, DCE, NCI). Results obtained to date include: (1) although bisulfite has been shown to be a potent transformation agent in vitro, acute bisulfite treatment (15 minutes, 10  $\mu$ g/ml) at neutral pH had no qualitative effect on gene expression; (2) similarly gene expression during "aging" (time in culture) was relatively stable from early passages (2nd) to later passages (10th); and (3) computer-assisted analysis of polypeptide differences between normal control cells (5th passage embryo cells) and various bisulfite transformed clones revealed both qualitative (six proteins) and quantitative polypeptide differences between untreated control cells and each of the individual transformed clones. Following transformation three polypeptides (pI 5.9, MW 55 kDa; pI 5.4/53 kDa; and pI 5.4/32 kDa) were either not expressed or appeared as a charge-shift variant in each of the transformed lines. In addition to the apparent loss of expression or charge-shift two new polypeptides (pI 6.55/45 kDa and pI 5.90/26 kDa) were expressed in all clones and one polypeptide (pI 5.62/45 kDa) was expressed in all clones except clone A following transformation with either bisulfite or benzo[a]pyrene (BP). One polypeptide (pI 6.36/28 kDa) was expressed only in the BP transformed clone.

(C) Analysis of polypeptide changes during cellular differentiation using mouse epidermal cells in culture (cooperating units: S. Yuspa and H. Hennings, Laboratory of Cellular Carcinogenesis and Tumor Promotion, DCE, NCI). Results obtained to date include: (1) mouse epidermal cells were cultured in the presence of either low calcium (0.02-0.1 mM) (mainly basal cells) or high calcium (1.2 mM) (mainly mature keratinocytes) and then treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) for 1, 4, or 24 hours. Two-dimensional gel analysis of total cellular polypeptides from cells grown in the presence of either low or high calcium revealed only quantitative polypeptide differences. (2) Similarly, only quantitative differences were detected following treatment of either low or high calcium cells with TPA (0.01 and 0.1  $\mu$ g/ml). In low calcium cells roughly 3 and 6%, respectively, of the total number of polypeptides (800 paired spots) from TPA-treated cells underwent quantitative changes greater than four-fold. In high calcium cells, however, TPA treatment had a much lesser effect on gene expression. Only 4/854 (0.5%) and 15/934 (1.6%) of the total number of polypeptides changed greater than four-fold following treatment. (3) Comparison of the common changing polypeptides that were observed following treatment with either TPA (0.1  $\mu$ g/ml) or during growth of cells in high calcium revealed 16 common changing polypeptides (four-fold differences). In 14/16 of the polypeptides the common changes were also in the same direction (e.g., if the polypeptide increased following treatment with TPA, the same

polypeptide also increased in the presence of high calcium and vice versa). Eight polypeptides were increased following treatment with TPA or growth in high calcium and six polypeptides decreased following both these treatments.

Hepatic Proliferation Inhibitors. The objectives of this project are to (1) identify and purify polypeptide factors from rat liver that inhibit proliferation of hepatocytes or hepatocyte cell lines, determining whether there is only one inhibitor or a family of cytostatic but non-cytotoxic hepatic proliferation inhibitors (HPI). The aim will be to obtain sufficient quantities of HPI(s) to allow detailed studies on the mechanism of action of these growth modulator(s). (2) Characterize the specificity of HPI(s) for inhibiting the proliferation of various cell types including normal hepatocytes from rats of different ages, preneoplastic and neoplastic hepatocytes, and cells from other tissues or species. (3) Determine whether HPI(s) are produced uniquely by hepatocytes or also by other normal, preneoplastic, or neoplastic cells. Results obtained so far include (i) development of methodology for (a) high resolution fractionation of tissue extracts by fast protein liquid chromatography (FPLC); (b) rapid assay, for cells maintained in 96-well microtiter plates, of the incorporation of tritiated thymidine into DNA. Cells are pulsed with [3-H]thymidine, released from the substratum by trypsinization, and collected with water washes onto glass fiber filters with a multi-channel cell harvester. Experiments with inhibitors of protein, DNA, and RNA synthesis indicate that the radioactivity retained on the filters selectively measures DNA synthesis. (c) Rapid fluorometric assay for DNA contained in the chromatin of cells attached to the wells of microtiter plates. In this assay, fluorescence resulting from interaction of chromatin DNA with Hoechst 33342 is measured automatically by a reflected light fluorometer. This fluorescence is directly proportional to cell or nucleus number and can be used for normalizing the incorporation of [3-H]thymidine into DNA described in (b) above. (ii) Demonstration that certain ethanol-precipitable fractions from adult rat livers cause a dose-dependent inhibition of DNA synthesis as assayed by the above methods.

Hepatic Asialoglycoprotein Receptor Mediated Gene Transfer. We have continued our studies attempting to construct a receptor mediated gene transfer system by utilizing the highly efficient endocytosis process whereby asialoglycoproteins are taken up by normal rodent hepatocytes. This is accomplished by covalently coupling the asialoglycoprotein to the DNA by using two reagents, N-acetyl-N'-(p-glyoxybenzoyl)cystamine and 2-iminothiolane. The former reacts specifically with unpaired guanine residues and upon reduction generates a free sulphhydryl group. The latter reacts with the protein to provide another sulphhydryl group which is subsequently conjugated to DNA by an intermolecular disulfide interchange reaction. The experimental model currently under study is the rat liver. The initial coupling has been done using two transformation specific viral DNAs, namely the bovine papillomavirus (BPV) DNA and the cDNA clone of the Harvey RNA tumor virus. Both tumor viruses have been well characterized in terms of transforming ability. The bovine papillomavirus DNA was prepared from pBR322 recombinants and tailed with approximately 50 residues of dGTP using terminal deoxytransferase. G-tailed viral DNA was coupled to modified asialofetuin by incubation under appropriate conditions. A similar procedure was used for conjugating the cDNA clone of the Harvey RNA tumor virus and asialofetuin. Initial analysis demonstrated the incorporation of both BPV DNA and Harvey cDNA into primary rat hepatocytes, however, both DNAs were rapidly degraded. Experiments designed to limit the endogenous nuclease activity are presently in progress.

The Biopolymer Chemistry Section plans and develops laboratory research on the chemical structure and conformational aspects of relevant biopolymers, of reactant chemical carcinogens and of carcinogen-biopolymer adducts using rigorous spectral and chemical methods such as mass spectrometry, nuclear magnetic resonance spectroscopy, circular dichroism, chemical synthesis and related techniques. The major efforts of the Section focus on (1) the isolation, characterization and mass spectral sequencing of polypeptides (proteins), that are determinants in the control of cell differentiation and proliferation; (2) the mechanism of action of these biopolymers; and (3) the synthesis and reactivity of carcinogens with biomolecules, particularly with DNA, and the structure, conformation and biological significance of the resulting modified products. Studies of the Section are aimed at a better understanding of the carcinogenic process at the molecular level in particular.

#### Structure and Physicochemical Studies of Proteins Relevant to Tumorigenesis.

This is a new project that involves studies on the chemical structure, molecular conformation, 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. Current emphasis is focused on proteins that play a role in cell growth regulation, cell transformation or differentiation, such as marker enzymes, hormones, growth factors and transforming factors, whose level of expression and/or molecular structure, is aberrantly modified during these biological processes. Emphasis is placed on applying the modern spectroscopic methodologies of mass spectrometry, nuclear magnetic resonance (NMR) and circular dichroism (CD) as well as the standard methods of protein sequencing to the solution of these problems. We have purified to homogeneity the heterodimeric glycoprotein, gamma-glutamyltransaminase (gamma-GT), a tumor marker enzyme of unknown structure. There are both enzyme level and gross structural differences in gamma-GT between the normal and tumorigenic tissue. Separation of the protein subunits and sequencing of the resulting components are in progress. The method of fast atom bombardment (FAB) mass spectrometry is being applied to determine the accurate structure of medium size polypeptides of up to 2600 molecular weight, for example of tryptic peptides. The sequencing information obtainable by this method was improved upon by group specific labeling of functional groups. This approach will be applied to the sequencing of gamma-GT and to the p21 transforming protein. Phenylalanine-containing polypeptides are being analyzed by CD methods in order to gain information about preferred conformations using the aromatic chromophore as a probe. Finally, in another model study using NMR methods it was found that formaldehyde, a suspected carcinogen, reacts relatively slowly with peptides under near physiological conditions.

Effect of Chemical Leukemogens on Hemopoietic Target Cells. This is also a new project in which the objective is to study the mechanism(s) of chemically-induced leukemia. Two major aspects are focused on (1) characterizing the covalent interactions of chemical carcinogens with cellular DNA in hemopoietic tissues both in vivo and in vitro, and (2) examining the effects of these covalent interactions of chemical carcinogens and cellular DNA on the proliferation and differentiation of hemopoietic target cells. Leukemogenic chemicals which are under investigation include 7,12-dimethylbenz[a]anthracene (DMBA), N-methylnitrosourea (MNU), derivatives of 2-acetylaminophenanthrene (AAP), representing the polycyclic hydrocarbons, direct alkylating agents, and aromatic amines, respectively. Results obtained so far are (1) detection of N-(guanine-8-yl)-2-aminofluorene (Gua-C8-AAF) in bone marrow and spleen cells of Fischer 344 rats

following an intravenous dose of N-OH-AAF or N-acetoxy-2-acetylaminophenanthrene (N-OAcAAP), and (2) development of a murine multipotential hemopoietic stem cell colony assay which consists of pure and mixed colonies (granulocyte, erythrocyte, megakaryocyte, macrophage and mast cells). Future studies will include (1) determination of in vivo formation of DNA adducts with N-OH-AAF and N-OAc-AAF which are more leukemogenic than the acetylaminofluorenes, (2) development of a murine hemopoietic blast cell colony assay which contains only undifferentiated cells, and (3) investigation of the biological interactions of DMBA, MNU, N-OAc-AAF, and N-OAc-AAF on hemopoietic target cells using these stem cell assays.

The Role of Fatty Acid Acylated Polypeptides in Cellular Transformation. Proteins modified by post-translational addition of fatty acids have been associated with both cellular oncogenic transformation and differentiation. In an effort to gain further knowledge about what roles these modified proteins play, two-dimensional gel electrophoresis patterns of 3H myristylated proteins have been obtained from HL60 cells. Because the HL60 cancer cell line can be manipulated to differentiate or cease proliferation without differentiation, it is a potential model for examining the changes in myristylated proteins after treatment with various inducing or inhibitory agents. Two-dimensional gel electrophoresis was used to assess the changes in myristylated proteins following these treatments. Four experiments were carried out; cells with no treatment, cells inhibited from proliferation using difluoromethyl ornithine (DFMO), cells differentiated into granulocytes with hexamethyl-bis-acetamide (HMBA), and cells differentiated into adherent macrophages with TPA. After these materials had their effects on the HL60 cells, they were incubated with 3H myristic acid, lysed with two-dimensional gel lysis buffer, and then applied to two-dimensional gel electrophoresis. Autoradiograms taken of the finished gels showed simple patterns that exhibited differences in some spots, depending on the agent present prior to incubation with labeled myristate, and others which remained constant, which provided internal standardization. Most changes in patterns were reflected as losses in spot intensities. For example, one major and several minor spots are subjected to down regulation whenever proliferation is halted, whether it be by DFMO or by differentiation, indicating that myristylated proteins are involved in the cell cycle. In another instance, what appeared to be a glycosylated, and thus presumably a cell surface protein, almost completely disappeared when the cells were caused to differentiate. Changes in patterns were also observed between cells differentiated into granulocytes or macrophages. In addition, several new spots appeared when differentiation occurred.

The Cell Biology Section plans and develops laboratory research aimed at defining the cellular events which mediate the conversion of normal cells into neoplastic or cancer cells. The major efforts of the Section are focused on (1) devising in vitro target cell assays which will permit a direct analysis of the effects of carcinogens on normal, differentiating cells at early, but well-defined, stages of development; (2) defining the hormonal requirements and sensitivities of a variety of cell types which have been transformed by chemicals, viruses, or unknown agents (i.e., spontaneously occurring neoplasms); and (3) reexamination at a cellular (clonal) level, rather than at a tissue level, of the processes involved in tumor promotion, progression and the relationship between benign and malignant neoplasias.

Normal Stem Cell Biology and Hemopoietic Regulation. This is a new project in which the overall objective is to define and characterize the normal stem cell biology with particular emphasis on the hemopoietic stem cell. The research is focused on the following areas: (1) employ current stem cell assays to identify the growth regulators which act upon these primitive cells; (2) continue to devise novel methods for both maintaining the stem cells in culture for longer periods of time and to improve the recognition of stem cells as well as further defining their progeny; and (3) purification, preparation of a panel of monoclonal antibodies and molecular cloning of erythropoietin (Epo). We previously reported that a single cell line derived from a mouse with erythroleukemia produced factors with erythropoietic activity. We have now isolated additional (new) erythroid lines and generated extensive evidence indicating that the erythropoietic activity produced by these lines is mouse Epo. Our characterization of the erythropoietic activity included (1) biological (active in nine biological assays), (2) immunological (four antierythropoietin sera), and (3) biochemical (enzyme treatments and multiple purification techniques) studies. The finding that a large percentage of cells became benzidine and spectrin-positive upon hemin induction indicated that most of the cells were erythroid and suggested autocrine production of Epo. Epo was detected in media from each of 20 lines derived from two cycles of single cell cloning, suggesting that, in these lines at least, the same cells which produce Epo also have erythroid developmental potential. Conditions have been found which allow production of more than 1 U/ml Epo in cultures that are free of serum and albumin.

Chemical Leukemogenesis. This project is highly integrated with other sections of the LEC. Our overall aim is to examine the interaction of chemical carcinogens with hemopoietic cells of rodents and primates to better define the target(s) for transformation and the sequence of events which mediate the progression of a chemically-induced neoplasm. A number of agents in the environment have been demonstrated to induce cancer in experimental model systems and have been strongly associated with spontaneous neoplasms in humans. During the past decade, methods have been devised which permit a direct analysis of the transformation of a variety of cell types by oncogenic viruses. While the events which accompany virus induction of leukemias and sarcomas can now be readily studied *in vitro*, many of the strains of transforming viruses have been highly selected for a particular property and may not have general relevance to carcinogenesis by naturally occurring viruses, chemicals or radiation. Furthermore, while the relevance of chemicals and radiation to cancer is clear, very few systems exist wherein one can directly analyze the early and late events in carcinogenesis induced by these agents. At present we have developed a method for recognizing small colonies of hemopoietic stem cells grown *in vitro* in semisolid cultures. These colonies have tremendous proliferative potential and give rise to secondary colonies which contain the progenitors of white blood cells, red blood cells and platelets.

Hormone Sensitivity and Growth Control of Tumor Cells. This is a new project in which the overall objective is to examine and characterize hormonal sensitivity and growth control of tumor cells in the context of a working hypothesis suggesting that oncogenic transformation may result from a selective increase in sensitivity to external regulators. Two different kinds of erythroleukemia populations have been analyzed. In the first, hematopoietic cells were infected *in vitro* with two strains of Friend virus. Both variants induce erythroid bursts that proliferated and differentiated without added Epo. However, while



the bursts induced by FVP were well "hemoglobinized" (i.e., most cells contained hemoglobin), the cells of FVA-induced bursts contained little or no hemoglobin. The nonhemoglobin bursts, induced by FVA, were established to be erythroid by cytochemistry, electron microscopy, and hormone sensitivity. FVA-induced cells appeared to be hypersensitive to Epo, since small concentrations of the hormone produced marked increases in hemoglobin production--even when the hormone was added to the cultures three days post-infection. Time-lapse photography documented that Epo-stimulated hemoglobin synthesis in virally transformed cells rather than uninfected erythroid precursors. Upon reexamination FVP-induced erythroid cells also were hypersensitive to Epo. These data are consistent with the hypothesis that oncogenic transformation may result from increased sensitivity of progenitor cells for natural, physiological regulators. The second erythroleukemia population was induced in vivo by Friend murine leukemia virus (F-MuLV). When placed into cultures these erythroleukemia cells need Epo for their growth. By reducing the Epo levels in vivo, survival times of the erythroleukemia could be greatly extended. These results suggest antihormone therapy may be efficacious for treatment of erythroleukemia.

Effect of Transforming Proteins of Oncogenic Viruses on Hemopoietic Cells. Our long-term goals with this project are (1) analyzing transforming genes in hemopoietic cells, (2) defining unknown functions of cellular genes, (3) globin gene regulation, and (4) examining the potential of genetic therapy. We have studied the effects of a cellular oncogene on hemopoietic cells in vitro. A virus chimera (EJ-1 virus) was constructed by ligating portions of Moloney leukemia virus and EJ-1, the transforming principle (encoding a ras p21) cloned from human bladder carcinoma cells. DNA of this replication-defective EJ-1 virus clone was transfected into psi-2 helper cells and transformed foci picked for expansion. Media was harvested from these expanded cultures and tested for transforming activity on 3T3 cells and for ras induced in vitro erythroid transformation. 3T3 cells were non-productively transformed since the culture media were negative for transforming activity, XC and reverse transcriptase. The p21 encoded by EJ-1 was not phosphorylated and migrated as a single band on polyacrylamide gel electrophoresis (PAGE). Thus, the EJ-1 protein could be distinguished from the p21 doublet observed in Harvey sarcoma virus infected cells. EJ-1 infection of murine hemopoietic cells induced large erythroid colonies (containing hemoglobin-positive and hemoglobin-negative cells) which appeared at 6-10 days post-infection. Picked erythroid cells were found positive for p21 by immunofluorescence. We have previously noted a variety of phenotypes of erythroid colonies induced by Friend, Harvey, Abelson, Rous and other transforming viruses. Evaluation of phenotypic characteristics of EJ-1 induced colonies with respect to time course, Epo sensitivity, proportion of differentiated cells, and replating potential, most closely resembled that of erythroid colonies induced by other ras-containing viruses.

The Hormone Action and Oncogenesis Section plans and develops laboratory research on the mechanisms of the regulation of gene expression by hormones and growth factors. The major efforts of the Section are focused on: (1) characterization of the regulation of mouse mammary tumor virus (MMTV) expression by glucocorticoid hormones using advanced recombinant DNA techniques; (2) developing methods for in vitro DNA mediated gene transfer; and (3) studies on mechanism of action of growth factors and other transformation related factors with particular emphasis on characterization of the cellular receptors.

Steroid Hormone Action and Oncogenesis. Previous analysis of molecular chimeras between the MMTV long terminal repeat (LTR) and the v-ras transformation gene from Harvey murine sarcoma virus (HaMuSV), and between the LTR and the chloramphenicol acetyl transferase (CAT) gene localized the steroid hormone regulatory sequences between 100 and 200 nucleotides upstream from the cap site in the LTR. Utilization of a competition assay with specific MMTV LTR fragments and total cellular DNA immobilized on cellulose has shown the preferential binding of the glucocorticoid receptor to fragments of LTR DNA containing the sequences identified in gene transfer experiments as important for hormone regulation. The addition of transcriptional activator sequences to the MMTV promoter indicates that the hormone regulatory sequence is capable of regulating the activity of the exogenous enhancer. These observations suggest a model for the mechanism of hormone action in which the regulatory sequence acts as a modulator of another cis-dominant positive element. The role of chromatin organization in hormone action has been addressed utilizing chimeras between the MMTV LTR and the BPV 69% transforming fragment. These chimeras replicate uniquely as episomal elements in murine fibroblasts, and maintain stable high-copy extrachromosomal copy numbers. It was found that nucleosomes are non-randomly organized on the sequences immediately upstream from the MMTV cap site, and that this phased structure is independent of hormone induction. A DNase I hypersensitive site is introduced into the chromatin structure upon hormone induction; the location of this site correlates precisely with the sequences required for transfer of hormone regulation in the biological assay. An S1 nuclease hypersensitive site also appears in the chromatin in a hormone-dependent manner. This site maps to the right side of the DNase I site. The phased organization of nucleosomes in the promoter region indicate that a highly organized nucleoprotein structure serves as template for hormone regulation in vivo. The specific changes induced in this structure upon hormone stimulation suggest that alteration of the chromatin template by interaction with the steroid receptor may play a critical role in the mechanisms of hormone action.

Analysis of Transcriptional Regulation of Murine Retroviruses. The MMTV LTR contains the target site for steroid hormone action associated with this virus. In order to directly address the molecular mechanisms by which hormone-receptor complex, RNA polymerase II and other nuclear protein components interact with the MMTV LTR to produce the observed biological phenomenon of elevated rates of transcription, we think that reconstitution of hormone regulated transcription in vitro will be required. We have previously introduced MMTV LTR into eukaryotic cells as an episome, by using the BPV as a vector. The MMTV LTR, present at 200 copies per cell, is still responsive to steroid-specific induction of transcription in this episomal form. The episomal elements can be extensively purified from cellular chromatin by selective extraction from the nuclear fraction, sedimentation through sucrose gradients, and concentration. The purified minichromosomes retain their association with 2-6 moles of glucocorticoid receptor subunit; this association is specific in that no bound receptor is found with control episomes not containing the MMTV LTR sequences. The fraction is highly enriched, with greater than 90% of the DNA in the fraction of episomal origin. These minichromosomes should serve as useful templates for in vitro transcription studies.

Receptor and Growth Factor Genes. Many important hormone and growth factor responses are mediated through the action of macromolecular receptors with high binding affinity for the respective hormone ligand. The glucocorticoid receptor

is a cytoplasmic receptor that migrates to the nucleus and induces a number of alterations in gene activity after binding a member of the glucocorticoid family. Most receptors are present at low concentrations in responsive tissues, and are difficult to purify in quantity. The characterization of various receptors, both in terms of gene organization, and in terms of protein structure and function, would benefit enormously from the availability of molecular clones for the receptor loci. Attempts are underway to isolate and characterize clones for the glucocorticoid receptor from human cells. Two approaches have been taken: one involves the preparation of cDNA expression libraries from receptor competent cells, followed by clone identification with antisera to the receptor; the other involves identification of the receptor genomic locus by insertional mutation with retrovirus vectors.

Hormone Regulatory Elements. Previous analysis of molecular chimeras between the MMTV LTR and the v-ras transformation gene from Harvey murine sarcoma virus (HaMuSV) localized the steroid hormone regulatory sequences between 100 and 200 nucleotides upstream from the cap site in the LTR. This test system has been transferred to the single-strand DNA virus, M13, to permit high-resolution mutational analysis of the hormone regulatory signals. It has been found that the assay (hormone-dependent appearance of v-ras dependent foci) remains functional in the new vector environment. In fact, the extent of hormone-dependence is even higher in the new system. A new method has been developed for the efficient and accurate introduction of mutations into regions of interest. This technique permits the oligonucleotide-directed introduction of mismatches as large as 10 nucleotides in a one-step procedure. The analysis of sequences required for the interaction of the hormone-receptor complex with target sequences in the DNA, and for the subsequent transcriptional response are in progress.

In addition to their intramural research efforts, 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 area of chemical carcinogenesis. Furthermore, the LEC scientists participate to a considerable degree in collaborative efforts with scientists both within the NCI and throughout the country.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04986-07 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Steroid Hormone Action; MMTV Oncogenesis; Viral Oncogenes and Transformation

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

PI: Gordon L. Hager Head, Hormone Action &amp; Oncogenesis Section LEC NCI

Others: Ronald G. Wolford Microbiologist LEC NCI  
 Diana S. Berard Microbiologist LEC NCI  
 Mark J. Miller Senior Staff Fellow LEC NCI  
 Peter J. Wirth Expert LEC NCI  
 M. Kessel Guest Researcher LMV NCI

## COOPERATING UNITS (if any)

Laboratory of Chemistry, NIADDK, NIH (S. Simmons and P. Miller)

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

Hormone Action and Oncogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

2.2

## PROFESSIONAL:

0.7

## 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.)

Previous analysis of molecular chimeras between the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) and the v-ras transformation gene from Harvey murine sarcoma virus (HaMuSV), and between the LTR and the chloramphenicol acetyl transferase (CAT) gene localized the steroid hormone regulatory sequences between 100 and 200 nucleotides upstream from the cap site in the LTR. Utilization of a competition assay with specific MMTV-LTR fragments and total cellular DNA immobilized on cellulose has shown the preferential binding of the glucocorticoid receptor to fragments of LTR DNA containing the sequences identified in gene transfer experiments as important for hormone regulation. The addition of transcriptional activator sequences to the MMTV promoter indicates that the hormone regulatory sequence is capable of regulating the activity of the exogenous enhancer. These observations suggest a model for the mechanism of hormone action in which the regulatory sequence acts as a modulator of another cis-dominant positive element. The role of chromatin organization in hormone action has been addressed utilizing chimeras between the MMTV-LTR and the bovine papilloma virus (BPV) 69% transforming fragment. These chimeras replicate uniquely as episomal elements in murine fibroblasts, and maintain stable high-copy extrachromosomal copy numbers. It was found that nucleosomes are non-randomly organized on the sequences immediately upstream from the MMTV cap site, and that this phased structure is independent of hormone induction. A DNase I hypersensitive site is introduced into the chromatin structure upon hormone induction; the location of this site correlates precisely with the sequences required for transfer of hormone regulation in the biological assay. An S1-nuclease hypersensitive site also appears in the chromatin in a hormone-dependent manner. This site maps to the right side of the DNase I site. Thus a highly organized nucleoprotein structure serves as a template for hormone regulation in vivo. The specific changes induced in this structure upon hormone stimulation suggest that alteration of the chromatin template by interaction with the steroid receptor may play a critical role in the mechanisms of hormone action.

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

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI
Ronald G. Wolford	Microbiologist	LEC	NCI
Diana S. Berard	Microbiologist	LEC	NCI
Mark J. Miller	Senior Staff Fellow	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
M. Kessel	Guest Researcher	LMV	NCI

Objectives:

(1) Analysis of hormone regulated transcription of mouse mammary tumor virus (MMTV); localization of hormone regulatory sequences involved in this regulation.

(2) Determination of the mechanism of hormone action in the up-regulated MMTV system; extension of the investigation of glucocorticoid regulation into systems where the expression is down-regulated.

(3) Application of tools developed for the study of hormone action to the study of other transcriptional regulatory systems important in cell growth, with initial emphasis on cell-cycle regulation of histone transcription.

(4) Structural analysis of the MMTV genome, utilizing full-length molecular clone of endogenous Unit V Mtv-1 proviral DNA and clones of other MMTV strains.

(5) Determination of the function of the newly discovered pLTR gene in MMTV; its potential role in MMTV induction of neoplasia; and the mechanism of mammary carcinogenesis.

(6) Investigation of the mechanism of cellular transformation mediated by the ras family of oncogenes; development of a conditional mutation system for the complementation analysis of oncogene action utilizing the regulated MMTV promoter.

Methods Employed:

Molecular chimeras between the MMTV long terminal repeat (LTR) and the v-ras gene of Harvey murine sarcoma virus (HaMuSV) are used in a hormone-dependent transfection assay to probe the regulatory regions involved in hormone induction of MMTV expression. Similar fusions between the LTR and the chloramphenicol acetyl transferase (CAT) gene from the bacterial Tn9 transposon will be tested in a transient expression assay. Deletion analysis of molecular chimeras will be performed to localize these regions.

Utilizing the S1 nuclease or mung bean nuclease mapping techniques, probes available from molecular clones of MMTV will be utilized to analyze steroid-dependent MMTV regulation.

Minichromosomes containing the MMTV LTR mobilized on BPV have been characterized. Nuclease sensitivity of the minichromosomes in isolated nuclei will be determined using micrococcal nuclease and pancreatic DNase I.

Initiation of transcription at the regulated MMTV promoter will be carried out in *Xenopus oocytes*. An analysis of the role of chromatin organization in hormone action will be carried out by comparing naked DNA templates and minichromosomes containing MMTV promoters as transcription templates in this system.

Transcriptional promoters responsive to other hormone effects (androgens) and other regulatory elements (histone-cell cycle) will be engineered into the v-ras transformation system to test for transfer of regulation.

Cell lines transformed with MMTV v-ras fusions will be established in serum-free media to facilitate the analysis of steroid regulation. Such lines will be subjected to mutagenesis to generate variants both in the transformation response and in the hormone response.

The protein expression profile in cell lines conditionally transformed with MMTV LTR v-ras fusions will be examined by high resolution two-dimensional gel electrophoresis for gene products uniquely present in response to the ras transforming gene product.

MMTV gene expression, particularly for the 3' terminal pLTR gene recently discovered in this laboratory, will be analyzed in MMTV-infected cells, and in MMTV-induced and chemically-induced mammary tumors. Methods will include Northern analysis of RNAs present, information content analysis of RNAs with subgenomic probes, and structural characterization by S1 analysis, cDNA cloning, and DNA sequencing.

The biological activity of full-length cloned Mtv-1 (Unit V) proviral DNA will be determined by transfection of DNA into appropriate cell lines and examination of viral expression.

#### Major Findings:

Regulatory signals involved in the control of MMTV transcription by glucocorticoids have been examined in detail in two independent expression systems. In the first, fusions between the v-ras gene of HaMuSV, the MMTV LTR, and an enhancer element from the HaMuSV LTR have allowed us to monitor steroid-inducible transcription from the MMTV LTR by a rapid transfection assay. Efficient transfection of NIH 3T3 cells to the transformed phenotype occurs only when glucocorticoids are present in the medium. In the second assay system, the CAT from the bacterial Tn9 transposon has been placed under control of the MMTV promoter; in this system, the elaboration of CAT enzyme activity was shown to be inducible by glucocorticoids during transient expression after acute DNA-mediated transfection. Using these assays, deletion analysis by molecular techniques has localized the site sufficient to confer hormone sensitivity on the MMTV LTR to within 100 nucleotides of the MMTV cap site.

In both of these assay systems, an increase in the uninduced, constitutive expression from the MMTV LTR was observed after deletion of the hormone responsive sequences, suggesting that the mechanism of hormone action may be more complex than a simple induction effect. It is now apparent that the glucocorticoid regulatory element can regulate the activity of an exogenous enhancer introduced into the fusion chimeras. A model is suggested in which the hormone regulatory sequence is actually composed of more than one element, a positive activator sequence, and another previously uncharacterized element that regulates the activity of the positive element.

A competition assay has been developed in which the interaction between glucocorticoid receptor protein and MMTV DNA containing the hormone target region can be detected. LTR sequences implicated in the hormone response by the gene transfer experiments described above have been shown to compete for receptor binding in receptor-containing cell-free extracts more efficiently than random DNA sequences. This assay serves as one measure of the interaction of the glucocorticoid receptor and its target. The single strand mutagenesis system (see Project Number Z01CE5118-05 LEC) has been constructed so that mutants identified by the biological response as containing lesions in critical sequences can be amplified and tested easily in this competition assay.

We have previously been unable to demonstrate specific initiation at the MMTV cap in cell-free extracts. An experimental system has now been established, however, for examining initiation of transcription at the MMTV promoter with defined templates, either naked DNA or more complex minichromosomes. Micro-injection of MMTV LTR-containing DNA into the nuclei of Xenopus oocytes leads to correct initiation of transcription at the normal MMTV cap. This represents an important step in our attempt to identify the critical elements involved in hormone regulation, and to reconstruct this system from its component parts.

Cell lines transformed with the MMTV v-ras fusions had previously been established in serum-free media. Since this medium is completely defined, and in particular is free of glucocorticoids, interaction of steroid receptors and the MMTV promoter can now be studied at the cellular level in a totally defined culture environment. The concentration curve for dexamethasone induced phenotype switch from normal to transformed parallels exactly the binding curve for dexamethasone affinity to its cellular receptor, confirming that induction of the cellular phenotype switch occurs via glucocorticoid receptor mediated regulation of p21 gene expression. Experiments are under way to derive mutants in which this phenotypic switch no longer occurs.

#### Significance to Biomedical Research and the Program of the Institute:

A major goal of the experimental program is to decipher the mechanisms involved in steroid hormone regulation as a model for the general problem of hormone action. Control of cell proliferation is a central issue in neoplastic transformation; hormones are directly implicated in many aspects of growth control. Steroid hormones are directly implicated in the biology of certain human neoplasms, such as breast cancer. A thorough knowledge of the mechanisms of hormone action is therefore necessary to our eventual understanding and control of the neoplastic process.

Proposed Course:

High-resolution mutants in the hormone response region will be examined in the *Xenopus oocyte* transcription system to determine their effect on the basic promoter structure of the MMTV LTR. Efforts will also be made to demonstrate regulation in the oocyte system by co-injection of extracts enriched in glucocorticoid receptors.

The role of chromatin structure in the hormone response will be investigated in the oocyte system by comparing the transcription initiation capacity of naked DNA templates and minichromosomes isolated from cells harboring bovine papilloma virus (BPV) MMTV LTR episomal chimeras.

Attempts will be continued to demonstrate correct initiation in cell-free transcription extracts, and to demonstrate regulation with receptor-enriched extracts, DNA and minichromosomal templates.

A further extrapolation of the tools developed for the study of hormone action in the MMTV system will be carried into the study of cell-cycle regulation of histone expression. Histone gene BPV chimeras have been prepared and characterized, and shown to support DNA synthesis-dependent regulation of message stability synthesized from the episomal element.

Cellular variants of the MMTV LTR v-ras transformed lines resistant to phenotype switching will be derived. Two classes of variants are expected, one in which cellular targets of the transformation protein are altered, and one in which elements of the hormone response pathway are modified. Both classes will be extensively characterized to identify the respective cellular targets.

High-resolution two-dimensional gel analysis of the proteins synthesized in MMTV LTR v-ras conditionally (hormone-dependent) transformed cell lines will be continued to identify gene products potentially expressed as a consequence of v-ras transforming protein expression.

Publications:

Hager, G. L.: Expression of a viral oncogene under control of the mouse mammary tumor virus promoter: A new system for the study of glucocorticoid regulation. Prog. Nucleic Acids Res. Mol. Biol. 29: 193-203, 1983.

Hager, G. L.: Regulation of transcription by glucocorticoids: Identification of the hormone regulatory element and analysis of mechanism of action with episomal fusions containing the MMTV promoter. In Scott, W. and Fazal, A. (Eds.): Advances in Gene Technology: Human Genetic Disorders. New York, Academic Press (In Press)

Hager, G. L., Lichtler, A. C. and Ostrowski, M. C.: The MMTV glucocorticoid regulatory sequence: A positive or negative element? In Gluzman, Y. and Shenk, T. (Eds.): Enhancers and Eukaryotic Gene Expression. New York, Cold Spring Harbor Laboratory, 1983, pp. 161-164.



Hager, G. L., Richard-Foy, H., Kessel, M., Lichtler, A. C. and Ostrowski, M. C.:  
The mouse mammary tumor virus model in studies of glucocorticoid regulation.  
Recent Prog. Hormone Res. 40: 121-139, 1984.

Miller, P. A., Ostrowski, M. C., Hager, G. L. and Simons, S. S., Jr.: Covalent  
and non-covalent receptor-glucocorticoid complexes preferentially bind to the  
same regions of the long terminal repeat of murine mammary tumor virus proviral  
DNA. Biochemistry (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05118-05 LEC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Analysis of Transcriptional Regulation of Murine Retroviruses

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

PI: Michael C. Ostrowski Senior Staff Fellow LEC NCI

Others: Gordon L. Hager Head, Hormone Action and Oncogenesis LEC NCI  
 Section

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

The mouse mammary tumor virus (MMTV) long terminal repeat (LTR) contains the target site for steroid hormone action associated with this virus. In order to directly address the molecular mechanisms by which hormone-receptor complex, RNA polymerase II and other nuclear protein components interact with the MMTV LTR to produce the observed biological phenomenon of elevated rates of transcription, we think that reconstitution of hormone-regulated transcription in vitro will be required. We have previously introduced MMTV LTR into eukaryotic cells as an episome, by using the bovine papillomavirus (BPV) as a vector. The MMTV LTR, present at 200 copies per cell, is still responsive to steroid-specific induction of transcription in this episomal form. The episomal elements can be extensively purified from cellular chromatin by selective extraction from the nuclear fraction, sedimentation through sucrose gradients, and concentration. The purified minichromosomes retain their association with 2 to 6 moles of glucocorticoid receptor subunit; this association is specific in that no bound receptor is found with control episomes not containing the MMTV LTR sequences. The fraction is highly enriched, with greater than 90% of the DNA in the fraction of episomal origin. These minichromosomes should serve as useful templates for in vitro transcription studies.

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

Michael C. Ostrowski	Senior Staff Fellow	LEC	NCI
Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI

Objectives:

Levels of mouse mammary tumor virus (MMTV)-initiated mRNA contained in cell lines harboring 200 copies per cell of bovine papilloma virus-long terminal repeat (BPV) (LTR) hybrids will be measured in either the presence or absence of glucocorticoids. This will be accomplished both by S1 mapping and by in vitro transcription run-off experiments. These run-off experiments will be performed on subnuclear extracts enriched for the minichromosomes.

Episomal chimeric DNA will be isolated from these cells so that nucleoprotein particles are obtained. These particles will be used in nuclease digestion studies as above to ensure that purification does not damage epigenetic structure.

The enriched minichromosomes will serve as templates for in vitro transcription experiments. Various nuclear extracts and purified glucocorticoid receptor will also be used in these transcription experiments. The cloned, naked DNA is used as a control.

Mutant MMTV LTR's will be made and combined in chimeras such as those described above. Comparison of the transcriptional ability of these mutants as compared to the parental LTR should yield additional insights into the mechanism of steroid hormone action.

Methods Employed:

Isolation of steady-state levels of RNA and S1 mapping of this RNA using end-labeled probes are accomplished using published procedures.

Minichromosomes used for run-off transcription are prepared from nuclei of cells containing LTR episomes by ammonium sulfate extraction. This procedure relies on the small size of these episomes as compared to chromosomal DNA. These particles are then incubated with radioactive ribonucleotides. The RNA produced is analyzed by hybridization to single-strand probes representing the sequences present in the chimeric BPV LTR episomes.

Minichromosomes to be used for nuclease digestion and in vitro transcription templates will be purified by low salt procedures that are less likely to damage chromatin structure. These procedures will depend on the small size of the episomes, but they must also be able to dissociate the higher orders of structure present in the nuclear matrix including so-called nuclear scaffolding. Chelators of specific ions may be useful in this process.

Various whole cell and nuclear extracts which contain factors required for transcription will be made according to published techniques.

Mutants in MMTV LTR sequences will be introduced using standard recombinant techniques and procedures previously reported. These mutants will then be introduced into murine cells using BPV as described previously.

#### Major Findings:

We have identified murine cells that are stably transformed by BPV-MMTV LTR chimeras following calcium phosphate transfection. These chimeras are found as episomes in the cells and not integrated into host information. The physical map of these episomes is not significantly different from the input recombinant DNA. The copy number of episomes can be as high as 200 copies per cell. More recently, we have seen that single-cell cloning of these lines after interferon treatment can increase the copy number of BPV LTR episomes five- to ten-fold in some of the clones obtained. We have also found by S1 mapping of total cellular RNA that steady-state levels of MMTV-initiated mRNA are increased in these cells when they are grown in the presence of glucocorticoid hormones. We have demonstrated that this increase in LTR-initiated RNA levels is dependent on transcriptional induction of the LTR promoter by transcription run-off experiments performed *in vitro*. These run-off studies, which measure the number of RNA polymerase II molecules already in place *in vivo*, were accomplished using subnuclear extracts 400-fold enriched for BPV LTR minichromosomes. These experiments constitute the first evidence conclusively demonstrating that MMTV LTR contains all of the information necessary for the steroid response independent of any contribution from host chromosomal information, and is also the first demonstration of any hormone-regulated promoter retaining its activity in a non-integrated, extrachromosomal form. The amount of MMTV-initiated RNA found, as determined by either S1 or run-off experiments, varies significantly in between independently obtained cell lines that have comparable copy numbers of episomes. Because of the episomal nature of the LTR in these cells, which precisely defines the genetic location of these sequences, we believe this variability in expression may reflect a major role for chromatin structure in the hormone response mechanism.

Procedures have been developed for the isolation of episomal elements as native nucleoprotein. These procedures can be employed on a scale sufficiently large to permit the purification of significant quantities of the minichromosomes. The purified episomes retain a specific association with the glucocorticoid receptor when isolated from hormone-induced cells. Control preparations of episomes that do not contain the LTR hormone-binding site do not have associated hormone receptor, showing the specificity of the interaction. Efforts have therefore been successful to isolate and purify for the first time a native chromatin element in association with its natural regulatory effector.

#### Significance to Biomedical Research and the Program of the Institute:

Genes homologous to the onc genes of retroviruses are present in normal vertebrate cells; in some cases these normal cellular genes can be activated to produce transformation. The mechanisms by which the expression of these genes

are regulated becomes of obvious importance to the understanding and ultimate control of oncogenic disease. Our efforts are aimed at the ultimate description in molecular terms of mammalian regulatory mechanisms.

#### Proposed Course:

Minichromosomes will be purified from cells based on their small size compared to chromosomal information after the disassembly of the normal nuclear matrix.

The structure of purified minichromosomes will be examined by nuclease digestion procedures and compared to the structure found in whole nuclei. Binding of glucocorticoid receptor present in crude cellular extracts to purified minichromosomes will be tested.

Whole cell and nuclear extracts that contain RNA polymerase II and various non-histone nuclear proteins will be prepared from rodent cells.

Attempts will be made to reconstitute faithful hormone-regulated transcription in vitro by recombining the nucleoprotein minichromosomes, prepared from cells grown both in the presence and absence of glucocorticoids, with appropriate extracts. S1 mapping will be employed to assay for successful initiation events.

Purified hormone receptor (obtained collaboratively) will be prepared and added to the in vitro extracts.

MMTV LTR mutants will be constructed and tested in the in vitro system.

When attempts at in vitro transcription are successful, classic biochemical purification of components from the crude extracts that are required for in vitro reconstitution will be undertaken.

#### Publications:

Ostrowski, M. C., Huang, A. L., Kessel, M., Wolford, R. G. and Hager, G. L.: Enhancement of the mouse mammary tumor virus promoter by sequences from the Harvey murine sarcoma virus LTR; a rapid, hormone-responsive transfection assay. EMBO J. (In Press)

Ostrowski, M. C., Richard-Foy, H., Wolford, R. G., Berard, D. S. and Hager, G. L.: Glucocorticoid regulation of transcription at an amplified, episomal promoter. Mol. Cell. Biol. 11: 2045-2057, 1983.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05260-03 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Regulation of Gene Expression and Differentiation in Neoplasia

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

PI: Carole A. Heilman Senior Staff Fellow LEC NCI

Others: Snorri S. Thorgeirsson Chief LEC NCI  
 Irene B. Glowinski Staff Fellow LEC NCI  
 Brian Huber PRAT Fellow, NIGMS LEC NCI  
 Mona E. Møller Visiting Associate LEC NCI  
 Jeff Cossman Senior Assistant Surgeon CP NCI  
 Eric Westin Expert LTB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

5.7

PROFESSIONAL:

3.7

OTHER:

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 object of this project is to examine the regulation of gene expression during neoplastic progression as well as during the normal growth and differentiation process. Our aim is to identify and characterize both cellular and genetic factors that are of critical importance in the oncogenic process. The experimental systems presently under study are (A) the human promyelocytic leukemia cell line, HL60; (B) a variety of undifferentiated human B cells and B-cell lymphomas; and (C) the rat hepatoma cell line and primary hepatocytes. The results obtained in the HL60 cell line study include (1) functional association of c-myc expression with the differentiated state of the cell, and (2) expression of the 2.2 kb N-ras specific transcript associated to some extent with the proliferative capacity of the cells. The results obtained and the experiments in progress in the human B-cell study include (1) chemical transformation of lymphoblastoid cell lines into high grade lymphomas, (2) transformation of NIH 3T3 cells by transfection of DNA obtained from these high grade lymphomas, (3) analysis of the transfection specific gene, and (4) two-dimensional gel analysis of proteins associated with the chemically-induced transformation state. The results obtained and the experiments in progress in the rat hepatoma and primary hepatocyte study include (1) partial construction of cDNA libraries which correspond to discrete stages in the neoplastic progression of rat hepatomas, (2) isolation of cDNA recombinant clones which are up-regulated in actively proliferating rat liver (i.e., following partial hepatectomy) as compared to normal adult liver, and (3) characterization of the change in expression of a differentiation specific gene.

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

Carole A. Heilman	Senior Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Irene B. Glowinski	Staff Fellow	LEC	NCI
Brian Huber	PRAT Fellow, NIGMS	LEC	NCI
Mona E. Møller	Visiting Associate	LEC	NCI
Jeff Cossman	Senior Assistant Surgeon	CP	NCI
Eric Westin	Expert	LTB	NCI

Objectives:

The objective of this project is to examine the regulation of gene expression and differentiation in neoplasia by employing both the techniques of molecular biology and quantitative measurement of total cellular proteins. Our aim is to identify and characterize both cellular and genetic factors that are important in the neoplastic process. The experimental systems currently under study are (1) the human promyelocytic leukemia cell line, HL60; (2) a variety of undifferentiated human B cells immortalized, in vitro or in vivo, with Epstein Barr Virus (EBV) as well as chemically-induced human B-cell lymphomas; and (3) the rat hepatoma cell lines or primary hepatocytes at different stages of neoplasia, differentiation or growth.

Methods Employed:

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

Major Findings:

(A) Studies on the Human Promyelocytic Leukemia Cell Line HL60: Independent Expression of c-myc and N-ras during Growth and Differentiation. The subject of somatic cell differentiation has been a focus of intense interest since a clear understanding of the mechanisms governing such processes should clarify some of the many unresolved problems concerning ontogeny and neoplastic transformation. The differential expression of certain cellular oncogenes during normal growth and development suggests a possible role of these genes in cellular differentiation. The ability to disassociate growth and differentiation specific functions in cultured human promyelocytic leukemia cells (HL60) provides a unique in vitro model to study the functional response of two HL60-associated oncogenes, c-myc and N-ras. The results demonstrate distinct functions associated with expression of these oncogenes. Specifically, expression of c-myc is almost exclusively associated with the promyelocytic status of the HL60

cells, and this expression is repressed during differentiation of the promyelocytes to monocytes. No change in c-myc expression is seen when promyelocytes are exposed to growth inhibitors such as difluoromethyl ornithine (DFMO) or reach plateau phase of growth. Conversely, the expression of N-ras is not dramatically altered when HL60 cells are treated with growth inhibitors (DFMO) or differentiation agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA). A consistent two- to three-fold repression of the 2.2 kb N-ras specific transcript was observed in both terminally differentiated monocytes as well as promyelocytes which have been growth inhibited. The expression of the 5.8 kb N-ras specific transcript, however, was modulated differently under these conditions. Whereas a two-fold repression at this transcript was observed in terminally differentiated monocytes, a two-fold enhancement in expression of the 5.8 kb N-ras transcript was observed in DFMO-treated promyelocytes.

(B) Studies on the Human B Cell Lines. (1) Chemical Transformation of Lymphoblastoid Cell Lines. Both EBV-positive and -negative Burkitt lymphoma cell lines have been extensively used to study the relationship between expression of retroviral oncogenes, chromosomal abnormalities and cellular transformation. To date, the role of chemical carcinogens in this process has not been fully investigated. Therefore, we have employed a model human B-cell system to investigate the potential correlation of oncogene expression and chromosomal abnormalities to chemical induction of tumorigenesis. EBV immortalized cord blood lymphocytes and B-cells derived from patients with infectious mononucleosis were treated with N-acetoxyacetylaminofluorene (N-Ac-AAF, 1 to 15  $\mu\text{g/ml}$  in 0.5% DMSO), a potent ultimate carcinogen and frameshift-inducing mutagen. Twenty population doublings post-treatment, treated and untreated cells ( $5 \times 10^6$ ) were subcutaneously injected into athymic mice. Rapidly proliferating tumors giving rise to high grade B-cell lymphomas were noted after one week in the treated lines. Karyotyping revealed model chromosome numbers ranging from diploid (untreated) to tetraploid (treated) with one tumorigenic line displaying a 6p-16p translocation. Oncogene expression of c-myc, c-myb, c-fes, H-ras, K-ras and N-ras were measured by Northern blot analysis, revealing no qualitative or quantitative differences in the level of expression.

(2) Transfection of Chemically-Induced High Grade Human Lymphomas into NIH 3T3 Cells. Eighty micrograms of mechanically sheared high molecular weight DNA derived from either CB23 lymphocyte cultures or the N-Ac-AAF-treated CB23 cultures were calcium phosphate precipitated into  $2 \times 10^6$  NIH 3T3 cells. Subsequently, the treated NIH 3T3 cells were subcultured once and observed for 21 days in vitro. At the end of this period, focal formation was scored and resulting foci were grown up into mass culture. The N-Ac-AAF-treated CB23 cells demonstrated a transformation efficiency of 0.1%, whereas the CB23 cells showed an efficiency of less than 0.005%. High molecular weight DNA was isolated from the various foci and analyzed for human DNA content by Southern restriction analysis. None of the CB23 foci were positive; however, all seven of the foci derived from the N-Ac-AAF-treated lines were. These seven foci were subsequently screened by Southern analysis for the presence of the ras family oncogenes (i.e., N-ras, H-ras, K-ras), Blym, and c-myc. None of these oncogenes appear to be pertinent to these transfected cells.



To further aid in the analysis of these transfected cells, a second round of transfection was carried out in hopes of deleting extraneous human DNA and concentrating the active transforming segment. Four clones derived from the original foci were retransfected. The transformation efficiency doubled this time (0.2%) and the resulting foci are being screened for the presence of high molecular weight human DNA and for the various oncogenes.

(3) Two-Dimensional Protein Gel Analysis of NIH 3T3 Cells Transformed with DNA from Chemically-Induced High Grade Human Lymphomas. Mid-log phase cultures of both normal and N-Ac-AAF-treated CB lines were metabolically labeled with 200  $\mu$ Ci of  $^{14}$ C amino acid mixture for 4 hours. At the end of this period, excess label was removed and the cells processed for two-dimensional gel electrophoresis. The cells were separated into nuclei, particulate and supernatant fractions, by differential centrifugation in the presence of the protease inhibitor phenylmethylsulfonyl fluoride. 400K dpm were applied to the first dimension gel for each fraction in an ampholyte range of pH 5 to 7. Second dimension slab gels were run and the dried gels were exposed to film for seven days. The resulting films are now being computer-analyzed, although "eyeballing" the films reveals differences between the normal and transformed cells. The same techniques of labeling, fractionation and analysis are being applied to the primary NIH 3T3 foci derived from the N-Ac-AAF-treated CB23 cell lines.

(C) Studies on Rat Hepatomas and Primary Hepatocytes. (1) Rat Liver cDNA Libraries. The rat liver is an excellent system to study the regulatory mechanisms that control differentiation and growth. Adult liver (non-dividing), fetal liver (developmental growth), regenerating liver (compensatory growth) and preneoplastic/neoplastic liver (uncontrolled growth at various stages of progression) are all stages which can be readily examined and experimentally controlled. We are presently optimizing the construction of cDNA libraries to each of the stages mentioned above in order to isolate and characterize specific genes which are "turned on" during that stage.

(2) Isolation of cDNA Recombinant Clones Which are Up-Regulated in Actively Proliferating Rat Liver. A cDNA library was constructed from poly(A) RNA obtained from 18 hours posthepatectomized (70%) Fischer rats. From this cDNA library a clone bank of 6,000 colonies was isolated and screened by the method of differential hybridization to identify clones that corresponded to genes which were specifically "turned on" 18 hours after 70% hepatectomy as compared to "resting" adult liver. No qualitative changes were seen in gene expression; however, four unique clones were isolated, containing cDNA inserts which varied from 600-2,000 base pairs (b.p.) which were up-regulated in regenerating liver. One of the clones has been initially characterized. It consists of approximately 1,080 b.p. and by slot blot analysis to poly(A) RNA has been shown to be three times more abundant in 18-hour posthepatectomized liver than sham-operated controls. In addition, Northern blot analysis has shown that this clone is also up-regulated in the livers of rats chronically fed phenobarbital or injected with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), two potent tumor promoter regimens. In contrast, this gene was down-regulated in frank liver tumors, preneoplastic nodules produced by the Solt-Farber method, in the rat hepatoma line 7777 and liver which has completely regenerated. Although this gene has not yet been identified, certain possibilities have been eliminated. Southern blot analysis of the cDNA insert has shown no homology to rat P-450 enzymes,

both inducible by 3-methylcholanthrene. Presently, phenobarbital-inducible rat P-450 and ornithine decarboxylase are also being investigated. Finally, the gene transcript is being characterized by the technique of hybrid selection, *in vitro* translation and two-dimensional protein analysis.

(3) Characterization of Changes in Transcriptional Levels of Genes Coding for Differentiation of Specific mRNA. (a)  $\gamma$ -Glutamyltranspeptidase (GGT) catalyzes the transfer of the  $\gamma$ -glutamyl moiety of glutathione (or artificial substrates) to amino acid or peptide acceptors. In rats or mice, GGT activity is high in fetal liver and is associated with the plasma membrane of the biliary tract. At birth, this activity begins to fall and by 6-10 days postnatal has reached the characteristically low levels of the adult hepatocyte. Certain noncarcinogenic manipulations such as portacaval anastomosis and phenobarbital pretreatment can cause two- to eight-fold elevations of hepatocyte GGT activity. However, in rats or mice maintained on diets of various carcinogens or after single high-dose carcinogen treatment, liver GGT activity can increase up to approximately 50-fold. Most transplantable hepatomas are strongly GGT positive and exhibit activities approximately 50-100 times that of normal liver. The rat hepatoma cell line, 7777, contains approximately 20- to 30-fold higher GGT activity than does the Reuber (H4-II-E) cell line. Of the various biochemical markers used to identify preneoplastic foci, GGT identifies a very high proportion when compared with other markers.

We are presently cloning the GGT gene in order to study its regulation and expression. Our original cDNA library, made from rat hepatoma cell line 7777 poly(A) mRNA, yielded approximately 30,000 recombinant-containing bacteria. Analysis of the library for the specific GGT clone was done by hybridization using  $^{32}\text{P}$ -cDNA-7777 versus  $^{32}\text{P}$ -cDNA-Reuber, hybrid selection and immunoprecipitation. The three clones obtained in this manner were not specific for GGT. A more detailed inspection of the library revealed very low molecular weights of all inserts (< 200 b.p.). Therefore, we have remade the cDNA libraries using RNA's made from both 7777 cells and preneoplastic liver nodules using an improved procedure (Gene 25: 263-269, 1983) which specifically selects for high molecular weight inserts. In addition we have obtained two specific antibodies against both the heavy and light chains of GGT, which may possibly allow us to select cDNA clones that are specific for one or the other chains of the enzyme.

(b) Modulation of Albumin Gene Expression in a Rat Hepatoma Line by Sodium Butyrate and ADP-ribosylation. Chromatin conformation plays a key role in the regulation of gene expression. The use of chemical compounds which alter the chromatin structure has helped to elucidate the relationship between conformational modifications and gene expression. To further understand this relationship with respect to specific genes, the effect of butyric acid (BA), a chemical which affects the postsynthetic acetylation activity of histone proteins on two liver specific genes, albumin and  $\alpha$ -fetoprotein (AFP), were investigated in the Reuber rat hepatoma cell line, H4-II-E. In this cell line both genes are expressed at steady-state levels and are not responsive to the synthetic glucocorticoid, dexamethasone. The two genes differ in that albumin is synthesized in relatively high quantities while AFP synthesis is extremely low as compared to other rat hepatoma cell lines. Butyric acid (3 mM) treatment of this cell line resulted in a 40-fold increase in albumin secretion, but no change in the

low level of AFP secretion as measured by radioimmunoassay. The effect of BA treatment on albumin secretion was determined by Northern blot analysis to be the direct result of an inhibition on the transcriptional level. No quantitative differences were seen for AFP-specific transcripts following BA treatment. The possibility that hyperacetylation might act in concert with other modulators of chromatin conformation to affect a given response was investigated. We chose to study two additional modulators of gene expression, poly-ADP ribosylation and base methylation. Methylation of base sequences, specifically GGCC was assayed by restriction endonuclease analysis of cellular DNAs using the isoschizomers Hpa 11 and Msp 1. No difference was seen in the methylation pattern of albumin or AFP in BA-treated or control cells. The effect of inhibitors of ADP-ribosylation (ADPRT) was next determined in cells maintained in the presence or absence of BA. Treatment of Reuber H4-II-E cells with 3 mM 3-aminobenzoic acid (3AB) dramatically reduced the expression of albumin-specific RNA. The decrease was also observed following treatment with 10 mM nicotinamide, another inhibitor of poly-ADPRT, although incubation in the presence of the noninhibitory analogue, meta-aminobenzoic acid, at 3 mM did not affect the expression of albumin-specific RNA. Interestingly, the inhibitory effect of 3AB was overcome to some extent when cells were incubated in the presence of 3AB and BA. No quantitative difference in AFP specific RNA was seen when cells were incubated in the presence of poly-ADPRT inhibitors.

The fact that both hyperacetylation and poly-ADP ribosylation show specificity in their mode of action for only one of the two steady-state genes examined suggests that the controlling elements governing gene expression are rigorously controlled. We are presently extending this study to another cell line, 7777, in which AFP synthesis is high and albumin synthesis low, in order to determine if these controlling elements are specific for certain genes (i.e., differentiation specific versus oncofetal) or if they are responsive to activity-transcribed genes.

#### Significance to Biomedical Research and the Program of the Institute:

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

#### Proposed Course:

Continue the course outlined under Objectives and Major Findings.

#### Publications:

Engel, L. W., Heilman, C. A. and Howley, P. M.: Transcriptional organization of bovine papillomavirus type I. J. Virol. 47: 516-528, 1983.

Heilman, C. A., Linder, L., Maguire, R. and Thorgeirsson, S. S.: Independent expression of c-myc and N-ras during growth and differentiation of HL60 cells. Science (In Press)

Tosato, G., Steinberg, A. D., Yarchoan, R., Heilman, C. A., Pike, S. E., Seau, V. and Blaese, R. M.: Abnormally elevated frequency of Epstein-Barr virus-infected B cells in the blood of patients with rheumatoid arthritis. J. Clin. Invest. (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05261-03 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Metabolism and Mutagenicity of Chemical Carcinogens

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

PI:	Snorri S. Thorgeirsson	Chief	LEC	NCI
Others:	Irene B. Glowinski	Staff Fellow	LEC	NCI
	Satoru Hayashi	Visiting Fellow	LEC	NCI
	Chehab Razzouk	Visiting Fellow	LEC	NCI
	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Mona E. Møller	Visiting Associate	LEC	NCI
	Preston H. Grantham	Chemist	LEC	NCI

## COOPERATING UNITS (if any)

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

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

2.90

## PROFESSIONAL:

2.70

## OTHER:

0.20

## CHECK APPROPRIATE BOX(ES)

- (a) 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 research is at present focused on the following areas: (1) the relative roles of metabolic activation and detoxification in determining both mutagenic and carcinogenic potential of aromatic amines and amides; (2) the relationship between DNA damage, measured by alkaline elution, and the formation of DNA adducts caused by aromatic amines; (3) the relationship between host cell DNA damage and bacterial mutation frequency of carcinogenic heterocyclic amines derived from pyrolysates of amino acids, meat and fish in the *Salmonella*/hepatocyte system; and (4) comparison of N- and C-hydroxylations of 2-acetylaminofluorene (AAF) with debrisoquine oxidation in samples of human liver. Results so far obtained include (1) dose-dependent DNA binding and formation of individual DNA adducts (Gua-C8-AAF, Gua-C8-AF and Gua-N2-AAF) were observed in rat and mouse primary hepatocytes following exposure to N-hydroxy-acetylaminofluorene (N-OH-AAF) and N-acetoxy-acetylaminofluorene (N-OAc-AAF). The patterns of DNA adducts formed in vitro for N-OH-AAF were similar to those found in vivo. A positive correlation was found between the extent of DNA strand breaks and the formation of either Gua-C8-AAF or Gua-C8-AF. (2) The data from studies of the heterocyclic amines (Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2 and IQ) indicate that cytochrome P-450-dependent N-hydroxylation of the heterocyclic amines is an obligatory step in the metabolic activation of these compounds in both subcellular and in whole cell systems; genotoxicity of these compounds quantitatively differ when measured in intact hepatocytes versus the *Salmonella* tester strain; and agents modulating the activity and the composition of the cytochrome P-450 system may greatly influence both toxicity and carcinogenicity of this compound in vivo. (3) The results from this study comparing the capacity of human liver microsomes of 28 individuals to metabolize debrisoquine, bufuralol, aldrin and AAF indicate that common cytochrome P-450 isoenzymes are involved in the formation of AAF metabolites while the metabolism of debrisoquine, bufuralol and aldrin is unrelated to the metabolism of this carcinogen in human liver.

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

Snorri S. Thorgeirsson	Chief	LEC	NCI
Irene B. Glowinski	Staff Fellow	LEC	NCI
Satoru Hayashi	Visiting Fellow	LEC	NCI
Chehab Razzouk	Visiting Fellow	LEC	NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Mona E. Møller	Visiting Associate	LEC	NCI
Preston H. Grantham	Chemist	LEC	NCI
Ronald F. Minchin	Visiting Associate	LETM	NCI

Objectives:

The main objectives of the project are (1) to define, in an intact cell system, the metabolic processing of chemical carcinogens, especially carcinogenic aromatic amines and amides, and to identify the metabolic pathways that are responsible for activation and detoxification of these compounds; and (2) to study the mechanism whereby carcinogenic aromatic amines and amides cause mutations and other types of genotoxicity in both microbial and mammalian cell systems.

Methods Employed:

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

Major Findings:

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

1. Human Study. The capacity of human liver microsomes from 28 individuals to metabolize debrisoquine and bufuralol, two drugs oxidized polymorphically in man, as well as the carcinogen, AAF, was determined. In addition, the cytochrome P-450 content and the capacity of these microsomes to carry out the epoxidation of aldrin were measured. Interindividual differences in debrisoquine 4-hydroxylation, bufuralol 1-hydroxylation and aldrin epoxidation were 12-, 20- and 2.4-fold, respectively. The metabolism of debrisoquine was not correlated with cytochrome P-450 content ( $r = 0.26$ ) whereas both the metabolism of bufuralol ( $r = 0.45$ ,  $r^2 = 0.20$ ) and the epoxidation of aldrin ( $r = 0.72$ ,  $r^2 = 0.52$ ) were correlated. Rates of debrisoquine and bufuralol metabolism were significantly

correlated ( $r = 0.73$ ) whereas only weak correlations existed between debrisoquine:aldrin ( $r = 0.49$ ) and bufuralol:aldrin ( $r = 0.51$ ). Because biphasic kinetics have been observed in human liver microsomes for the 7- and 5-hydroxylation of AAF, two concentrations of this substrate were used. The disappearance of AAF at either  $0.37 \mu\text{M}$  or  $50 \mu\text{M}$  was not correlated with debrisoquine, bufuralol or aldrin metabolism. Similarly, at  $0.37 \mu\text{M}$  AAF no correlation existed between the formation of N-, 1-, 3-, 5-, 7- and 9-hydroxylation products of AAF and debrisoquine, bufuralol or aldrin metabolism. At  $50 \mu\text{M}$  AAF only the 7-hydroxylation of this substrate correlated with bufuralol metabolism ( $r = 0.47$ ). This lack of, or weak correlation between pathways leading to metabolic activation (N-hydroxylation) or detoxification (C-hydroxylation) of the carcinogens, AAF and debrisoquine, bufuralol and aldrin metabolism strongly suggests that different forms of cytochrome P-450 are involved in these pathways. In contrast, exceptionally high correlations ( $r > 0.94$ ) existed between N-OH-AAF: 1-OH-AAF, N-OH-AAF:7-OH-AAF, and 7-OH-AAF:1-OH-AAF at the low concentration of aminofluorene (AF) and imply that similar forms of cytochrome P-450 produce these metabolites. However, at  $50 \mu\text{M}$  AAF, these correlations are considerably weaker and explain less than 35% of the variance in the data. It is concluded, based on these multiple cross-correlations, that common cytochrome P-450 isoenzymes are involved in the formation of AAF metabolites while the metabolism of debrisoquine, bufuralol and aldrin is unrelated to the metabolism of this carcinogen in human liver microsomes.

2. Rabbit Study. Kinetic analysis of oxidative metabolism of AAF was studied in control and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced microsomes and with six highly purified cytochrome P-450 isoenzymes from rabbit liver. Kinetic parameters were defined for 7-, 5-, 3-, 1- and N-hydroxylations of AAF. 7-Hydroxylation was best defined by a two enzyme system, displaying a high affinity and relatively low capacity and a low affinity high capacity component in both control and TCDD-induced microsomes. The kinetic parameters for 7-hydroxylation were  $K_{m1} 0.53 \pm 0.07 (\mu\text{M})$ ,  $V_{max1} 938 \pm 47 \text{ pmol mg}^{-1}\text{min}^{-1}$  and  $K_{m2} 253 \pm 73$   $253 \pm 73 (\mu\text{M})$ ,  $V_{max2} 3610 \pm 531 \text{ pmol mg}^{-1}\text{min}^{-1}$  for control microsomes and  $K_{m1} 0.11 \pm 0.02 (\mu\text{M})$ ,  $V_{max1} 473 \pm 35 \text{ pmol mg}^{-1}\text{min}^{-1}$  and  $K_{m2} 147 \pm 48 (\mu\text{M})$ ,  $V_{max2} 3884 \pm 694 \text{ pmol mg}^{-1}\text{min}^{-1}$  for TCDD microsomes. All of the purified cytochrome P-450 isoenzymes were capable of catalyzing the 7-hydroxylation of AAF and, with the exception of form 4, this was the only oxidation on the AAF molecule catalyzed by these forms. The kinetic parameters [ $K_{ms} (\mu\text{M})$  and  $V_{maxs} (\text{pmol unit P-450}^{-1}\text{min}^{-1})$ ] for the 7-hydroxylation were:  $3.5 \pm 0.5$ ,  $256 \pm 15$ ;  $72 \pm 13$ ,  $39 \pm 6$ ;  $36 \pm 4$ ,  $106 \pm 7$ ;  $161 \pm 38$ ,  $175 \pm 31$ ;  $0.50 \pm 0.10$ ,  $8 \pm 1$ ; and  $0.40 \pm 0.04$ ,  $45 \pm 2$  for forms 1, 3b, 3c, 3v, 4 and 6, respectively. Forms 1, 4 and 6 accounted for a substantial part (> 25%) of total metabolic capacity corresponding to the high affinity component of 7-hydroxylation, whereas forms 3b and 3c accounted for less than 5% of the metabolic capacity displayed by the low affinity component in control microsomes. Forms 4 and 6 could account for over 90% of the metabolic capacity of the high affinity component of 7-hydroxylation in TCDD microsomes, whereas the form(s) responsible for the metabolic capacity of the low affinity component was not identified. Each of the 1-, 3-, 5- and N-hydroxylations was best defined by a single enzyme system in both control and TCDD microsomes (3- and 5-hydroxylations could not be defined in TCDD microsomes). Close agreements were found between the apparent  $K_m$  for N-hydroxylation in control ( $K_m = 0.36 \pm 0.03 \mu\text{M}$ ,  $V_{max} = 46 \pm 2 \text{ pmol mg}^{-1}\text{min}^{-1}$ ), TCDD induced

( $K_m = 0.25 + 0.03 \mu\text{M}$ ,  $V_{max} = 737 + 37 \text{ pmol mg}^{-1}\text{min}^{-1}$ ) and with form 4 ( $K_m = 0.35 \pm 0.02$ ,  $V_{max} = 31 + 2 \text{ pmol unit P-450}^{-1}\text{min}^{-1}$ ).  $\alpha$ -Naphthoflavone inhibited AAF N-hydroxylation to a similar extent in control and TCDD microsomes and in form 4. These data indicate that (1) a subpopulation of cytochrome P-450 isoenzymes, which includes all of the purified P-450 forms tested in the present study, is solely involved in detoxification (i.e., 7-hydroxylation) of AF, and as such probably behaves as a functional unit in vivo; (2) modulation of cytochrome P-450 content by inducers such as TCDD results in emergence of relatively few cytochrome P-450 isoenzymes that can account for most of the oxidative metabolism of AAF; and (3) a single cytochrome P-450 isoenzyme (i.e., form 4) is responsible for catalyzing N-hydroxylation of AAF, the first and the obligatory step in the metabolic activation of this carcinogen.

**B. Epoxide Hydrolase.** The expression of epoxide hydrolase was studied in cultured rat hepatocytes and hepatoma cell lines. Styrene 7,8-oxide and benzo[a]pyrene 4,5-oxide were used as substrates for microsomal epoxide hydrolase and trans-stilbene oxide for the cytosolic form of this enzyme. In freshly isolated hepatocytes from control rats, microsomal epoxide hydrolase activity was 7.7 and 10.8 nmol/mg cellular protein/min with benzo[a]pyrene 4,5-oxide and styrene 7,8-oxide as substrates, respectively. This enzyme activity increased by more than two-fold in hepatocytes after 24 hours in culture and remained elevated throughout 96 hours using both substrates. In cultured hepatocytes from rats pretreated in vivo with phenobarbital, trans-stilbene oxide, AAF and N-hydroxy-2-acetylaminofluorene (N-OH-AAF) both benzo[a]pyrene 4,5-oxide and styrene 7,8-oxide hydrolase activities were increased > 1.8 relative to controls. Hepatocytes from AAF pretreated animals at 24 hours in culture had approximately nine-fold higher activities than control hepatocytes. In marked contrast to microsomal epoxide hydrolase activity, the cytosolic enzyme showed an initial activity of 191 pmol/mg cellular protein/min in freshly isolated hepatocytes, decreased by 75% after 24 hours in culture and was barely detectable at 96 hours. A similar trend was apparent in hepatocytes from pretreated animals. In vitro treatment of hepatocytes with trans-stilbene oxide and phenobarbital increased microsomal epoxide hydrolase, while this activity was refractory to AAF treatment. Styrene 7,8-oxide hydrolase activity was increased in the McA-RH-7777 rat hepatoma cell line by phenobarbital, trans-stilbene oxide and AAF treatment. Similarly, benzo[a]pyrene 4,5-oxide hydrolase activity was also increased in this cell line by treatment with phenobarbital and trans-stilbene oxide but not by AAF. Microsomal epoxide hydrolase activity in rat H4-II-E hepatoma cells was refractory to induction, except by trans-stilbene oxide treatment, which caused a 70% increase in benzo[a]pyrene 4,5-oxide activity.

**C. Genotoxicity of N-hydroxy-2-acetylaminofluorene (N-OH-AAF), N-hydroxyphenacetin (N-OH-P) in Reuber (H4-II-E) Hepatoma Cells.** Derivatives of both N-OH-AAF and N-OH-P were tested for their ability to cause DNA damage in H4-II-E cells using the alkaline elution technique. Reuber cells are devoid of N-OH-AAF deacylase, N,O-acyltransferase, and sulfotransferase activities. The hydroxamic acids themselves caused very little DNA damage, while N-hydroxy-2-aminofluorene (20 to 100  $\mu\text{M}$ ), N-OH-P (20 to 200  $\mu\text{M}$ ), and p-nitrosophenetole (10 to 100  $\mu\text{M}$ ) all caused dose-dependent damage. The dose-dependent DNA damage caused by N-acetoxy-2-acetylaminofluorene (5 to 25  $\mu\text{M}$ ) was completely inhibited by the deacylase inhibitor paraoxon (100  $\mu\text{M}$ ). In the presence of both partially



purified rabbit liver cytosolic, N,O-acyltransferase and guinea pig liver microsomal deacylase, N-OH-AAF was genotoxic. Neither paraoxon nor tRNA had any effect on the DNA damage induced by N-OH-AAF in the presence of N,O-acyltransferase, while paraoxon completely inhibited the damage when N-OH-AAF was incubated in the presence of guinea pig deacylase, and N-OH-P only caused slight DNA damage at higher concentrations of enzyme. In addition, partially purified guinea pig liver deacylase and N-OH-AAF (25  $\mu\text{M}$ ) caused 2600 revertants in the *Salmonella* test system, while only 380 revertants were seen with a 40-fold greater concentration of N-OH-P (1000  $\mu\text{M}$ ). The mutagenicity of both N-OH-AAF and N-OH-P was completely inhibited by paraoxon.

Thus, it is clear that metabolites of N-OH-AAF formed outside the cell are capable of passing both the cellular and nuclear membranes to cause genotoxicity. Metabolic activation of N-OH-AAF by either the membrane-bound deacylase or the cytosolic N,O-acyltransferase caused genotoxicity via a deacylation process. Metabolic activation of N-OH-P by guinea pig deacylase caused low levels of DNA damage, whereas activation by N,O-acyltransferase was not sufficient to cause genotoxicity.

D. Aromatic Amine Induced DNA Damage in Mouse Hepatocytes. The capacity of the chemical carcinogen, 2-acetylaminofluorene (AAF), and its derivatives to cause DNA damage in primary mouse hepatocytes from arylhydrocarbon responsive C57BL/6 and non-responsive DBA/2 mice was studied using the alkaline elution technique. Low levels of DNA damage were observed after exposure of hepatocytes to either AAF or 2-aminofluorene (AF) (50-100  $\mu\text{M}$ ). Quantitation of metabolites produced from AAF in hepatocytes from untreated C57BL/6 and DBA/2 mice using HPLC showed a similar metabolic profile with respect to C- and N-hydroxylations. After *in vivo* pretreatment with the potent monooxygenase inducer TCDD (50  $\mu\text{g}/\text{kg}$ ), N-hydroxylation in the C57BL/6- and DBA/2-derived hepatocytes increased 25- and 5-fold, respectively. However, the C-hydroxylation pathways are still responsible for approximately 90% of the metabolism in cells from both strains. This may explain why only a slight increase in the DNA damage was observed in C57BL/6 mouse hepatocytes after incubation with AF or AAF and no increase in DNA damage was seen in the DBA/2 hepatocytes isolated from TCDD-treated animals. Both N-hydroxy-2-acetylaminofluorene (N-OH-AAF) and N-acetoxy-2-acetylaminofluorene (N-OAc-AAF) caused clear dose-dependent increases in DNA strand breaks (5-100  $\mu\text{M}$ ), suggesting that N-hydroxylation was the rate limiting step in the activation process of AAF leading to the DNA damage. Treatment of hepatocytes with paraoxon, an inhibitor of microsomal deacetylase activity, prior to exposure to either N-OH-AAF or N-OAc-AAF completely inhibited the damage caused by N-OH-AAF, while the damage caused by N-OAc-AAF was only partially inhibited. This suggests that these compounds are causing genotoxic effects after deacetylation. In accordance with this, N-hydroxy-2-aminofluorene (N-OH-AF), the deacetylated metabolite of N-OH-AAF, was an effective genotoxic agent, causing DNA strand breaks at low doses. Depletion of cellular glutathione by pretreatment with diethyl maleate increased the sensitivity of the cells to the damage induced by N-OH-AF. These data indicate that glutathione may play an important role in the detoxification of N-OH-AF in mouse hepatocytes.

E. Genotoxicity of Heterocyclic Amines in the Salmonella/Hepatocyte System. Several of the heterocyclic amines found in pyrolysates of amino acids and proteins or isolated from broiled fish or beef have been shown to induce

hepatomas in mice (Matsukura *et al.*, *Carcinogenesis* 5: 921-924, 1984; Sugimura *Cancer* 49: 1970-1984, 1982). These compounds are also found to be extremely potent mutagens in the *Salmonella* assay. A common activation pathway for creating the ultimate mutagens and possibly also the ultimate carcinogens from the heterocyclic amines is considered to be a cytochrome P-448 dependent N-hydroxylation.

In order to study the relative role of metabolic activation versus detoxification pathways in the potential genotoxicity of heterocyclic amines in mouse liver, we have used the *Salmonella*/hepatocyte system. This system is based upon coinoculation of isolated hepatocytes with the *Salmonella* tester strains, and both the mutation frequency in bacteria as well as the DNA damage in the hepatocytes are measured. Hepatocytes from arylhydrocarbon responsive C57BL/6N (B6) mice were used for this study.

The heterocyclic amines, 3-amino-1,4-dimethyl-5H-pyrido-[4,3-b]indole (Trp-P-1), 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2) showed very low mutagenic activity in the *Salmonella* strain TA98 after coinoculation with mouse hepatocytes for 30 minutes. In contrast 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) showed a clear mutagenic effect (up to 1  $\mu\text{M}$ ). Only low levels of DNA damage (measured by the alkaline elution technique) were observed after exposure of the hepatocytes to these heterocyclic amines (60 minute incubation).

In vivo pretreatment of the mice with the cytochrome P-448 inducer, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), markedly increased both the mutagenic effect in the bacteria and the DNA damage in the hepatocytes. Employing hepatocytes or microsomes from TCDD pretreated mice, IQ and Trp-P-2 were the most mutagenic in the *Salmonella* assay while Glu-P-2 showed the lowest mutagenic effect. In contrast, Glu-P-2 induced more DNA strand breaks at low concentrations (1-5  $\mu\text{M}$ ) than the other compounds. The mutagenic activation in both microsomes and hepatocytes of all the heterocyclic amines tested was completely blocked by the microsomal monooxygenase inhibitor  $\alpha$ -naphthoflavone (ANF). These data indicate that (1) cytochrome P-450 dependent N-hydroxylation of the heterocyclic amines is an obligatory step in the metabolic activation of these compounds in both subcellular and in whole cell systems, (2) genotoxicity of these compounds quantitatively differs when measured in intact hepatocytes versus *Salmonella* tester strain, and (3) agents modulating the activity and the composition of the cytochrome P-450 system may greatly influence both toxicity and carcinogenicity of this compound in vivo.

**F. Nitropyrene Induced DNA Damage in Primary Mouse Hepatocytes and Rat Hepatoma Cells.** The capacity of nitropyrenes to cause DNA damage in primary mouse hepatocytes (C57BL/6N mice) and rat H4-II-E hepatoma cells was studied using the alkaline elution technique. 1-Nitropyrene (10-200  $\mu\text{M}$ ) caused clear dose-dependent increases in DNA strand breaks in both cell types, whereas no increase in DNA damage was observed in hepatocytes treated with 1,3-, 1,6-, 1,8-dinitropyrene, 1,3,6-trinitropyrene and 1,3,6,8-tetranitropyrene under standard assay conditions (5-20  $\mu\text{M}$ , 30 minute incubation). However, 1,8-dinitropyrene (1,8-DNP) caused dose-dependent increases in DNA strand breaks when incubated with the H4-II-E cells for 48 hours, while 1,6-dinitropyrene (1,6-DNP) caused no DNA

damage under the same conditions. Neither 1,6-DNP nor 1,8-DNP induced DNA cross-links in the H4-II-E cells. These data indicate that substrate specificity exists in the metabolic activation of nitropyrenes in murine liver.

G. Association between DNA Strand Breaks and Specific DNA Adducts in Murine Hepatocytes Following In Vivo and In Vitro Exposure to N-Hydroxy-2-Acetylaminofluorene (N-OH-AAF) and N-Acetoxy-2-Acetylaminofluorene (N-OAc-AAF). N-OH-AAF and N-OAc-AAF have previously been shown to induce dose-dependent DNA strand breaks in primary hepatocytes from mice and rats. The deacetylase inhibitor, paraoxon ( $10^{-4}$  M) completely inhibited DNA damage induced by N-OH-AAF in mouse and partially in rat hepatocytes while DNA damage caused by N-OAc-AAF was only partially inhibited by paraoxon ( $10^{-4}$  M) in both species. In an attempt to determine the relationship between the extent of DNA strand breaks and the formation of specific DNA-carcinogen bound adducts in murine liver, the capability of N-OH-AAF and N-OAc-AAF to induce both DNA single strand breaks and adduct formation in vivo and in primary hepatocytes was measured. N-OH-AAF induced a low level of DNA damage in the rat (10 mg/kg, i.p.) and in the mouse (40 mg/kg, i.p.) 4 hours after treatment. The DNA adducts identified in vivo were N-(guanine-8-yl)-2-acetylaminofluorene (Gua-C8-AAF), 55% versus 11%, N-(guanine-8-yl)-2-aminofluorene (Gua-C8-AF), 34% versus 67% and 3-(guanine- $N^2$ -yl)2-acetylaminofluorene (Gua- $N^2$ -AAF), 11% versus 10%, respectively, for rat and mouse liver. An additional unknown adduct (12%) was detected in mouse liver. Dose-dependent DNA binding and formation of individual DNA adducts were observed in rat and mouse primary hepatocytes following one hour exposure to [ring- $^3$ H]-N-OH-AAF (0.1-2-  $\mu$ M) and [ring- $^3$ H]-NOAc-AAF (5-20  $\mu$ M). The patterns of DNA adducts in mouse and rat primary hepatocytes exposed to N-OH-AAF were similar to those in vivo. Paraoxon, at low concentrations ( $10^{-8}$ - $10^{-5}$  M), did not alter either the level of DNA binding or the pattern of adduct formation in rat hepatocytes treated with N-OH-AAF. However, at  $10^{-4}$  M, paraoxon partially blocked DNA binding (60%) and the formation of Gua-C8-AAF (95%) and Gua- $N^2$ -AAF (80%) while Gua-C8-AF was increased two-fold. In mouse hepatocytes paraoxon pretreatment ( $10^{-8}$ - $10^{-4}$  M) caused a dose-dependent reduction of the covalent binding of N-OH-AAF to DNA and complete inhibition of the formation of the unknown adduct and Gua-C8-AF by 70%. Gua-C8-AAF and Gua- $N^2$ -AAF were also inhibited but only at  $10^{-4}$  paraoxon. Rat and mouse hepatocytes treated with N-OAc-AAF displayed a similar pattern of adduct formation consisting of Gua-C8-AAF (72% vs. 39%), Gua-C8-AF (20% vs. 57%) and Gua- $N^2$ -AAF (8% vs. 4%). Paraoxon ( $10^{-8}$ - $10^{-4}$  M) pretreatment induced dose-dependent partial inhibition of the covalent binding of N-OAc-AAF to rat DNA and the formation of all guanine adducts. In the mouse, paraoxon partially blocked DNA binding of N-OAc-AAF only at high concentrations ( $10^{-5}$  and  $10^{-4}$  M) and inhibited the formation of Gua-C8-AF. These results indicate that a positive correlation exists between the extent of DNA strand breaks and the formation of either Gua-C8-AAF or Gua-C8-AF.

#### Significance to Biomedical Research and the Program of the Institute:

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

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

- Mattison, D. R., Chang, L., Thorgeirsson, S. S. and Shiromizu, K.: The effects of cyclophosphamide, azathioprine, and 6-mercaptopurine on oocyte and follicle number in C57BL/6N mice. Fertil. Steril. (In Press)
- McManus, M. E., Minchin, R. F., Sanderson, N. D., Wirth, P. J. and Thorgeirsson, S. S.: Kinetic evidence for the involvement of multiple forms of human cytochrome P-450 in the metabolism of acetylaminofluorene. Carcinogenesis 4: 693-698, 1983.
- McManus, M. E., Minchin, R. F., Sanderson, N., Wirth, P. J. and Thorgeirsson, S. S.: Kinetics of N- and C-hydroxylations of 2-acetylaminofluorene in Sprague-Dawley rat liver microsomes: Implications for carcinogenesis. Cancer Res. 43: 3720-3724, 1983.
- Møller, M., Glowinski, I. B. and Thorgeirsson, S. S.: Aromatic amine induced DNA damage in mouse hepatocytes. In Rydström, J., Montelius, J. and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogenesis. Amsterdam, Elsevier/North-Holland Biomedical Press, 1983, pp. 507-508.
- Shiromizu, K., Thorgeirsson, S. S. and Mattison, D. R.: The effect of cyclophosphamide on oocyte and follicle number in Sprague-Dawley rats, C57BL/6N and DBA/2N mice. Cancer Res. (In Press)
- Thorgeirsson, S. S.: Metabolic determinants in the carcinogenicity of aromatic amines. In Greim, H., Juna, R., Kramer, M., Marquardt, H. and Oesch, F. (Eds.): Biochemical Basis for Chemical Carcinogenesis. New York, Raven Press, 1984, pp. 47-56.
- Thorgeirsson, S. S., McManus, M. E. and Glowinski, I. B.: Metabolic processing of aromatic amines. In Mitchell, J. R. and Horning, M. G. (Eds.): Drug Metabolism and Drug Toxicity. New York, Raven Press, 1984, pp. 183-197.
- Thorgeirsson, S. S., Wirth, P. J. and Smith, C. L.: Genetics and induction of cytochrome P-450 dependent monooxygenases. In Flamm, W. G., Lorentzen, R. J. and Andrews, L. S. (Eds.): Handbook of Experimental Pharmacology. New York, Springer-Verlag (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05262-03 LEC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Mechanism of Chemically-Induced Murine Hepatomas

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

PI:	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Peter J. Wirth	Expert	LEC	NGI
	Carole A. Heilman	Senior Staff Fellow	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

2.15

PROFESSIONAL:

1.15

OTHER:

1.00

CHECK APPROPRIATE BOX(ES)

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

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

The goal of this project is to study the mechanism of chemically-induced murine hepatomas, and to identify and characterize endogenous and exogenous factors that may control initiation, promotion and progression of these tumors. Topics of present interest are (1) isolation and characterization of preneoplastic liver cell populations; (2) characterization of transplantation and growth of normal, preneoplastic and neoplastic rat hepatocytes in the anterior chamber of the eye of the isogenic host; and (3) modulation of cell surface receptors during chemically-induced hepatoma formation in the rat. Results obtained so far include: (1) immunohistochemical studies using antibodies against the asialoglycoprotein surface receptor of normal rat hepatocytes have confirmed the lack of this receptor in preneoplastic areas in rat liver. The areas lacking the asialoglycoprotein receptor are entirely superimposable with glucose-6-phosphatase-deficient areas and partially overlapped with the gamma-glutamyltranspeptidase positive areas in serial liver sections. (2) Due to the lack of asialoglycoprotein receptor on the surface of preneoplastic hepatocytes, an efficient method for separation of these cells from normal hepatocytes was developed using tissue culture plates coated with asialofetuin. Only normal cells attach, whereas preneoplastic and neoplastic cells do not. (3) In contrast to the reduction of asialoglycoprotein receptors during early stages of hepatocarcinogenesis, the surface receptor protein for transferrin is present at an increased level. (4) The transplantation to the anterior chamber of the rat eye offers a promising model to study attachment and growth of normal, preneoplastic, neoplastic and in vitro carcinogen treated liver cells.

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

Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Carole A. Heilman	Senior Staff Fellow	LEC	NCI

Objectives:

The objective of this project is to study the mechanism of chemically-induced murine hepatomas, and to identify and characterize endogenous and exogenous factors that may control initiation, promotion and progression of these tumors. Topics of present interest are (1) isolation and characterization of preneoplastic liver cell populations; (2) the time-course of chemically-induced hepatoma formation, and changes in cell surface receptor during this process; and (3) characterization of transplantation and growth of normal preneoplastic and neoplastic rat hepatocytes in the anterior chamber of the eye of the isogenic host.

Methods Employed:

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

Major Findings:

(1) We have used the anterior chamber of the eye for attachment and growth of normal, preneoplastic, neoplastic and in vitro carcinogen-treated liver cells. Short exposure, either in vivo or in vitro, of hepatocytes to carcinogens prior to the implantation to the eye offers a promising model to study the sequence of events during carcinogenesis. Our ultimate goal is to use this model for histological, biological and biochemical studies for early events in carcinogenesis. Thymidine uptake by the cells is used as a criteria for cell proliferation and different histochemical and immunohistochemical methods for identification of transformed liver cells.

(2) Studies on surface receptors of preneoplastic and neoplastic liver cells have revealed a significant reduction of surface receptors for asialoglycoproteins during early stages of carcinogenesis. These receptors are entirely lacking in neoplastic cell lines. On the contrary, the surface receptor protein for transferrin is present at an increased level on the surface of tumor cell lines. Further studies on transferrin receptors and on iron metabolism in preneoplastic and neoplastic cells induced by chemical carcinogens will be continued. Comparisons are being made with hepatoma cell lines.

(3) Due to the lack of asialoglycoprotein receptor on the surface of the preneoplastic cells, a method for separation of these cells from normal cells was developed using tissue culture plates coated with asialofetuin. Only normal cells attach, whereas preneoplastic cells do not.

(4) Immunohistochemical studies using antibodies against asialoglycoprotein surface receptor confirmed the lack of this receptor in preneoplastic areas. These areas were entirely superimposable with glucose-6-phosphatase deficient areas and partially overlapped the  $\gamma$ -glutamyltranspeptidase positive areas in serial liver sections.

#### Significance to Biomedical Research and the Program of the Institute:

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

#### Proposed Course:

Continue the course outlined under Objectives and Major Findings.

#### Publications:

Evarts, R. P., Marsden, E., Hanna, P., Wirth, P. J. and Thorgeirsson, S. S.: Isolation of preneoplastic rat liver cells by centrifugal elutriation and binding to asialofetuin. Cancer Res. (In Press)

Evarts, R. P., Marsden, E. and Thorgeirsson, S. S.: Regulation of heme metabolism and cytochrome P-450 levels in primary culture of rat hepatocytes in a defined medium. Biochem. Pharmacol. 33: 565-569, 1984.

Thorgeirsson, S. S., Wirth, P. J. and Evarts, R. P.: Early changes in gene expression during hepatocarcinogenesis. In Rydström, J., Montelius, J. and Bengtsson, M. (Eds.): Extrahepatic Drug Metabolism and Chemical Carcinogenesis. Amsterdam, Elsevier/North Holland Biomedical Press, 1983, pp. 541-546.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05263-03 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Arthur D. Olson	Computer Programmer	LEC	NCI
	Timothy Benjamin	Chemist	LEC	NCI

## COOPERATING UNITS (if any)

Laboratory of Biochemical Genetics (M. Nirenberg and K. E. Kruger) and  
Molecular Disease Branch (D. Sprecher), NHLBI, NIH

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

Chemical Carcinogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.1

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

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

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

The main objective of this project is to study the mechanism of chemical carcinogenesis by employing the technique of quantitative two-dimensional gel electrophoresis of total cellular protein. Since this technique allows for the simultaneous separation of total cellular polypeptides on a single polyacrylamide gel, it is possible to follow changes in the rate of synthesis of individual proteins as well as qualitative changes in the protein patterns as the cell undergoes malignant transformation. Our aim is to identify and characterize those proteins that are associated with the transformed phenotype. We have acquired, and have significantly revised, a computer-based system to automatically analyze autoradiograms produced from these gels. We have also been successful in analyzing silver-stained gels. This system automatically finds and measures the intensity of any polypeptide resolved by these electrophoretograms. Newly developed programs automatically match together the spot patterns found in different gels. Still other programs link together a series of gels which may constitute an experiment, allowing the investigator to quantitatively follow the synthesis of any resolvable protein through that experiment, or series of experiments. The investigator may specify various parameters and ask the computer to list those spots whose pattern of synthesis may lie within or without those parameters. Finally, several sophisticated computer-graphics programs allow the investigator to visually compare and follow various polypeptides which may be matched on a virtually unlimited number of electrophoretograms. The ultimate aim of this facility is to develop a gel analysis system that is as completely automatic as possible in analyzing the gels involved in an experiment.



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

Mark J. Miller	Senior Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Arthur D. Olson	Computer Programmer	LEC	NCI
Timothy Benjamin	Chemist	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 qualitative changes in the protein patterns as the cell undergoes malignant 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 techniques, (3) computer-based quantitation of autoradiograms, and (4) radioisotope measurements.

Major Findings:

In the past year, the major effort within the Laboratory's computer facility has been to further expand and develop the two-dimensional gel analysis system. An increasing portion of our time has been spent in the analysis of experiments involving these gels. In the past year, we have written, or revised, over 95 programs and subroutine packages. We have been careful to document these programs and keep the documentation up-to-date. A brief description of the major hardware and software developments follows.

(A) Hardware. In order to convert a two-dimensional image into a form a computer can manipulate, it is necessary to digitize that image. Last year we obtained an Eikonix Model 791 optical digitizer for this purpose. The Eikonix consists of a 2048-element photodiode array mounted behind a lens. The array scans across the focal plane of the lens, digitizing the incident light into one of 4096 discrete levels (gray levels) corresponding from 0 to 1.6 optical density units. Each element of this array can be independently calibrated with respect to background subtraction and gain control so as to produce a uniform response. In addition, a look-up table within the Eikonix can be utilized to convert the data from linear transmission format to reverse transmission or optical density format. In the past year, so-called "User Friendly" programs have been developed to aid investigators in scanning gels using the Eikonix.

These programs are menu driven and guide the user through the various steps and options involved in digitizing a set of gels. Also, improvements have been made in the precision and reproducibility of the scans.

(B) Software. The ultimate aim of this facility is to develop a gel analysis system that is as completely automatic as possible in analyzing the gels involved in an experiment. Equally important is the development of "tools" which aid investigators in the interpretation of their results. A major effort has been made to make the gel analysis software more robust and accurate. Almost all of the programs have been modified to improve their usability. A brief outline of the major advances follows.

(1) Findpeaks and findareas. These programs find and quantitate the intensity of each spot in a gel. Findpeaks utilizes a new spot finding algorithm which detects poorly resolved shoulders on spots. This is done by calculating the second derivative of the surface of gel in both the X and the Y directions. A least squares convolution template is used to smooth the data and calculate the second derivative in one operation. Regions where the second derivative becomes negative identify the peak of a spot. Findareas measures the total intensity of each spot and generates a "segment list" which defines the spot's shape. This segment list specifies regions of the gel that are uniquely associated with each spot and is used by several other programs, mostly for matching spot patterns or for visualizing the spots on an image processing device.

(2) Automatch. This program automatically matches the spot patterns resolved on two-dimensional gel electrophoretograms. The program does not require hand-matched, landmark matches for initial alignment of the spot patterns. The matching algorithm is based on a hierarchical nearest neighbor analysis. Starting with the most intense spots in the two films, the program determines if spots are equivalent by attempting to match a list of all nearby spots (a "cluster") from each film. "Clusters" are considered to be aligned if there is a low probability that the pairing of spots between them is due to a random process. The match is further tested by requiring that secondary clusters (i.e., those clusters that surround the spots matched between the central clusters) can also be aligned. Pairings found by cluster matching are checked for consistency and matches that are out of alignment with the majority of other matches are eliminated. Finally, the program tries to match the remaining spots by mapping the coordinates of an unmatched spot in one gel into the coordinate system of the other using the matched spots in the cluster as landmarks. The formulas used for the transformation allow for localized rotation and stretching of the coordinate systems.

This program is a tremendous saver of investigator time and effort. If high quality gels are used, the program will find on average, 95% of the spots that are matchable between the gels, and requires about 4.3 seconds per match. Most of the errors are found at the extreme edges of the gels.

(3) Library routines. We have created a series of subroutine packages that handle most of the common manipulation protocols used in our analysis system. Our plan is to isolate any set of subroutines that are used in two or more programs into an archived library. For portability reasons, we do the same with any set of subroutines that must utilize equipment specific to this

installation. Thus, if in future years, we should decide to change the format of any of the data files, or obtain new equipment, we will need only to modify the subroutine packages on which our programs depend, and not a large number of individual programs. Likewise, other installations which may wish to utilize our programs, but do not have the same equipment, need concentrate only on rewriting the subroutines and not all of the programs. A brief description of some of the more significant packages is outlined below.

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

(b) Pair. These functions operate on the files that list pairs of spots matched between two films, and are designed to allow a program to create, read, write, and edit the contents of any number of these files simultaneously. Also, given a point within the coordinate space of any film, these routines will find the contour, if any, to which that point belongs.

(c) Experiment. These functions let you get at data associated with experiments that involved multiple gels. The subroutines partition the spots in gels into 'groups,' where each spot in a group is paired with some other spot in the group and no spot in any group is paired with any other group. Parameters can be specified to flag certain groups. For example, one could ask for all groups that are found only in a certain subset of gels in an experiment, or for all groups where at least one spot in the group was at least 10-fold more intense than one of the others.

In addition the following collaborations are in progress: Dr. Marshall Nirenberg and Dr. Karl E. Kruger (Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute [NHLBI]) on a study of cAMP-dependent changes in the glycoproteins of NG108-15 cells associated with synaptogenesis, and Dr. Dennis Sprecher (NHLBI) on a study of apolipoproteins by two-dimensional gel electrophoresis.

Several laboratories have obtained copies of our analytical system. In some cases, this is part of a collaborative study. These laboratories are: Dr. E. P. Geiduschek and Dr. N.-H. Xuong, Departments of Physics and Biology, University of California at San Diego; Dr. Dale Deutsch, Department of Pathology, SUNY at Stony Brook; Dr. Philip L. Bloch, Department of Microbiology and Immunology, University of Michigan; Dr. Dennis Hochstrasser, Department of Medicine, Geneva University Hospital, Switzerland; Dr. Arthur Toga, Department of Neurology, Washington University School of Medicine; and Kenneth R. Erikson, Nuclear Systems Operations, Beckman Instruments, Inc.

#### Significance to Biomedical Research and the Program of the Institute:

The technique of two-dimensional gel electrophoresis provides a virtual "snapshot" of the metabolic activity of a cell under a specific set of environmental conditions. Our Laboratory's computer system gives us the capability of analyzing and cataloging the synthesis of any protein resolved by such gels. We are involved in studies which will analyze, catalog, and compare the capacity of a cell to synthesize its various proteins during both normal development and chemical transformation and to identify and characterize those

proteins that are highly associated with the malignant phenotype. These studies should provide clues as to the biochemical nature of the malignant process and provide a means to identify the number of genes involved in this process.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

Miller, M. J. and Olson, A. D.: Automatic analysis of two-dimensional gel electrophoretograms: The processing of multiple gels. Electrophoresis '84 (In Press)

Miller, M. J., Olson, A. D., and Thorgeirsson, S. S.: Computer analysis of two-dimensional gels: Automatic matching. Electrophoresis (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05313-02 LEC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Early Events in Chemically Induced Hepatocarcinogenesis

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

PI:	Peter J. Wirth	Expert	LEC	NCI
Others:	Timothy Benjamin	Chemist	LEC	NCI
	Brian Huber	PRAT Fellow, NIGMS	LEC	NCI
	Dolores M. Schwartz	Biologist	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The project was initiated to study the regulation of growth and differentiation of normal and chemically transformed cells using the rat hepatocarcinogenesis model in combination with quantitative two-dimensional electrophoresis. Results obtained to date include (1) using the Solt-Farber initiation promotion protocol, early preneoplastic hepatocytes were induced in male rats, isolated, and then separated into "small" and "large" cell populations using elutriation centrifugation. Following carcinogen treatment two major constitutive polypeptides (pI 5.7, MW 50 kDa and pI 6.6/49 kDa) were not detected in either "small" or "large" cells. In contrast, three new polypeptides (pI 6.5/53 kDa; pI 6.6/51 kDa; and pI 6.8/52 kDa) were expressed in both "small" and "large" cells but not in untreated control cells. "Small" and "large" cells showed no qualitative polypeptide differences. In contrast 8-10% of the 600-800 readily detected proteins were undergoing quantitative changes of at least four-fold during chemical carcinogenesis. (2) Individual hyperplastic liver nodules were dissected, and then classified histologically as being either early preneoplastic, preneoplastic, or neoplastic. Following carcinogen treatment one minor cytosolic polypeptide (pI 6.75/31 kDa) was not expressed in either preneoplastic or neoplastic nodules. However, four new cytosolic (pI 6.35/75 kDa; pI 6.8/57 kDa; pI 5.55/50 kDa; pI 6.1-5.7/57-60 kDa) and three membrane associated polypeptides (pI 6.25/48 kDa; pI 6.75/26 kDa; pI 6.05/24 kDa) were expressed in both neoplastic and preneoplastic nodules but not in normal liver. Numerous quantitative differences were also detected among the various cell types. (3) Investigations of the homogeneity/heterogeneity of gene expression among individual nodules isolated from separate animals have revealed marked similarities among the tissues analyzed.

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

Peter J. Wirth	Expert	LEC	NCI
Timothy Benjamin	Chemist	LEC	NCI
Brian Huber	PRAT Fellow, NIGMS	LEC	NCI
Dolores M. Schwartz	Biologist	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The process of chemical carcinogenesis has traditionally been divided into three phases: initiation, promotion, and progression. Although the mouse skin was the first experimental model used to delineate these phases, the rodent liver is another tissue in which the initiation and promotion stages of chemical carcinogenesis can be clearly stated *in vivo*. The initiation events are generally believed to be the covalent interaction of a carcinogen with critical cellular macromolecules (e.g., DNA, RNA, and proteins) in the target tissues which results in alteration of the normal functions within the cell. These alterations may result in changes in both gene expression and phenotypic characteristics and under appropriate conditions, namely following promotion, develop into malignant tumor cells. Little is known concerning either the early cellular events in the initiation process or the gene products that control and maintain the initiated cells or how these products may change as a function of time during the promotion and progression phases of the carcinogenic process. Therefore the main objectives of this project are to characterize the early biochemical events and subsequent changes in gene expression which occur at each of the stages (initiation, promotion, and progression) of hepatocarcinogenesis. Using the Solt-Farber initiation-promotion hepatocarcinogenesis protocol initial studies have focused on the isolation and characterization (histologically, histochemically, and biochemically) of early preneoplastic, preneoplastic and neoplastic hepatocyte populations and on examining differences in gene expression in these populations using quantitative two-dimensional gel electrophoresis of total cellular proteins.

Methods Employed:

The principal methods employed are (1) tissue culture techniques; (2) cell separation techniques-elutriation centrifugation; (3) histochemical staining; (4) enzyme assays involving radiometric (tritium, carbon-14, sulfur-35, phosphorus-32, and iodine-125) assays; (5) chemical and radiochemical synthesis; (6) differential centrifugation and chromatographic techniques; (7) autoradiography and fluorography; (8) two-dimensional gel electrophoresis; and (9) computer-assisted quantitation of autoradiograms and silver-stained gels.

Major Findings:

(1) Preneoplastic and neoplastic changes were induced in adult male Fischer 344 rat liver using the Solt-Farber technique which selects for the formation of "resistant hepatocytes" that proliferate to form hyperplastic nodules,

some of which serve as sites for the development of malignant cells. As a selection process for the isolation of early preneoplastic hepatocytes, cells were separated on the basis of size into "small" and "large" cell populations using elutriation centrifugation. After separation both "small" and "large" cells plated with the same efficiency on collagen-coated dishes and both incorporated [14-C] amino acids with the same efficiency. Approximately 10-15% of both "small" and "large" cells stained positive for  $\gamma$ -glutamyltranspeptidase (GGT) activity. In contrast none of the untreated control hepatocytes stained for GGT activity. Two-dimensional electrophoretic analysis of hepatic polypeptide patterns from treated and untreated control animals revealed both qualitative and quantitative spot differences among untreated "small" and "large" cell populations. Following carcinogen treatment two major constitutive polypeptides (pI 5.7, MW 50 kDa; and pI 6.6/49 kDa) representing 0.61 and 0.65%, respectively, of the total amount of cellular protein (actin constitutes approximately 2-3% of the total) were not detected and apparently not expressed in either "small" or "large" cell populations. In addition to the apparent loss of expression of these proteins during hepatocarcinogenesis, three new proteins (pI 6.5/53 kDa, 0.35-0.46%; pI 6.6/51 kDa, 0.48-0.60%; and pI 6.8/52 kDa, 0.56-0.72%) were expressed in both "small" and "large" cell populations that were not expressed in untreated control hepatocytes. Comparison of "small" and "large" cells revealed no qualitative polypeptide differences. In addition to these qualitative protein changes, more numerous quantitative protein changes were also observed during hepatocarcinogenesis. Comparison of polypeptides from untreated cell populations to those from either "small" or "large" cells showed that approximately 8-10% of the total polypeptides (400-500 polypeptides were quantitated and compared) were undergoing quantitative changes greater than four-fold during chemical carcinogenesis. Unlike the qualitative changes which occurred in a relatively narrow molecular weight range of 49-53 kDa, the quantitative changes occurred in proteins at all molecular weights and isoelectric point ranges. In a majority of the polypeptides (27/37) a quantitative polypeptide change in one cell type (e.g., small cell) is paralleled by a similar change both in magnitude and direction, either increase or decrease, in the corresponding polypeptide of the large cell as compared to control hepatocytes. Of the 37 polypeptides which exhibited quantitative changes greater than four-fold, roughly 67% (24/37) increased following carcinogen treatment. Polypeptides from the "small" cell populations appear to show a slightly greater modulation of gene expression during hepatocarcinogenesis than do those from the large cells. At present nothing is known concerning the identity of any of the polypeptides which are apparently lost during chemical carcinogenesis or which are expressed in both "small" and "large" cells following carcinogen treatment. We are currently attempting to do this.

(2) During the course of hepatocarcinogenesis one of the most important processes in the neoplastic transformation of the liver is the development of focal lesions of proliferative hepatocytes during or shortly after initiation. After further carcinogen treatment or after promotion these lesions enlarge to form grossly visible hyperplastic nodules. These nodules have two options: the majority (90-98%) "redifferentiate" back to normal appearing liver while a few persist, enlarge further, and may serve as sites for the formation of the ultimate hepatocellular carcinomas. Since the hyperplastic nodule serves as a critical point in the formation of cancer we have recently begun a study concerning the biochemical nature of these nodules. Male Fischer rats were subjected to the

standard Solt-Farber procedure for the induction of the formation of liver hyperplastic nodules. Individual nodules of approximately the same size were removed and classified as being either preneoplastic or neoplastic on the basis of histological examination. All nodules, both preneoplastic and neoplastic, stained strongly for GGT activity. Two-dimensional electrophoretic (2D) separation of silver-stained polypeptides from normal untreated rat liver tissue and from a neoplastic hyperplastic nodule were very similar although numerous qualitative and quantitative polypeptide differences were readily detected. Approximately 1100-1200 polypeptides were readily visible on each electrophoretogram. To aid in analysis, tissue samples (untreated control liver, preneoplastic, and neoplastic nodules) were fractionated into cytosolic and crude membrane preparations prior to 2D analysis. Approximately 1000-1100 membrane and 800-1000 cytosolic polypeptides were readily resolved on each electrophoretogram. Following carcinogen treatment one minor cytosolic polypeptide (pI 6.75, MW 31 kDa) was not detected and apparently not expressed in either preneoplastic or neoplastic nodules. In addition to the apparent loss of expression of this protein during hepatocarcinogenesis, three new polypeptides (pI 6.35/ 75 kD; pI 6.80/57 kDa, and pI 5.55/50 kDa) and a polypeptide (pI 6.10-5.70, 57-60 kDa) composed of a group of seven spots most likely representing either glycosylated or sialic acid modified isoelectric point variants of a single polypeptide were detected in both preneoplastic and neoplastic nodules but not in untreated control liver samples. No qualitative spot differences (either in cytosolic or membrane fractions) were observed among preneoplastic nodules and/or neoplastic nodules themselves. Examination of membrane preparations following carcinogen treatment revealed the expression of two relatively major polypeptides (pI 6.25/48 kDa and pI 6.75/26 kDa) and one minor polypeptide (pI 6.05/24 kDa) in all neoplastic and preneoplastic nodules (10 total) compared. Numerous quantitative polypeptide differences were also detected among the various cell types. Quantitative comparisons of polypeptides within the same type of tissues (e.g., untreated versus untreated; neoplastic versus neoplastic; or preneoplastic versus preneoplastic) revealed a relatively tight quantitative correspondence between paired spots in each of the same tissue types in both cytosolic and membrane fractions. Greater scattering of polypeptide densities was observed, however, when normal untreated liver samples were compared to either preneoplastic or neoplastic nodules. Of particular interest are a group of cytosolic polypeptides (pI 6.80-7.10, MW 55-57 kDa) which were markedly increased in cytosolic preparations from both preneoplastic and neoplastic nodules as compared to untreated control liver. Recently, Lindahl and coworkers (Proc. Am. Assoc. Cancer Res. 25: 139, 1984) have reported the isolation and purification of an Inducible form of aldehydeNAD(P) oxidoreductase from hyperplastic liver nodules and preliminary data suggest that these two polypeptides may be the same. Various workers have also reported that other cytosolic proteins, such as the glutathione-S-transferase(s), epoxide hydrase, DT-diaphorase, and a 21 kDa polypeptide are selectively increased in hyperplastic nodules following carcinogen treatment and we are currently trying to demonstrate their presence on our 2D electrophoretograms. In addition to these cytosolic proteins we are currently attempting to identify some of the changes in the membrane associated polypeptides such as the cytochrome P450 isozymes which are markedly decreased in hyperplastic nodules or some of the polypeptides which are markedly increased such as  $\gamma$ -glutamyltranspeptidase.



(3) One of the main characteristics of cancer cells is their marked heterogeneity with respect to cellular structure, biochemistry, immunology, etc., and one hypothesis states that this heterogeneity appears early in the carcinogenic process, possibly during initiation. Others feel, however, that this heterogeneity occurs only late in the process. In an attempt to address this problem we have begun to investigate the heterogeneity/homogeneity of gene expression in preneoplastic and neoplastic nodules isolated from different animals undergoing hepatocarcinogenesis. Hyperplastic nodules were induced in male Fischer rats (Solt-Farber technique) and 6 hours prior to sacrifice and isolation of hyperplastic nodules animals were treated with [<sup>35</sup>S]-methionine (1 mCi). Nodules were dissected, sections histologically scored as either preneoplastic or neoplastic and the two-dimensional electrophoretic analysis of the cytosolic and crude membrane polypeptides performed. Electrophoretograms were both silver-stained to analyze for differences in constitutive polypeptide levels and then subjected to fluorography to analyze for differences in turnover rates of the individual polypeptides. Preliminary data obtained from the analysis of silver-stained gels revealed marked homogeneity among the individual nodules isolated from separate animals (three animals). We are currently analyzing the [<sup>35</sup>S] labeled polypeptides for differences in turnover rates for the individual polypeptides among the various nodules.

(4) Work has been initiated towards the construction of a "liver-polypeptide" map. We have begun a systematic study dealing with the subcellular isolation of the various liver fractions (e.g., nuclei, mitochondria, plasma membranes, nuclear membranes, cytosolic proteins, gap proteins, etc.) and the 2D electrophoretic analysis of their constitutive polypeptides. Once constructed this map will allow one to focus more closely on those polypeptides which are critically involved in the carcinogenic process(es).

#### Significance to Biomedical Research and the Program of the Institute:

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

#### Proposed Course:

Continue as outlined under Objectives and Findings.

#### Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05315-02 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Regulation of Cell Surface Protein Expression in Normal and Neoplastic Cells

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

PI:	William L. Richards	Senior Staff Fellow	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Mark J. Miller	Senior Staff Fellow	LEC	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

Cell Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

0.6

## PROFESSIONAL:

0.6

## 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.)

The purpose of this project is to identify, primarily by two-dimensional gel electrophoresis, polypeptides that characterize the cell surface in normal and neoplastic cells. Studies on normal hepatocytes from rats at different stages of development and from regenerating livers will be compared to studies on preneoplastic and neoplastic liver cell populations in order to identify alterations in cell surface polypeptide patterns that distinguish the malignant cell from the normal cell. Major findings are as follows: (1) The optimum method for the surface iodination of cells maintained as monolayers in 55 mm diameter tissue culture dishes utilized 1 mCi of Na[125-I] per dish and the direct addition of hydrogen peroxide to cells bathed in a lactoperoxidase-PBS-Na[125-I] solution at 2-4°C. (2) Transferrin-Sepharose beads were used to extract the transferrin receptor from a Triton X-100 extract of cells that had been surface-labeled with radioactive iodine. Two-dimensional gel electrophoresis of the isolated transferrin receptor and of the complete mixture of labeled cell surface proteins allowed identification of the position of this receptor on the autoradiogram of the complete mixture. (3) Preliminary manual analysis of autoradiograms of the two-dimensional gel electrophoresis patterns of radioiodinated cell surface proteins from normal and preneoplastic cells indicated that, with respect to control hepatocytes, preneoplastic hepatocyte cell surface patterns gain four spot/spot groups and lose one spot.

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

William L. Richards	Senior Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Mark J. Miller	Senior Staff Fellow	LEC	NCI

Objectives:

The objective of this program is to compare, primarily by two-dimensional gel electrophoresis, the regulation of cell surface protein expression in normal, preneoplastic, and neoplastic cells. Our aim is to identify alterations in cell cycle surface protein patterns that distinguish the malignant cell from the normal cell, and to examine the regulation of those surface proteins that are highly associated with the neoplastic process.

Methods Employed:

Methods used in these studies include: (1) examination of liver cells from rats at different stages of development, (2) examination of preneoplastic and neoplastic cell populations, (3) examination of hepatocytes from regenerating livers, (4) maintenance of primary cell cultures and cell lines in hormonally-defined media, (5) use of synchronous cell cultures, (6) study of growth factor-mediated stimulation of DNA synthesis in density-inhibited cell cultures, (7) use of [3-H]-thymidine incorporation into DNA to measure DNA synthesis or percent of cells entering S-phase, (8) two-dimensional polyacrylamide gel electrophoresis (2D PAGE) of radiolabeled proteins with detection by autoradiography. The incorporation of [14-C] or [3-H]-amino acids into proteins is used to examine the synthesis of individual proteins in the whole cell, in isolated cell organelles, or in secreted proteins. Lactoperoxidase-catalyzed iodination by [125-I] is used to label plasma membrane proteins. The synthesis of plasma membrane proteins is examined by parallel processing of replicate samples labeled with either the radioactive amino acids or iodine, subjected to 2D PAGE, and detected by autoradiography. (9) Identification of cell surface polypeptide hormone receptors in 2D PAGE patterns, and (10) use of cell mutants that are altered in the complement or concentration of hormones required to stimulate growth.

Major Findings:

(A) Three methods for radioiodination of the hepatocyte cell surface were compared. The methods yielded comparable two-dimensional autoradiograms, each revealing about 100 major presumptive cell surface proteins. The method of direct addition of hydrogen peroxide to cell monolayers in a lactoperoxidase-PBS-Na[125-I] solution at 2-4°C yielded optimal labeling. The optimum concentration of Na[125-I] was 1 mCi per 55 mm diameter plate.

(B) To begin to identify cell surface polypeptide hormone receptors in 2D PAGE patterns, the method of Felsted and Gupta was used to identify the transferrin receptor in two rat hepatoma cell lines. Surface proteins were radioiodinated in log phase H4-II-E and McA-RH 7777 cells. A portion of each sample was subjected to 2D PAGE to obtain the complete pattern of labeled surface proteins. The remainder of each sample was extracted with Triton X-100 to solubilize membrane proteins and the extract was incubated with transferrin-Sepharose beads. After the beads were washed extensively to remove nonadsorbed material the adsorbed material, presumably the transferrin receptor, was eluted into a denaturing buffer and subjected to 2D PAGE. In both cell lines, the presumptive receptor proteins formed two rows of 4 or 5 spots differing by unit charge and falling in the pH 6.2-6.5 range in the isoelectric focusing dimension. The estimated molecular weights of the rows of spots were 77K and 80K for the H4-II-E line and 87K and 90K for the McA-RH 7777 line. We anticipate that this method can be used to identify other cell surface polypeptide hormone receptors in 2D PAGE patterns.

(C) Replicate samples of normal and preneoplastic hepatocytes were treated with labeled amino acids or by the cell surface iodination technique and subjected to 2D PAGE using both equilibrium and nonequilibrium conditions. Autoradiograms of the resultant gels have been scanned and await computer analysis. Preliminary manual comparison of the gel indicates that, with respect to control hepatocytes, preneoplastic hepatocyte cell surface patterns gain four spots/spot groups and lose one spot.

#### Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying cell surface patterns that distinguish the malignant from the normal cell. The results of these studies could provide a basis for analyzing cancer cause and for selecting strategies designed to treat or prevent cancer.

#### Proposed Course:

Continue the course outlined under Objectives, Methods Employed and Major Findings.

#### Publications

Peraino, C., Richards, W. L. and Stevens, F. J.: Multistage hepatocarcinogenesis. In Sraga, T. (Ed.): Tumor Promotion and Carcinogenesis in Internal Organs. Cleveland, CRC Uniscience, 1984, pp. 1-53.

Richards, W. L.: Gamma-glutamyl transpeptidase expression in neoplasia and development. In Milman, H. and Sell, S. (Eds.): Application of Biological Markers to Carcinogen Testing. New York, Plenum Press, 1983, pp. 199-200.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05316-02 LEC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Hepatic Asialoglycoprotein Receptor Mediated Gene Transfer

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

PI:	Snorri S. Thorgeirsson	Chief	LEC	NCI
Others:	Carole A. Heilman	Senior Staff Fellow	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

The objective of this project is to construct an effective in vivo and in vitro gene transfer system by utilizing the highly efficient endocytosis process whereby asialoglycoproteins are taken up by normal hepatocytes. This should enable us to examine the biological effects, particularly with respect to oncogenesis, of introducing specific genes, both under in vivo and in vitro conditions into a normal highly differentiated cell. This is accomplished by covalently coupling the asialoglycoprotein to the DNA by using two reagents, N-acetyl-N'-(p-glyoxy-benzoyl)cystamine and 2-iminothiolane. The former reacts specifically with non-paired guanine residues and upon reduction generates a free sulfhydryl group. The latter reacts with the protein to provide another sulfhydryl group which is subsequently conjugated to DNA by an intermolecular disulfide interchange reaction. The experimental model currently under study is the rat liver. The initial coupling has been done using two transformation specific viral DNAs, namely the bovine papillomavirus DNA and the cDNA clone of the Harvey RNA tumor virus. Both tumor viruses have been well characterized in terms of transforming ability. The bovine papillomavirus DNA was prepared from pBR322 recombinants and tailed with approximately 50 residues of dGTP using terminal deoxytransferase. G-tailed viral DNA was coupled to modified asialofetuin by incubation under appropriate conditions. A similar procedure was used for conjugating the cDNA clone of the Harvey RNA of both bovine papillomavirus DNA and Harvey cDNA into primary rat hepatocytes, however, both DNAs were rapidly degraded. Experiments designed to limit the endogenous nuclease activity are presently in progress.

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

Snorri S. Thorgeirsson	Chief	LEC	NCI
Carole A. Heilman	Senior Staff Fellow	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI

Objectives:

The objective of this project is to construct an effective in vivo and in vitro gene transfer system by utilizing the highly efficient endocytosis process whereby asialoglycoproteins are taken up by normal hepatocytes. This should enable us to examine the biological effects of "transfecting" specific genes. This is accomplished by covalently coupling the asialoglycoprotein to the DNA by using two reagents, N-acetyl-N'-(p-glyoxybenzoyl)cystamine and 2-iminothiolane. The former reacts specifically with nonpaired guanine residues and upon reduction generates a free sulphydryl group. The latter reacts with a protein to provide another sulphydryl group which is subsequently conjugated to DNA by an intermolecular disulfide interchange in the reaction. The experimental model currently under study is the rat liver both in vivo and in vitro.

Methods Employed:

Methods used in these studies include chemical synthesis, recombinant DNA techniques, two-dimensional gel electrophoresis, radioisotope measurements using tritium, 32-P, carbon-14, and iodine-125, radioimmunoassays, phase contrast microscopy and tissue culture techniques.

Major Findings:

Cheng et al. (Nucleic Acids Res. 11: 659, 1983) have recently described a method which may prove quite useful in ligand-directed gene transfer studies. The procedure involves the attachment of DNA to a protein molecule that is selectively bound by a specific cell surface receptor. The receptor protein DNA complex is then transported into the cell via receptor mediated endocytosis. The method utilizes two reagents, N-acetyl-N'-(p-glyoxybenzoyl)cystamine (Gbz-Cyn 2-Ac) and 2-iminothiolane. DNA of interest is tailed with homopolymer tracts of deoxyguanosine and then reacted with Gbz-Cyn 2-Ac. Gbz-Cyn 2-Ac reacts specifically with nonpaired guanine residues and is easily reduced to yield a free sulphydryl group. Protein is similarly derivatized via reaction with iminothiolane to yield an intermediate which also contains a free sulphydryl group. The derivatized protein is then conjugated to the glyoxylated DNA via an intermolecular disulfide exchange reaction.

Recently isolated rat hepatocytes have been shown to bind desialylated serum glycoproteins very rapidly via an asialoglycoprotein receptor which is efficiently internalized along with the asialoglycoprotein ligand during endocytosis.

Utilizing this property of the hepatocytes we have attempted to introduce into the normal hepatocytes selected genes (BPV, ras, etc.) which have been implicated in the oncogenic process. Gbz-Cyn 2-Ac was synthesized in a three-step sequence starting from 4-acetylbenzoic. Acetylbenzoic acid was oxidized to p-carboxy-phenylglyoxal with selenium dioxide with a 50% yield. Condensation of p-carboxy-phenylglyoxal with cystamine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide yielded N-(p-glyoxybenzoyl)cystamine (Gbz-Cyn 2). Gbz-Cyn 2, which is unstable, was then converted to the acetyl derivative, N-acetyl-N'-(p-glyoxybenzoyl)cystamine (Gbz-Cyn 2-Ac) with acetic anhydride.

Preparation of derivatized asialofetuin. Dilute acid hydrolysis (0.1 N H<sub>2</sub>SO<sub>4</sub>-80°C-1 hour) of fetuin (Gibco) yielded asialofetuin (ASF). ASF was incubated with 2-iminothiolane at 0°C for 10 minutes. Excess 2-iminothiolane was separated from protein by a Pharmacia PD-10 column. The derivatized ASF (free sulphhydryl group) was then converted to the mixed disulfide with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in phosphate buffer. The degree of derivatization of protein (mixed disulfide form) was determined by measuring absorbance at 412 nm. Excess DTNB and 2-nitro-5-thiobenzoic acid were separated from protein on a PD-10 column.

Addition of homopolymer tracts to DNA. Bovine papillomavirus DNA and cDNA clone of the Harvey RNA tumor virus were tailed with dGTP using terminal transferase.

Conjugation of asialofetuin (ASF) to G-tailed DNA. Derivatized ASF was incubated with 32-P G-tailed DNA in triethanolamine-boric acid buffer at 23°C for 2 hours. The DNA was then precipitated twice with ethanol, dried, and then reduced to the free thio with dithiothreitol in triethanolamine-boric acid buffer. The reduced DNA was precipitated twice with ethanol, dried, and then added to the derivatized ASF and incubated at 23°C for 18 hours.

Initial coupling was done using two transformation specific viral DNAs, the bovine papillomavirus (BPV) DNA and the cDNA clone of the Harvey RNA tumor virus. Both tumor viruses have been well characterized in terms of transforming ability. The BPV viral DNA was prepared from pBR322 recombinants and tailed with approximately 50 residues of dGTP using terminal deoxytransferase. G-tailed viral DNA was linked to the modified ASF by incubation under appropriate conditions. A similar procedure was used for conjugating the cDNA clone of Harvey RNA tumor virus with ASF. Primary rat hepatocytes and the ASF-linked DNA were incubated for one hour in Hanks balanced salt solution and 5 mM CaCl<sub>2</sub> and then plated on collagen-coated plates in the presence of hormone supplemented defined media. Cellular DNA was prepared at various times post-transfection and analyzed for the presence of BPV viral DNA using Southern blotting analysis and hybridization with in vitro 32-P labeled BPV probe. Initial analysis demonstrated the presence of incorporated but degraded BPV DNA and Harvey cDNA in the hepatocyte cultures. Experiments designed to limit the endogenous nuclease activity are presently in progress.

#### Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying critical and specific genetic factors that are important in the oncogenic process. The information obtained from these studies could provide a basis for a better definition of the factors involved in cancer cause and may help in formulating an effective cancer prevention program.

Proposed Course:

Continue as outlined under Objectives and Major Findings.

Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE5369-01 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Chemical Leukemogenesis: A New Approach

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

PI:	W. David Hankins	Expert	LEC	NCI
Others:	S. Peter Klincken	Visiting Fellow	LEC	NCI
	Rou-Lan Qian	Visiting Associate	LEC	NCI
	Snorri S. Thorgeirsson	Acting Head, Cell Biology Section	LEC	NCI
	Vanessa T. Vu	Staff Fellow	LEC	NCI
	Susan Sieber	Deputy Director	OD (DCE)	NCI

## COOPERATING UNITS (if any)

University of South Carolina, Charleston, SC (M. Ogawa)

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

Cell Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.35

## PROFESSIONAL:

1.35

## 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.)

Our overall aim is to examine the interaction of chemical carcinogens with hemopoietic cells of rodents and primates to better define the target(s) for transformation and the sequence of events which mediate the progression of a chemically induced neoplasm. A number of agents in the environment have been demonstrated to induce cancer in experimental model systems and have been strongly associated with spontaneous neoplasms in humans. During the past decade, methods have been devised which permit a direct analysis of the transformation of a variety of cell types by oncogenic viruses. While the events which accompany virus induction of leukemia and sarcomas can now be readily studied in vitro, many of the strains of transforming viruses have been highly selected for a particular property and may not have general relevance to carcinogenesis by naturally occurring viruses, chemicals or radiation. Furthermore, while the relevance of chemicals and radiation to cancer is clear, very few systems exist wherein one can directly analyze the early and late events in carcinogenesis induced by these agents. At present we have developed a method for recognizing small colonies of hemopoietic stem cells grown in vitro in semisolid cultures. These colonies have tremendous proliferative potential and give rise to secondary colonies which contain the progenitors of white blood cells, red blood cells and platelets. We will culture these stem cells in the presence of a variety of chemicals to test their effect on cellular proliferation, differentiation and hormone sensitivity. In addition, chemically-treated stem cells will be inoculated into irradiated mice which will then be monitored for normal differentiation or hemopoiesis or leukemogenesis. We are hopeful that we will be able to define the target cell and the specific interactions which lead to leukemia.

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

W. David Hankins	Expert	LEC	NCI
Peter Klincken	Visiting Fellow	LEC	NCI
Rou-Lan Qian	Visiting Associate	LEC	NCI
Kay Chin	Medical Technologist	LEC	NCI
Nancy Sanderson	Chemist	LEC	NCI
Snorri S. Thorgeirsson	Acting Chief, Cell Biology Section	LEC	NCI
Vanessa T. Vu	Staff Fellow	LEC	NCI
Susan Sieber	Deputy Director	OD (DCE)	NCI

Objectives:

The project has four objectives: (1) to analyze direct chemical interactions with the DNA of partially purified hemopoietic "target" cells, (2) to initiate chemical transformation in vitro and follow the progression of leukemogenesis by transplanted cells in vivo, (3) to define the earliest detectable changes in hemopoietic progenitors which indicate the onset of leukemia, and (4) to study the growth properties and hormonal requirements of hemopoietic cells exposed to chemicals in vitro or in vivo.

Methods Employed:

(1) In vitro cultures of hemopoietic stem cells, (2) immunochemistry to define specific cell populations, (3) light and electron microscopy, (4) bone marrow transplantation in irradiated rats, and (5) long-term marrow cultures for extended chemical treatment.

Major Findings:

Historically, investigators have administered carcinogens to animals or, at best, partially purified cell populations in vitro and observed various effects or cancers which developed. Since transformed cells were identified in multiple differentiation lineages, it was interpreted in retrospect that the disease had resulted from transformation of a primitive multipotential "stem" cell. About a year ago, we began a study to define the cellular targets of chemical carcinogens in leukemogenesis. This study is being conducted in collaboration with Dr. Makio Ogawa of the University of South Carolina who has recently discovered a method for recognizing small colonies of hemopoietic stem cells grown in vitro in semisolid cultures. These colonies have tremendous proliferative potential and give rise to secondary colonies which contain the progenitors of white blood cells, red blood cells and platelets. We have now developed a completely new approach to assess the effects of a variety of environmental carcinogens (chemicals, viruses and radiation) on the earliest identifiable progenitors of the blood-forming elements. We have already demonstrated that with certain retroviral vectors we can transfer cellular genes into these multipotential stem cells.

Significance to Biomedical Research and the Program of the Institute:

Hematopoiesis permits *in vivo* and *in vitro* studies of readily identifiable self-renewing and differentiating cell populations. Since blood cells and their progenitors are amenable to various types of investigations, studies of hematopoiesis have long been at the scientific forefront in cell biology. Consequently, a high percentage of fundamental concepts of biology (cell regulation, protein synthesis, molecular evolution and molecular disease) have been derived in this model system. It is not surprising, therefore, that hematopoiesis has been successfully employed for a number of years to elucidate the mechanisms by which viruses bring about cellular transformation and altered growth. It is ironic, however, that even though there is a clear association of environmental chemicals with carcinogenesis, there has been little exploration of the direct effects of these carcinogens on specific hemopoietic cell types. We have, therefore, initiated such a study in an attempt to fill this research void.

Proposed Course:

First, to continue our efforts to establish an integrated program to study the effects of different classes of carcinogens in an effort to develop and test unifying concepts of carcinogenesis. The specific projects analyzing the effects of chemicals on hemopoietic cells *in vitro* are described elsewhere in this report.

Second, by assessing growth and differentiation of hemopoietic progenitors at various stages of chemically-induced leukemogenesis *in vivo*, we will define the earliest growth alterations which accompany and perhaps mediate the leukemia development.

Third, we will attempt to define new markers of chemically-induced transformation such as expression of specific genes (oncogenes?), or chromosomal breaks, deletions or translocations. In addition to providing new conceptual information, these studies may be of diagnostic value.

Fourth, if chemically-induced leukemia can be shown to be clonal, as are human leukemias, it may be possible to distinguish *in vitro*, stem cells from the normal clone and the abnormal (leukemic) clone. If so, this would make feasible studies to regenerate a leukemic animal's hemopoietic compartment with normal stem cells derived from the same individual (i.e., homologous transplant). This type of therapeutic procedure would have notable advantages over heterologous transplants which are generally accompanied by rejection and/or graft-versus-host disease.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05370-01 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

Normal Stem Cell Biology and Hemopoietic Regulation

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

PI: W. David Hankins Expert LEC NCI

Others: S. Peter Klinken Visiting Fellow LEC NCI  
 Rou-Lan Qian Visiting Associate LEC NCI  
 Kay Chin Medical Technologist LEC NCI  
 Nancy Sanderson Chemist LEC NCI

## COOPERATING UNITS (# any)

American Red Cross Laboratory, Bethesda, MD (C. Eastment); University of California at Berkeley (J. Schooley)

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

Cell Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

0.75

## OTHER:

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 overall objective of this project is to define and characterize normal stem cell biology with particular emphasis on the hemopoietic stem cell. The research is focused on the following areas: (1) employ current stem cell assays to identify the growth regulators which act upon these primitive cells; (2) continue to devise novel methods for both maintaining the stem cells in culture for longer periods of time and to improve the recognition of stem cells as well as further defining their progeny; and (3) purification, preparation of a panel of monoclonal antibodies and molecular cloning of erythropoietin. We previously reported that a single cell line derived from a mouse with erythroleukemia produced factors with erythropoietic activity. We have recently found that other lines, isolated several years ago, also produced erythropoietin. Extensive evidence indicate that erythropoietic activity produced by these lines is mouse erythropoietin. Our characterization of the erythropoietic activity included (A) biological (active in nine biological assays), (B) immunological (four anti-erythropoietin sera), and (C) biochemical (enzyme treatments and multiple purification techniques) studies. The finding that a large percentage of cells became benzidine- and spectrin-positive upon hemin induction indicated that most of the cells were erythroid and suggested autocrine production of erythropoietin. We therefore tested cloned lines for heterogeneity with respect to erythropoietin production. Since erythropoietin was detected in media from each of the 20 lines derived by 2 cycles of single cell cloning, it is likely that, in these lines at least, the same cells which produce erythropoietin also have erythroid developmental potential. Conditions have been found which allow production of more than 1 U/ml erythropoietin in cultures that are free of serum and albumin.

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

W. David Hankins	Expert	LEC	NCI
Peter Klinken	Visiting Fellow	LEC	NCI
Rou-Lan Qian	Visiting Associate	LEC	NCI
Kay Chin	Medical Technologist	LEC	NCI
Nancy Sanderson	Chemist	LEC	NCI

Objectives:

The research is focused on the following areas: (1) employ current stem cell assays to identify the growth regulators which act upon these primitive cells; (2) continue to devise novel methods for both maintaining the stem cells in culture for longer periods of time and to improve the recognition of stem cells as well as further defining their progeny; and (3) purification, preparation of a panel of monoclonal antibodies and molecular cloning of erythropoietin.

Methods Employed:

(1) Methycellulose cultures, (2) hematology staining methodology, (3) tissue culture of adherent and non-adherent cells, (4) protein purification procedures (PAGE, gel filtration, affinity columns, etc.), and (5) tritiated thymidine uptake erythropoietin assay.

Major Findings:

With regard to assays of hemopoietic stem cells most of efforts have been directed toward setting up and standardizing conventional assays. Although little new information has been obtained from these necessary studies, culture systems which allow growth of all of the progenitors of blood-forming elements are now available for use in studies of carcinogenesis and normal physiology.

On the other hand, we have made a substantial breakthrough in our erythropoietic studies. The hormone which regulates physiologic red cell production is erythropoietin and it is generally accepted that this hormone is made in the kidney. We have noted that several previously established erythroleukemia lines, several of the lines release large quantities of erythropoietin. We have begun to characterize the differentiation status of these erythroid lines and they appear to be relatively early erythroblasts, at or about the proerythroblast stage. We have proven biologically, immunologically, and biochemically that the hormone released by the erythroid cells is erythropoietin. Conditions have been devised to permit hormone production in serum-free medium. The hormone has been partially purified by chromatography on phenylsepharose, wheat germ agglutinin, ion exchange and molecular sieving columns. The active molecule is recognized by several antibodies raised against human erythropoietin, and is a glycoprotein which has a molecular weight of 35,000 daltons.

Significance to Biomedical Research and the Program of the Institute:

To realize the full potential of our chemical and viral transformation studies, it is essential to continue to improve our understanding of the regulation of growth and differentiation of normal blood-forming cells. The experiments in this project represent an attempt by Section members to achieve and maintain a hematology research program which is at the "forefront" in the area of normal hemopoietic physiology. Consequently, it is presumed that any information gained from such studies will be useful in further attempts to unravel the events of carcinogenesis.

The erythropoietin-secreting cell lines have practical, conceptual and clinical implications. Thus, a much needed source of large amounts of hormone is now available. Once purified, the hormone will certainly be useful for receptor and mechanistic studies. In addition, although appropriate caution must be exercised, our finding that the hormone stimulates erythropoietic development of human cells suggests that clinical trials with large quantities of purified erythropoietin can now at least be contemplated as feasible.

Proposed Course:

First, we will attempt to exploit current stem cell assays to identify the growth regulators which act on these primitive cells. These growth factors stimulate stem cells into division or inhibit their growth to negatively regulate the "stem" cell compartment. Alternatively, such factors may specifically induce differentiation of these cells to expand a particular hemopoietic pathway.

Second, we will continue to devise novel methods for maintaining the stem cells in culture for a longer period of time, improving the recognition of stem cells and further defining their progeny.

Third, a series of experiments have been initiated to purify, molecularly clone and prepare a panel of monoclonal antibodies to erythropoietin.

Fourth, when homogeneous erythropoietin is at hand, we will conduct studies to define its receptor and mechanism of action.

Publications:

Hankins, W. D. and Kaminchik, J.: Modification of erythropoiesis and hormone sensitivity by RNA tumor viruses. In Young, N., Levine, A. and Humphries, K. (Eds.): Aplastic Anemia: Stem Cell Biology and Advances in Treatment. New York, Alan R. Liss, Inc., 1984, pp. 141-152.

Hankins, W. D., Kaminchik, J. and Luna, J. A.: Transformation of adult and fetal hemopoietic tissues with RNA tumor viruses. In Neinhuis, A. and Stamatoyannopoulos, G. (Eds.): Globin Gene Expression and Hematopoietic Differentiation. New York, Alan R. Liss, Inc., 1983, pp. 245-261.

Langdon, W. Y., Ruscetti, S. K., Silver, J. E., Hankins, W. D., Buckler, C. E. and Morse, H. C.: Cas spleen focus-forming viruses. II. Further biological and biochemical characterization. J. Virol. 47: 329-334, 1983.

Lee, E. J., Kaminchik, J. and Hankins, W. D.: Expression of xenotropic-like env RNA sequences in normal DBA/2 and NZB mouse tissues. J. Virol. (In Press)

Tambourin, P., Casadevall, N., Choppin, J., Lacombe, C., Heard, J. M., Fichelson, S., Wendling, F., Hankins, W. D. and Varet, B.: Production of erythropoietin-like activity by a murine erythroleukemia cell line. Proc. Natl. Acad. Sci. USA 80: 6269-6273, 1983.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05371-01 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Effect of Transforming Proteins of Oncogenic Viruses on Hemopoietic Cells

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

PI:	W. David Hankins	Expert	LEC	NCI
Others:	S. Peter Klinken	Visiting Fellow	LEC	NCI
	Rou-Lan Qian	Visiting Associate	LEC	NCI
	Kay Chin	Medical Technologist	LEC	NCI
	Nancy Sanderson	Chemist	LEC	NCI

## COOPERATING UNITS (if any)

Massachusetts Institute of Technology, Cambridge, MA (R. A. Weinberg and R. Mulligan)

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

Cell Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

0.75

## OTHER:

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.)

With the long-term goals of (1) analyzing transforming genes in hemopoietic cells, (2) defining unknown functions of cellular genes, (3) studying globin gene regulation, and (4) examining the potential of genetic therapy, we have examined the effects of a cellular oncogene on hemopoietic cells in vitro. A virus chimera (EJ-1 virus) was constructed by ligating portions of Moloney leukemia virus and EJ-1, the transforming principle (encoding a ras p21) cloned from human bladder carcinoma cells. DNA of this replication-defective EJ-1 virus clone was transfected into psi-2 helper cells and transformed foci picked for expansion. Media was harvested from these expanded cultures and tested for transforming activity on 3T3 cells and for ras-induced in vitro erythroid transformation. 3T3 cells were non-productively transformed since the culture media were negative for transforming activity, XC and reverse transcriptase. The p21 encoded by EJ-1 was not phosphorylated and migrated as a single band on polyacrylamide gel electrophoresis (PAGE). Thus, the EJ-1 protein could be distinguished from the p21 doublet observed in Harvey sarcoma virus-infected cells. EJ-1 infection of murine hemopoietic cells induced large erythroid colonies (containing hemoglobin-positive and hemoglobin-negative cells) which appeared at 6-10 days post-infection. Picked erythroid cells were found positive for p21 by immunofluorescence. We have previously noted a variety of phenotypes of erythroid colonies induced by Friend, Harvey, Abelson, Rous and other transforming viruses. Evaluation of phenotypic characteristics of EJ-1 induced colonies with respect to time course, erythropoietin sensitivity, proportion of differentiated cells, and replating potential, most closely resembled that of erythroid colonies induced by other ras-containing viruses.



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

W. David Hankins	Expert	LEC	NCI
Peter Klinken	Visiting Fellow	LEC	NCI
Rou-Lan Qian	Visiting Associate	LEC	NCI
Kay Chin	Medical Technologist	LEC	NCI
Nancy Sanderson	Chemist	LEC	NCI

Objectives:

The long-term goals of this project are to (1) analyze transforming genes in hemopoietic cells, (2) define unknown functions of cellular genes, (3) study globin gene regulation, and (4) examine genetic therapy.

Methods Employed:

(1) Northern, Southern, and Western blotting techniques to identify transforming DNA, RNA or protein, respectively; (2) preparation of retroviral vectors carrying transforming sequences; (3) in vivo and in vitro hemopoietic stem cell assays; (4) gene cloning procedures; (5) in situ hybridization; and (6) in vitro virus-transformation assays.

Major Findings:

A virus chimera (EJ-1 virus) was constructed by ligating portions of Moloney leukemia virus and EJ-1, the transforming principle (encoding a ras p21) cloned from human bladder carcinoma cells. DNA of this replication-defective EJ-1 virus clone was transfected into psi-2 helper cells and transformed foci picked for expansion. Media was harvested from these expanded cultures and tested for transforming activity on 3T3 cells and for ras-induced in vitro erythroid transformation. 3T3 cells were non-productively transformed since the culture media were negative for transforming activity, XC and reverse transcriptase. The p21 encoded by EJ-1 was not phosphorylated and migrated as a single band on polyacrylamide gel electrophoresis (PAGE). Thus, the EJ-1 protein could be distinguished from the p21 doublet observed in Harvey sarcoma virus-infected cells. EJ-1 infection of murine hemopoietic cells induced large erythroid colonies (containing hemoglobin-positive and hemoglobin-negative cells) which appeared at 6-10 days post-infection. Picked erythroid cells were found positive for p21 by immunofluorescence. We have previously noted a variety of phenotypes of erythroid colonies induced by Friend, Harvey, Abelson, Rous and other transforming viruses. Evaluation of phenotypic characteristics of EJ-1 induced colonies with respect to time course, erythropoietin sensitivity, proportion of differentiated cells, and replating potential, most closely resembled that of erythroid colonies induced by other ras-containing viruses.

Significance to Biomedical Research and the Program of the Institute:

Transforming genes (oncogenes) encode proteins that convey a growth advantage to cells which permit their expression. Their derivation in relation to cell physiology and cancer remain obscure. This study represents a successful attempt to develop methodology with which one can directly analyze the growth promoting effects on a variety of cells. The ability to transfer cellular information via retroviral vectors should greatly facilitate our efforts to study transforming genes.

Proposed Course:

First, we will further analyze the growth characteristics and hormone requirements of erythroid cells transformed by vectors carrying the activated cellular ras gene.

Second, we will construct new vectors carrying other oncogenes and envelope sequences which appear to be responsible for the growth-inducing activity of HTLV and several murine retroviruses.

Third, we will attempt to infect hemopoietic stem cells with retroviruses carrying specific beta globin genes. These cells will be assessed for their ability to reconstitute thalassemic and normal mice and to give rise to progeny which, upon differentiation, synthesizes the transferred globin gene products.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05372-01 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Hormone Sensitivity and Growth Control of Tumor Cells

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

PI:	W. David Hankins	Expert	LEC	NCI
Others:	Rou-Lan Qian	Visiting Associate	LEC	NCI
	S. Peter Klinken	Visiting Fellow	LEC	NCI
	Kay Chin	Medical Technologist	LEC	NCI
	Nancy Sanderson	Chemist	LEC	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

Cell Biology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

0.75

## OTHER:

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 overall goal of this project is to examine and characterize hormonal sensitivity and growth control of tumor cells in the context of a working hypothesis suggesting that oncogenic transformation may result from a selective increase in sensitivity to external regulators. Two different kinds of erythroleukemia populations have been analyzed. In the first, hematopoietic cells were infected in vitro with two strains of Friend virus. Both variants induce erythroid bursts that proliferated and differentiated without added erythropoietin (Epo). However, while the bursts induced by FVP were well "hemoglobinized" (i.e. most cells contained hemoglobin), the cells of FVA-induced bursts contained little or no hemoglobin. The nonhemoglobin bursts, induced by FVA, were established to be erythroid by cytochemistry, electron microscopy, and hormone sensitivity. FVA-induced cells appeared to be hypersensitive to Epo since small concentrations of the hormone produced marked increases in hemoglobin production--even when the hormone was added to the cultures three days post-infection. Time-lapse photography documented that Epo-stimulated hemoglobin synthesis in virally transformed cells rather than uninfected erythroid precursors. Upon reexamination FVP-induced erythroid cells also were hypersensitive to Epo. These data are consistent with the hypothesis that oncogenic transformation may result from increased sensitivity of progenitor cells for natural, physiological regulators. The second erythroleukemia population was induced in vitro by Friend murine leukemia virus (F-MuLV). When placed into cultures these erythroleukemia cells need Epo for their growth. By reducing the Epo levels in vivo, survival time of the erythroleukemia could be greatly extended. These results suggest antihormone therapy may be efficacious for treatment of erythroleukemia.

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

W. David Hankins	Expert	LEC	NCI
Peter Klinken	Visiting Fellow	LEC	NCI
Rou-Lan Qian	Visiting Associate	LEC	NCI
Kay Chin	Medical Technologist	LEC	NCI
Nancy Sanderson	Chemist	LEC	NCI

Objectives:

The overall objective of this project is to examine and characterize the hormonal sensitivity and growth control of tumor cells. Specifically, we wish to better understand the reasons for a growth advantage in leukemic cells in two different kinds of erythroleukemia. Another aim of this project is to develop improved therapeutic reagents for treatment of cancer.

Methods Employed:

Several tissue culture systems will be employed in this study. These include: "aggregate" cultures, Dexter long-term marrow cultures, macroburst cultures (mixed colony assays), and "cannonball" assays.

Major Findings:

First, transformed erythroid cells from mice or cells infected with Friend polycythemia as anemia virus were found to be hypersensitive to erythropoietin. These cells had previously been assumed to be independent of the hormone for growth.

Second, transplantable leukemic cells were also shown to be sensitive in vitro to erythropoietin.

Third, transfusion of mature erythrocytes into mice which were near death with erythroleukemia led to a rapid and profound remission of the disease, which has not relapsed in some cases for at least four months. While other interpretations are possible, our working hypothesis is that the erythroleukemic cells are still dependent (perhaps hypersensitive) on the hormone, erythropoietin, and that this hormone is being reduced by the transfusion of erythrocytes (i.e., physiologic feedback inhibition). We are, therefore, pursuing this model to develop anti-hormone therapy of leukemia and other tumors.

Significance to Biomedical Research and the Program of the Institute:

The studies have suggested a new rational approach to cancer treatment based on the observed sensitivity of transformed cells to their natural regulators (hormones). This work should stimulate further investigation into the hormonal requirements of tumor populations. It should also lead to the development of anti-cancer agents by exploiting the hormone sensitivity of cancer cells.

Proposed Course:

- (1) We will continue to study the hormonal requirements of erythroleukemia cells in vitro and in vivo.
- (2) We will prepare antibodies against erythropoietin or its receptor and initiate therapeutic trials with these reagents in animals. .
- (3) We will culture primary explants from other tumors in various hormonal cocktails in an effort to identify the growth factors required for their survival.

Publications:

Hankins W. D., Cohen, S., Scolnick, E. and Furth, M.: Human epidermoid cells (A431) retain epidermal growth factor (EGF) receptors and hormone sensitivity following transformation by Kirsten sarcoma virus. In Rich, M. A. and Yohn, D. S. (Eds.): Leukemia Reviews International. New York, Marcel Dekker, Inc., 1984, pp.180-181.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05373-01 LEC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Hepatic Proliferation Inhibitors

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

PI:	William L. Richards	Senior Staff Fellow	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Henry C. Krutzsch	Expert	LEC	NCI
	Min-Kyung H. Song	Visiting Fellow	LEC	NCI
	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

The objectives of this project are to (1) identify and purify polypeptide factors from rat liver that inhibit proliferation of hepatocytes or hepatocyte cell lines, determining whether there is only one inhibitor or a family of cytostatic but non-cytotoxic hepatic proliferation inhibitors (HPI). The aim will be to obtain sufficient quantities of HPI(s) to allow detailed studies on the mechanism of action of these growth modulator(s). (2) Characterize the specificity of HPI(s) for inhibiting the proliferation of various cell types including normal hepatocytes from rats of different ages, preneoplastic and neoplastic hepatocytes, and cells from other tissues or species. (3) Determine whether HPI(s) are produced uniquely by hepatocytes or also by other normal, preneoplastic, or neoplastic cells. Results obtained so far include (1) development of methodology for (a) high resolution fractionation of tissue extracts by fast protein/peptide liquid chromatography; (b) rapid assay, for cells maintained in 96-well microtiter plates, of the incorporation of tritiated thymidine into DNA. Cells are pulsed with [3-H]-thymidine, released from the substratum by trypsinization, and collected with water washes onto glass fiber filters with a multi-channel cell harvester. Experiments with inhibitors of protein, DNA, and RNA synthesis indicate that the radioactivity retained on the filters selectively measures DNA synthesis. (c) Rapid fluorometric assay for DNA contained in the chromatin of cells attached to the wells of microtiter plates. In this assay, fluorescence resulting from interaction of chromatin DNA with Hoechst 33342 is measured automatically by a reflected light fluorometer. This fluorescence is directly proportional to cell or nucleus number and can be used for normalizing the incorporation of [3-H]thymidine into DNA described in (b), above. (2) Demonstration that certain ethanol-precipitable fractions from adult rat livers cause a dose-dependent inhibition of DNA synthesis as assayed by the above methods.

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

William L. Richards	Senior Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Henry C. Krutzsch	Expert	LEC	NCI
Min-Kyung H. Song	Visiting Fellow	LEC	NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI

Objectives:

The objective of this project is to isolate and characterize, both biologically and structurally, polypeptides and proteins that inhibit proliferation of hepatocytes or hepatocyte cell lines. We will primarily focus our studies on the hepatic proliferation inhibitor (HPI) described by Iype and his colleagues but will use a newer high resolution method fast protein/peptide liquid chromatography (FPLC) for fractionation of cell extracts and will thus be able to determine whether HPI can be resolved into a family of proteins. The latter will also be studied by subjecting inhibitor-positive fractions to two-dimensional polyacrylamide gel electrophoresis (2D PAGE). The initial goal will be to obtain sufficient quantities of HPI(s) to allow detailed studies of specificity, mechanism of action, amino acid sequence and other structural features, and tissue sources of these inhibitors. In addition we will prepare antibodies to HPI(s) to facilitate the above studies as well as anticipated future studies of the molecular biology of these inhibitors. Our aim is to investigate the mechanism of action of HPI(s) in terms of the following questions: (a) Is the antiproliferative activity of HPI(s) mediated by a plasma membrane receptor or by some other receptor and can such a receptor be isolated for further characterization? (b) Does HPI efficacy require intact cells as targets? (c) In 2D PAGE studies of polypeptide patterns and protein synthesis, can specific changes be mediated by HPI(s)? (d) Does the antiproliferative activity of HPI(s) depend upon interaction with the metabolic pathways altered by mitogens such as epidermal growth factor (EGF) or insulin, or do the growth inhibitor(s) and mitogens induce effects by independent and noninteracting pathways?

Methods Employed:

Methods used in these studies include (a) use of ethanol precipitation, ultrafiltration, and FPLC (fast protein/peptide liquid chromatography) to purify tissue HPI(s); (b) new rapid assays, developed in this laboratory, for the fluorometric assay of cellular DNA and assay of the cellular incorporation of tritiated thymidine into the DNA of cells grown or maintained in 96-well microtiter plates; (c) study of various cell types including normal hepatocytes from rats of different ages, preneoplastic and neoplastic hepatocytes, and cells from other tissues or species to determine the specificity of HPI(s) for inhibiting various cell types and to determine whether HPI(s) are produced uniquely by hepatocytes or also by other cells; (d) binding and degradation studies utilizing [<sup>125</sup>I]-labeled HPI(s) to determine whether the antiproliferative activity

of HPI(s) is mediated by a plasma membrane receptor or by some other receptor; (e) affinity chromatography, if there is evidence for an HPI receptor, to isolate the putative receptor; (f) examination of DNA synthesis in isolated nuclei versus intact cells to determine whether HPI efficacy requires intact cells as targets; (g) two-dimensional polyacrylamide gel electrophoresis studies of protein synthesis to determine whether HPI(s) mediate specific changes in the synthesis patterns; (h) investigation of the metabolic pathways modified by mitogens, e.g., EGF-induced phosphorylation of specific cell surface and intracellular proteins, to determine whether the antiproliferative activity of HPI(s) depends upon interaction with such pathways or whether the HPI(s) and mitogens induce effects by independent and non-interacting pathways; (i) amino acid sequencing; and (j) antibody production.

#### Major Findings:

Our initial studies have emphasized development of the following methods for the high resolution fractionation of tissue extracts and for the rapid assay of large numbers of fractions for their ability to inhibit DNA synthesis. (a) The initial isolation of HPI(s) followed previously used procedures for extraction from rat liver. After crude material was isolated from liver by homogenization and ethanol precipitation, it was fractionated into broad molecular weight cuts of 2K-10K, 10K-30K and 30K+ daltons by ultrafiltration. The particulate material remaining from the ethanol precipitation was extracted by pH 4.0/37°C treatment and similar molecular weight cuts were made on the material derived from this source after ethanol precipitation. These six pools were tested in the bioassays described in (b) and (c) below. The results indicated that HPI activity was present in both 2K-10K and 10K-30K molecular weight cuts, with somewhat more activity present in the mild acid extracted material. Material from the ethanol precipitation of the initial liver homogenate was further purified by FPLC and fractions were bioassayed; both the 2K-10K and 10K-30K cuts were so treated. Preliminary results of FPLC fractionation indicate that the 10K-30K material behaved the same during chromatography and bioassay as determined by other investigators, yielding one pool of stimulatory activity and one of inhibitory activity. The results from FPLC of the 2K-10K material showed several pools containing inhibitory activity and several containing stimulatory activity; no earlier work similar to this has been done on this molecular weight cut. (b) A specific and rapid method for assaying the incorporation of [3-H]-thymidine into the DNA of adherent cells maintained in 96-well microtiter plates has been developed. In this method, cells are pulsed with [3-H]-thymidine for an appropriate interval, released from the substratum by trypsinization, and collected with water washes onto glass fiber filters with a multi-channel cell harvester. Liquid scintillation counting is then used to determine radioactivity of chromatin retained on the filters. Experiments with inhibitors of protein, DNA, and RNA synthesis indicate that this method selectively measures DNA synthesis. (c) A fluorometric assay for DNA contained in the chromatin of cells attached to the wells of 96-well microtiter plates was developed. In this assay, fluorescence resulting from interaction of chromatin DNA with Hoechst 33342 is measured by a reflected light fluorometer that rapidly and automatically reads each well. We demonstrate that this fluorescence is directly proportional to cell number or nucleus number, can be used as a direct measure of cell proliferation, and can be used for normalizing the incorporation of tritiated thymidine into DNA described in (b), above. (d) An autoradiography method for determining the



percentage of nuclei labeled by [3-H]-thymidine, an index of the entry of cells into the S-phase of the cell cycle, was developed for use with 96-well microtiter plates.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying alterations in hepatic growth inhibitor production or responsiveness that distinguish malignant from normal cells. The results of these studies could provide a basis for analyzing cancer cause and for selecting strategies designed to treat or prevent cancer.

Proposed Course:

Continue as outlined under Objectives and Major Findings.

Publications:

None

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Structural and Physicochemical Studies of Proteins Relevant to Tumorigenesis

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:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	James L. Cone	Chemist	LEC	NCI
	Preston H. Grantham	Chemist	LEC	NCI
	Irene B. Glowinski	Staff Fellow	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

2.55

PROFESSIONAL:

1.05

OTHER:

1.50

CHECK APPROPRIATE BOX(ES)

- (a) 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, molecular conformation, 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. Current emphasis is focused on proteins that play a role in cell growth regulation, cell transformation or differentiation, such as marker enzymes, hormones, growth factors and transforming factors, whose level of expression and/or molecular structure, is aberrantly modified during these biological processes. Emphasis is placed on applying the modern spectroscopic methodologies of mass spectrometry, nuclear magnetic resonance (NMR) and circular dichroism (CD) as well as the standard methods of protein sequencing to the solution of these problems. We have purified to homogeneity the heterodimeric glycoprotein, gamma-glutamyl transaminase (gamma-GT), a tumor marker enzyme of unknown structure. There are both enzyme level and gross structural differences in gamma-GT between the normal and tumorigenic tissue. Separation of the protein subunits and sequencing of the resulting components is in progress. The method of fast atom bombardment (FAB) mass spectrometry is being applied to determine the accurate structure of medium size polypeptides of up to 2600 molecular weight, for example of tryptic peptides. The sequencing information obtainable by this method was improved upon by group specific labeling of functional groups. This approach will be applied to the sequencing of gamma-GT and to the p21 transforming protein. Conformation in the microenvironment determines the binding site to drugs, substrates and cofactors, although the overall secondary and tertiary structure of proteins is an important determinant also. Phenylalanine-containing polypeptides are being analyzed by CD methods in order to gain information about preferred conformations using the aromatic chromophore as a probe. Finally, in another model study using NMR methods it was found that formaldehyde, a suspected carcinogen, reacts relatively slowly with peptides under near physiological conditions.

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

Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
James L. Cone	Chemist	LEC	NCI
Preston H. Grantham	Chemist	LEC	NCI
Irene B. Glowinski	Staff Fellow	LEC	NCI

Objectives:

(1) To study in some detail the chemical structure, molecular conformation and physicochemical characteristics of certain natural biopolymeric materials, with current emphasis on proteins that play a role in cell growth regulation and cell transformation or differentiation, such as enzymes, hormones, growth factors and transforming factors, whose level of expression, and/or molecular structure is aberrantly modified during these biological processes. (2) Development and applications of modern fast atom bombardment mass spectrometric methods of protein sequence and structure determination as a complementary tool to the Classical methodologies. (3) To study the secondary and tertiary structure of proteins and other biopolymers and the changes in the conformation of these molecules caused by carcinogens, external agents that bind to them, or by minor modifications in the structure, using modern spectroscopic methods. (4) To relate the physicochemical characteristics of macromolecules to their biological functions.

Methods Employed:

(1) Fast atom bombardment negative and positive ion mass spectrometry; (2) proton and carbon-13 nuclear magnetic resonance spectrometry on samples in the solution state; (3) circular dichroisms spectropolarimetry; (4) spectrophotometry; (5) chemical modification and derivatization of peptides and proteins; (6) high pressure liquid chromatography, gel filtration, partition and affinity chromatographies; (7) gel electrophoresis; (8) enzymatic proteolysis of glycoproteins; and (9) Edman sequencing of proteins.

Major Findings:

(1) Chemical structure studies on  $\gamma$ -glutamyltranspeptidase.  $\gamma$ -Glutamyltranspeptidase ( $\gamma$ -GT) is a membrane bound glycoprotein enzyme of unknown structure that is involved in the degradative metabolism of glutathione and the uptake of some amino acids. In rodents  $\gamma$ -GT activity is especially high in fetal liver and in adult kidney but not in adult liver. The low liver enzyme activity is inducible by certain drugs and carcinogens such as azo dyes. For example, elevated levels of  $\gamma$ -GT are found in most hepatomas. This enzyme is thus expected to be a marker of neoplastic transformation, especially in hepatoma cells. It is also known that there are structural differences between the enzymes found in tumorigenic tissue and the corresponding normal tissues. We have set out to purify sufficient amounts of this enzyme to perform analysis of

the peptide sequence on materials both from normal and from tumorigenic tissues. The source of materials was normal rat kidney and tumor tissue derived from transplanted 7777 hepatoma cells. The membrane bound enzyme was dissociated either with Triton or by papain digestion. After a series of purification steps and chromatographies, purified material was obtained that showed two major components on the basis of gel electrophoresis, and each in turn exhibited a series of homologous components on the basis of isoelectric focusing. This observation confirms the earlier known finding that the enzyme is made up of two protein subunits of approximate molecular weight of 51,000 and 22,000, and both of these subunits are extensively conjugated with a set of homologous series of carbohydrates. Part of this purified enzyme will be utilized for preparation of antibodies. In structural studies, current efforts focus on the preparative separation of lightly bound subunits, selective enzymatic removal of carbohydrate side-chains, Edman sequencing of the polypeptide backbone, assisted with proteolytic fragmentation of proteins and analysis of the resulting polypeptides by classical sequencing methods and by fast atom bombardment mass spectrometry.

(2) Peptide sequencing by fast atom bombardment mass spectrometry. The classical Edman sequencing of proteins and peptides is a time proven technique for structural analysis. There are numerous situations, however, where this technique fails or where the results are ambiguous. In those cases the rapidly developing technique of fast atom bombardment (FAB) mass spectrometry can serve as an invaluable alternative. FAB mass spectrometric measurements allow determination of molecular weights to within 1 mass unit or better on peptides with amino acid residues of 25 and possibly more, a molecular weight range eminently suited, for example, for tryptic peptide analysis. The sequence information in these spectra, especially when obtained on subnanomole amounts of sample, is not satisfactory. In an effort to overcome this limitation we are developing methods with model peptides, using group specific reagents to increase the predictable charge localization in the molecule once it is ionized, and also to simplify the fragmentation pattern in an effort to give more reliable sequence information.

Good results were obtained by specifically tagging the polypeptides at various sites prior to running the spectral measurements. Under carefully controlled conditions the amino terminal end of peptides can be converted to the p-bromobenzamides. The bromine atom can act as a marker, since it consists of an even mixture of two isotopes two mass units apart, and thus ions containing the amino terminal segment of the peptide will show up as doublets. In addition the bromine isotopes are mass deficient by 0.1 mass units and also the bromophenyl group assists in localization of charge. Application of this technique, for example, to the hexapeptide gly-leu-ala-gly-leu-ala and the tetrapeptide pro-phe-gly-lys yield positive FAB spectra that exhibit ordered sequential fragmentation of the molecule with most of the ion current being carried by the amino terminal-containing fragments. The lysine group of the tetrapeptide can also be derivatized in addition to the N-terminal end. This difunctionalized derivative also has a set of bromine containing fragments indicative of the sequence from the carboxy terminal end of the peptide. Interestingly, the decapeptide, angiotensin I, (MW 1295.7) with potentially five reactive sites for bromobenzoylation produces only a monobromobenzamide. Pentafluorobenzoylation of peptides was another avenue we explored. We have been able to increase the fragmentation efficiency in this manner, and it is hoped that the sensitivity of

the method can be improved using this electro-negative end group, especially in running negative ion FAB spectra. Other group specific alterations are planned on model polypeptides with the aim of applying these methods to the structural studies of relevant proteins. The FAB approach of sequencing will be a method of choice when the amino terminal is blocked, where the protein is conjugated to phosphate, carbohydrates or is otherwise branched, or where modified or unnatural amino acids are present in the sequence. An inviting situation occurs when the primary structures have been deduced from the base sequences of their corresponding genes, but where errors in translation on the sites of post-translational modifications, which may influence their functional role, introduce uncertainties in the definition of structure. A case in point is the *ras* gene product, the p21 protein, that is expressed in closely related forms both virally and also in certain mammalian carcinomas. Our methods will be applied for structure analysis of these transforming gene products.

(3) Physicochemical studies related to peptides. (a) Formaldehyde is a widely used industrial chemical which can be toxic at increased concentrations and it is a suspected human carcinogen. Its physiological mode of action is not clear although it is known that it preferentially modifies single stranded bases in DNA. It also reacts with proteins but the nature of these interactions still awaits elucidation. We have examined the progress of reaction of simple polypeptides with formaldehyde by  $^{13}\text{C}$  NMR spectroscopy at various pH values including the near physiological range of pH 7.5 and find essentially a single product forming but at a slower than expected rate. This product could be a bridged amino product coupling the peptide at the terminal amino groups, a dihydroxymethyleneamine or an autoreduction product. Whether this reaction is reversible is unknown. (b) Circular dichroism (CD) spectropolarimetric measurements of biopolymers, such as proteins and homopolymeric amino acids provides useful information about the overall orderedness of structure, for example whether they are  $\alpha$ -helical,  $\beta$ -sheet or random in conformation. However, it is more difficult to assign accurate conformational parameters to the microenvironment of even shorter chain biopolymers. Our experiments in this area are aimed at using the chromophoric phenylalanines as indicators of conformational variations. Aromatic chromophores can interact in an asymmetric environment depending on their relative orientations, according to a phenomena called "exciton splitting." We have analyzed to date the CD spectra of polypeptides containing several phenylalanines separated by zero, one and two amino acids measured in lipophilic solvents and have not observed any indications of chromophore interactions, however a number of parameters can be varied in hopes of developing this approach as a useful conformational method on polypeptides.

#### Significance to Biomedical Research and the Program of the Institute:

Establishment of molecular structure and conformation of relevant biomolecules by up-to-date spectroscopic and other methods is a necessary prerequisite in a modern approach for ultimate understanding of the complex molecular transformations taking place in living systems, particularly in cancer causation mechanisms. We are applying existing methodologies to solve the structural and functional roles of biopolymers, particularly of proteins and marker enzymes that are causative or indicative of the neoplastic transformation. We are also developing methodologies with the intention of applying these to relevant problems.

Proposed Course:

Continue the course outlined under Objectives and Major Findings and hopefully expand the application of methodologies to a wider variety of problems.

Publications:

Frolk, C. A., Roller, P. P., Cone, J. L., Dart, L. L., Smith, D. M. and Sporn, M. B.: Inhibition of transforming growth factor-induced cell growth in soft agar by oxidized polyamines. Arch. Biochem. Biophys. 230: 93-102, 1984.

Hwang, K., Stelzig, D. A., Barnett, H. L., Roller, P. P. and Kelsey, M. I.: Purification of the growth factor mycotrophein. Mycologia (In Press)

Schmuff, N. R., Phillips, J. K., Burkholder, W. E., Fales, H. M., Chen, C.-W., Roller, P. P. and Ma, M.: The chemical identification of the rice weevil and maize aggregation pheromone. Tetrahedron Lett. 25: 1533-1534, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05375-01 LEC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Effect of Chemical Leukemogens on Hemopoietic Target Cells

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

PI: Vanessa T. Vu Staff Fellow LEC NCI

Others: Snorri S. Thorgeirsson Chief LEC NCI  
 Peter P. Roller Head, Biopolymer Chemistry Section LEC NCI  
 W. David Hankins Expert LEC NCI  
 Preston H. Grantham Chemist LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.3

PROFESSIONAL:

0.8

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The objective of this project is to study the mechanism(s) of chemically induced leukemia. Two major aspects are focused on: (1) characterizing the covalent interactions of chemical carcinogens with cellular DNA in hemopoietic tissues both in vivo and in vitro, and (2) examining the effects of these covalent interactions of chemical carcinogens and cellular DNA on the proliferation and differentiation of hemopoietic target cells. Leukemogenic chemicals which are under investigation include 7,12-dimethylbenz[a]anthracene (DMBA), N-methylnitrosourea (MNU), derivatives of 2-acetylaminophenanthrene (AAP), representing the polycyclic hydrocarbons, direct alkylating agents, and aromatic amines, respectively. Results obtained so far are (1) detection of N-(guanin-8-yl)-2-aminofluorene (Gua-C8-AAF) in bone marrow and spleen cells of Fischer 344 rats following an intravenous dose of N-hydroxy-acetylaminofluorene (N-OH-AAF) or N-acetoxy-2-acetylaminophenanthrene (N-OAc-AAF), (2) development of a murine multipotential hemopoietic stem cell colony assay which consists of pure and mixed colonies (granulocyte, erythrocyte, megakaryocyte, macrophage and mast cells). Future studies will include (1) determination of in vivo formation of DNA adducts with N-OH-AAF and N-OAc-AAF which are more leukemogenic than the acetylaminofluorenes, (2) development of a murine hemopoietic blast cell colony assay which contains only undifferentiated cells, and (3) investigation of the biological interactions of DMBA, MNU, N-acetylaminofluorene (N-OAc-AAF), and N-OAc-AAF on hemopoietic target cells using these stem cell assays.

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

Vanessa T. Vu	Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Peter P. Roller	Section Head	LEC	NCI
W. David Hankins	Expert	LEC	NCI
Preston H. Grantham	Chemist	LEC	NCI

Objectives:

A number of chemicals have been shown to induce leukemia in human and experimental animals. Previous *in vivo* studies indicate that certain chemicals elicit only specific types of leukemia (e.g., erythroid, myeloid, lymphoid) depending on species, strain, sex, and age of the animals, suggesting that chemical leukemogens may act on different target cells. This project has recently been initiated using direct approaches to study the mechanism(s) of chemically induced leukemia. Two major aspects are focused on: (1) characterization of covalent interactions of chemical carcinogens with cellular DNA in hemopoietic tissues and (2) examining the effects of these covalent interactions of chemical carcinogens and cellular DNA on the proliferation and differentiation of specific hemopoietic target cells. Leukemogenic chemicals which are under investigation include 7,12-dimethylbenz[a]anthracene (DMBA), N-methylnitrosourea (MNU), derivatives of 2-acetylaminofluorene (AAF) and 2-acetylaminophenanthrene (AAP), representing the polycyclic hydrocarbons, direct alkylating agents, and aromatic amines, respectively.

Methods Employed:

(1) HPLC analysis of carcinogen-DNA adducts using radiolabeled substrates, (2) UV, NMR, mass spectroscopy (MS) for structural confirmation of synthetic nucleoside and nucleobase adducts, and (3) hemopoietic stem cell colony assay using bone marrow and spleen cells from normal and 5-fluorouracil treated mice and rats in methylcellulose culture in the presence of medium conditioned by pokeweed mitogen-stimulated mouse spleen cells.

Major Findings:

(1) We have completed the study on the interactions of 2-acetylaminofluorene compounds in hemopoietic tissues. Aminofluorene-DNA adduct(s) were detected in Fischer 344 rat bone marrow, spleen, liver and kidney following an intravenous dose of the proximate carcinogen, N-hydroxy-acetylaminofluorene (N-OH-AAF) and its reactive derivative, N-acetylaminofluorene (N-OAc-AAF). The only DNA adduct detected in bone marrow and spleen is the deacetylated product, N-(guanin-8-yl)-2-aminofluorene (Gua-C8-AF), while the liver and the kidney contain two additional adducts, N-(guanin-8-yl)-2-acetylaminofluorene (Gua-C8-AAF) and 3-(guanin-N<sup>2</sup>-yl)-2-acetylaminofluorene (Gua-N<sup>2</sup>-AAF), in varying amounts. The mutagenic and carcinogenic adduct Gua-C8-AF persists in the spleen, kidney and liver but is rapidly lost from bone marrow.



(2) The hemopoietic stem cell colony assay(s) is being developed to study the effect of chemical carcinogens on hemopoietic differentiation. A multipotential hemopoietic stem cell colony assay which consists of pure and mixed colonies (granulocyte, erythrocyte, macrophage, megakaryocyte, mast) from mouse and rat spleen cells has recently been developed. Furthermore, a blast cell colony assay which contains only undifferentiated cells is currently being set up. These blast cells have been recently reported to be more primitive than CFU-GEMM (colony forming unit-granulocyte-erythrocyte-megakaryocyte-macrophage) since upon replating they reveal their self-renewal capacity and their extensive ability to generate secondary colonies, many of which are multipotential hemopoietic colonies.

Significance to Biomedical Research and the Program of the Institute:

This study is aimed at developing new approaches toward a better understanding of chemical carcinogenesis. The hemopoietic system offers an extremely valuable model by allowing one to study the direct effect of leukemogenic carcinogens on specific target cells.

Proposed Course:

Continue the course as outlined under Objectives and Major Findings.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05376-01 LEC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

The Role of Fatty Acid Acylated Polypeptides in Cellular Transformation

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

PI:	Henry C. Krutzsch	Expert	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Min-Kyung H. Song	Visiting Fellow	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.9

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.)

Proteins modified by post-translational addition of fatty acid have been associated with both cellular oncogenic transformation and differentiation. In an effort to gain further knowledge about what roles these modified proteins play, two-dimensional gel electrophoresis patterns of 3H myristylated proteins have been obtained from HL60 cells after experiments were performed with untreated cells, with cells blocked from proliferation, or with cells differentiated to either granulocytes or macrophages. Changes were noted when proliferation was halted, indicating that myristylated proteins are involved in the cell cycle, and when differentiation occurred. Most variations observed were manifested as partial or total decreases in spot intensities on gel autoradiograms when compared to those of untreated cells.

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

Henry C. Krutzsch	Expert	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Min-Kyung H. Song	Visiting Fellow	LEC	NCI

Objectives:

The objective of this project is to study the biochemical roles and mechanisms of action of fatty acid acylated polypeptides and proteins in normal and neo-plastic cellular development and proliferation.

Methods Employed:

The principle methods employed are various tissue culture techniques, high resolution ion exchange and reverse phase chromatography, two-dimensional gel electrophoresis, and various protein structural analysis procedures.

Major Findings:

Myristylated Proteins in HL60 Cells after Treatment with Various Agents. It has been shown that proteins modified by addition of myristic acid may be associated with cellular differentiation, proliferation and transformation. Because the HL60 cancer cell line can be manipulated to differentiate or cease proliferation without differentiation, it is a good potential model for examining the changes in myristylated proteins after treatment with various inducing or inhibitory agents. Two-dimensional gel electrophoresis was used to assess the changes in myristylated proteins following these treatments. Four experiments were carried out: cells with no treatment, cells inhibited from proliferation using difluoromethylornithine (DFMO), cells differentiated into granulocytes with hexamethylbis-acetamide (HMBA), and cells differentiated into adherent macrophages with 12-O-tetradecanoylphorbol-13-acetate (TPA). After these materials had their effect on the HL60 cells, they were incubated with 3H myristic acid, and lysed with two-dimensional gel lysis buffer, which was then applied to two-dimensional gel electrophoresis. Autoradiograms taken of the finished gels showed simple patterns that exhibited differences in some spots, depending on the agent present prior to incubation with labeled myristate, and others which remained constant, which provided internal standardization. Most changes in patterns were reflected as losses in spot intensities. For example, one major and several minor spots are subjected to down regulation whenever proliferation is halted, whether it be by DFMO or by differentiation, indicating that myristylated proteins are involved in the cell cycle. In another instance, what appeared to be a glycosylated, and thus presumably a cell surface protein, almost completely disappeared when the cells were caused to differentiate. Changes in patterns were also observed between cells differentiated into granulocytes or macrophages. In addition, several new spots appeared when differentiation occurred.

Significance to Biomedical Research and the Program of the Institute:

This research project is aimed at increasing the understanding about polypeptide and protein factors that control or are involved in normal and neoplastic cell proliferation and transformation. This knowledge should provide further insight into the oncogenic process and give clues to its control.

Proposed Course:

Continue as outlined under Objectives and Major Findings.

Publications:

Deibler, G. E., Martenson, R. E., Krutzsch, H. C. and Kies, M. W.: Sequence of guinea pig myelin basic protein. J. Neurochem. (In Press)

Dizdaroglu, M. and Krutzsch, H. C.: A comparison of weak ion exchange and reverse-phase HPLC for peptide separation. J. Chromatogr. 264: 223-229, 1983.

Fairwell, T., Krutzsch, H., Hempel, J., Jeffrey, J. and Jornvall, H.: Acetyl-blocked N-terminal structures of sorbitol and aldehyde dehydrogenases. FEBS Lett. (In Press)

Gajewski, E., Dizdaroglu, M., Krutzsch, H. C. and Simic, M. G.: OH radical induced cross-links of methionine peptides. Int. J. Radiat. Biol. (In Press)

Kira, J.-I., Deibler, G. E., Krutzsch, H. C. and Martenson, R. E.: Amino acid sequence of porcine myelin basic protein. J. Neurochem. (In Press)

Krutzsch, H. C.: Polypeptide sequence analysis using as chromatography-mass spectroscopy. In Shively, J. E. (Ed.): Microcharacterization of Polypeptides: A Practical Manual. New Jersey, Humana Press. (In Press)

Marasco, W. A., Phan, S. H., Krutzsch, H., Showell, H. J., Feltner, D. E., Nairn, R., Becker, E. L. and Ward, P. A.: Purification and identification of formyl-methionyl-(ency)-phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. J. Biol. Chem. (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05377-01 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Receptor and Growth Factor Genes: Cloning and Characterization

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

PI: Michael G. Cordingley Visiting Fellow LEC NCI

Others: Diana S. Berard Microbiologist LEC NCI  
 Gordon L. Hager Head, Hormone Action and Oncogenesis Section 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 20205

## TOTAL MAN-YEARS:

1.45

## PROFESSIONAL:

1.20

## OTHER:

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.)

Many important hormone and growth factor responses are mediated through the action of macromolecular receptors with high binding affinity for the respective hormone ligand. The glucocorticoid receptor is a cytoplasmic receptor that migrates to the nucleus and induces a number of alterations in gene activity after binding a member of the glucocorticoid family. Most receptors are present at low concentrations in responsive tissues, and are difficult to purify in quantity. The characterization of various receptors, both in terms of gene organization, and in terms of protein structure and function, would benefit enormously from the availability of molecular clones for the receptor loci. Attempts are underway to isolate and characterize clones for the glucocorticoid receptor from human cells. Two approaches have been taken; one involves the preparation of cDNA expression libraries from receptor competent cells, followed by clone identification with antisera to the receptor; the other involves identification of the receptor genomic locus by insertional mutation with retrovirus vectors.

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

Michael G. Cordingley	Visiting Fellow	LEC	NCI
Diana S. Berard	Microbiologist	LEC	NCI
Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI

Objectives:

Molecular cloning and characterization of the macromolecular receptor for glucocorticoid hormones.

Other growth factor receptors, such as insulin-like growth factor II will be cloned by the same approach.

Methods Employed:

cDNA libraries will be prepared from tissue culture cells that harbor relatively high concentrations of glucocorticoid receptor.

The libraries will be prepared in bacterial expression vectors, permitting the expression of part of the receptor polypeptide in lambda lysogens of bacteria.

Clones expressing fragments of the receptor sequence will be identified by plaque detection with polyclonal antibodies to the receptor protein.

Retroviral vectors capable of conferring positive selection on infected mammalian cells will be prepared. These vectors will be utilized as insertional mutagens in S49 lymphoma cells.

Induction of forward mutations to glucocorticoid resistance (known to correlate with loss of functional glucocorticoid receptor) will be accomplished by retroviral vector insertional mutagenesis.

Major Findings:

A series of cDNA libraries have been prepared with cells containing functional receptors for the glucocorticoid hormones, and for insulin-like growth factor II (IGF-II). High-titer representative libraries with complexities sufficient to include low-copy messages have been characterized in each case.

Lysogens that express polypeptides reactive with the anti-glucocorticoid receptor antisera have been identified and are being characterized.

Significance to Biomedical Research and the Program of the Institute:

The aberrant expression of a variety of growth factor and/or growth factor receptors has been implicated recently in several neoplastic events. Our understanding of the role of hormone and growth factor receptors in mammalian

cell metabolism, particularly in growth regulation of normal and transformed cells, is becoming of major importance. The experiments presented in this project will greatly increase our knowledge of the structure and function of two types of receptors.

Proposed Course:

A program has been initiated to molecularly clone the structural gene for the glucocorticoid receptor. The approach will be to utilize retroviruses and/or retroviral based vectors as insertional mutagens to inactivate the receptor locus in cells hemizygous for the receptor allele. This is a well characterized system, and will permit a rigorous test of the potential of retrovirus vectors as useful insertional mutagens.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05378-01 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Hormone Regulatory Elements: Structure and Function

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

PI:	Alexander C. Lichtler	Guest Researcher	LEC	NCI
Others:	Diana S. Berard	Microbiologist	LEC	NCI
	Gordon L. Hager	Head, Hormone Action and Oncogenesis Section	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 20205

## TOTAL MAN-YEARS:

1.45

## PROFESSIONAL:

1.20

## OTHER:

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.)

previous analysis of molecular chimeras between the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) and the v-ras transformation gene from Harvey murine sarcoma virus (HaMuSV) localized the steroid hormone regulatory sequences between 100 and 200 nucleotides upstream from the cap site in the LTR. This test system has been transferred to the single-strand DNA virus, M13, to permit high-resolution mutational analysis of the hormone regulatory signals. It has been found that the assay (hormone dependent appearance of v-ras dependent foci) remains functional in the new vector environment. In fact, the extent of hormone-dependence is even higher in the new system. A new method has been developed for the efficient and accurate introduction of mutations into regions of interest. This technique permits the oligonucleotide-directed introduction of mismatches as large as 10 nucleotides in a one-step procedure. The analysis of sequences required for the interaction of the hormone-receptor complex with target sequences in the DNA, and for the subsequent transcriptional response are in progress.



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

Alexander C. Lichtler	Guest Researcher	LEC	NCI
Diana S. Berard	Microbiologist	LEC	NCI
Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI

Objectives:

Analysis of hormone regulated transcription of mouse mammary tumor virus (MMTV). Localization of hormone regulatory sequences involved in this regulation.

High-resolution mutagenesis of sequences involved in hormone regulation for the purpose of characterizing elements responsible for the interaction of the glucocorticoid receptor complex with the chromatin target, and to identify other sequences necessary for this complex regulatory response.

Methods Employed:

Molecular chimeras between the MMTV long terminal repeat (LTR) and the v-ras gene of Harvey murine sarcoma virus (HaMuSv) will be used in a hormone-dependent transfection assay to probe the regulatory regions involved in hormone induction of MMTV expression.

This transfection assay will be adapted to the M13 single-stranded virus system to permit efficient and directed isolation of appropriate mutations.

Heteroduplex molecules will be prepared containing gaps to permit site-directed mutagenesis of regions of interest.

Mutants will be introduced into regions previously identified as necessary for the hormone response by oligo-directed mutagenesis and single-strand specific chemical mutagenesis.

Major Findings:

The v-ras MMTV fusion system has been transferred into the M13 bacteriophage, permitting high-resolution site-directed mutagenesis in gapped molecules created by heteroduplex formation between single-stranded molecules containing complete LTR regions and duplex molecules deleted for selected areas of the regulatory sequence. The test system after transfer to the M13 vector remains highly inducible to the action of glucocorticoid hormones, manifesting a 200-fold response in the number of foci recovered in the presence of hormone compared to the absence of hormone.

A new technology has been derived for the rapid isolation of extensively mismatched mutants in regions of interest. Evidence from physical characterization of nucleoprotein structure at the regulated promoter indicates that chromatin is highly organized in this region. Deletion mutagenesis thus

suffers a serious weakness in that large-scale removal of sequences probably alters several parameters of structure simultaneously. Alternatively, single-base mutants usually do not impair the structure sufficiently to score a clear phenotype in the test system.

The new technique permits the simultaneous change of as many as 10 base pairs without changing the relative position of non-mutated sequences with respect to each other, or with respect to other elements of the promoter.

Preliminary results indicate that individual binding sites for the receptor complex can be eliminated without seriously impairing the hormone response, as expected from earlier results. A long-range, high-resolution analysis of the various elements critical for the hormone response is in progress.

#### Significance to Biomedical Research and the Program of the Institute:

Experimentation in the past five years clearly indicates that the aberrant expression of genetic information, particularly elements referred to as oncogenes, is a major determinant in the development of neoplastic transformation, both in animal and in human systems. A detailed knowledge of the mechanisms involved in the control of mammalian genes is therefore paramount in the effort to understand and control cancer. The experiments conducted in this project will considerably aid in our understanding of how hormones regulate gene expression in mammalian tissues.

#### Proposed Course:

Site-directed molecular mutagenesis will be applied with the M13 MMTV LTR v-ras fusion system to precisely characterize the hormone regulatory sequences at the single nucleotide level.

High-resolution mutants in the hormone response region will be transferred into amplification vectors for study in cell-free binding to the glucocorticoid receptor.

High-resolution mutants in the hormone response region will be transferred into bovine papilloma virus episomal fusions containing the MMTV LTR driving the v-ras sequence. The effect of mutants on the hormone response can therefore be tested in the normal chromatin environment, as opposed to naked DNA.

#### Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05379-01 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Analysis of Polypeptide Changes During Cellular Differentiation

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

PI:	Peter J. Wirth	Expert	LEC	NCI
Others:	Timothy Benjamin	Chemist	LEC	NCI
	Dolores M. Schwartz	Biologist	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Stuart H. Yuspa	Chief	LCCTP	NCI
	Henry Hennings	Expert	LCCTP	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.4

## OTHER:

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.)

This project was initiated to analyze, both qualitatively and quantitatively, changes in total cellular protein patterns during cellular differentiation using the technique of quantitative two-dimensional electrophoresis. Results obtained to date include: (1) mouse epidermal cells were cultured in the presence of either low calcium (0.02-0.1 mM) (mainly basal cells) or high calcium (1.2 mM) (mainly mature keratinocytes) and then treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) for 1, 4, or 24 hours. Two-dimensional analysis of total cellular polypeptides from cells grown in the presence of either low or high calcium revealed only quantitative polypeptide differences. (2) Similarly, only quantitative differences were detected following treatment of either low or high calcium cells with TPA (0.01 and 0.1 microgram/ml). In low calcium cells, roughly 3 and 6%, respectively, of the total number of polypeptides (800 paired spots) from TPA-treated cells underwent quantitative changes greater than four-fold. In high calcium cells, however, TPA treatment had a much lesser effect on gene expression. Only 4/854 (0.5%) and 15/934 (1.6%) of the total number of polypeptides changed greater than four-fold following TPA treatment. (3) Comparison of the common changing polypeptides that were observed following treatment with either TPA (0.1 microgram/ml) or during exposure of cells to high calcium revealed 16 common changing polypeptides (four-fold differences). In 14/16 of the polypeptides the common changes were also in the same direction (e.g., if the polypeptide increased following treatment with TPA, the same polypeptide also increased in the presence of high calcium and vice versa). Eight polypeptides were increased following treatment with TPA or growth in high calcium and six decreased following treatment.

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

Peter J. Wirth	Expert	LEC	NCI
Timothy Benjamin	Chemist	LEC	NCI
Dolores M. Schwartz	Biologist	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Stuart H. Yuspa	Chief	LCCTP	NCI
Henry Hennings	Expert	LCCTP	NCI

Objectives:

The overall objective of this project is to employ the computer-based two-dimensional electrophoresis of total cellular proteins to analyze, both qualitatively and quantitatively, the changes in the protein patterns during cellular differentiation. We plan to use this experimental technique to critically examine the hypothesis that neoplasia results from a "block" in normal cellular differentiation. The experimental system that we are currently examining is the mouse skin, but we plan to analyze several other experimental systems, in particular, hemopoietic cell systems in this context of blocked differentiation versus neoplasia.

Methods Employed:

The principle methods employed are (1) tissue culture techniques, (2) histochemical staining, (3) differential centrifugation, (4) autoradiography and fluorography, (5) two-dimensional electrophoresis, and (6) computer-assisted quantitation of autoradiograms and silver-stained gels.

Major Findings:

(1) Mouse epidermal cells were cultured in the presence of either low (0.02-0.09 mM) or high (1.2 mM)  $Ca^{+2}$  and then treated with the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) (0.01 and 0.1  $\mu\text{g/ml}$ ) for 1, 4, and 24 hours. Cells were then pulse-labeled with [ $^{14}\text{C}$ ] amino acids for 4 hours (except 1-hour TPA-treated cells which were labeled for only 1 hour). Two-dimensional electrophoretic analysis of total cellular polypeptides from epidermal cells grown in the presence of either low calcium (mainly basal cells) or high calcium (mainly keratinocytes) revealed only quantitative polypeptide differences. Similarly only quantitative differences could be detected following treatment of either low calcium cells or high calcium cells with TPA. Comparison of quantitative polypeptide differences following a 4 hour treatment of low calcium cells with either 0.01  $\mu\text{g}$  or 0.1  $\mu\text{g/ml}$  of TPA revealed that roughly 3% and 6%, respectively, of the total number of polypeptides (800 total paired polypeptide spots) were undergoing quantitative changes of at least four-fold following treatment with TPA. Comparison of polypeptide changes in high calcium TPA-treated cells revealed even fewer quantitative differences. In high calcium cells only (4/854, 0.5% and 15/934, 1.6%) of the total number of polypeptides were undergoing quantitative changes greater than four-fold.

A comparison of the common changes that were observed following treatment with either TPA (0.1  $\mu\text{g/ml}$ ) or exposure of cells to high calcium revealed 16 common changing polypeptides (four-fold). In 14/16 polypeptides the common changes were also in the same direction (e.g., if the polypeptide increased following treatment with TPA, it also increased in the presence of high  $\text{Ca}^{+2}$  and vice versa). Eight polypeptides were increased either in the presence of high calcium or following treatment with TPA and six polypeptides decreased following treatment.

(2) Epidermal cells were grown in either low or high calcium and then were treated for one hour with TPA (0.1  $\mu\text{g/ml}$ ). Cells were fractionated into soluble and crude membrane fractions (mainly keratin proteins) prior to two-dimensional electrophoresis. Comparison of low  $\text{Ca}^{+2}$ /DMSO; low  $\text{Ca}^{+2}$ /TPA, high  $\text{Ca}^{+2}$ /Dimethyl sulfoxide (DMSO), and high  $\text{Ca}^{+2}$ /TPA revealed the formation of a polypeptide doublet (pI 7.20/57 kDa and pI 7.25/57 kDa) in TPA-treated cells (either low or high  $\text{Ca}^{+2}$ ). In untreated cells both polypeptides were also present but the pI 7.20 polypeptide was markedly decreased.

(3) We are currently investigating differences in the protein phosphorylation modulated by calcium concentration and TPA treatment. Cells were grown in the presence of either low or high  $\text{Ca}^{+2}$  +2 and treated for 1 hour with TPA and then pulse-labeled (15 minutes) with [ $^{32}\text{P}$ ] phosphate. The two-dimensional electrophoresis gels are now being analyzed for polypeptide differences.

#### Significance to Biomedical Research and the Program of the Institute:

Cultured mouse epidermal cells offered an important in vitro model for the study of normal differentiation and malignant transformation. It is hoped that analysis of polypeptide changes accompanying normal differentiation and in cells undergoing malignant transformation may allow one to identify those gene products critically involved in the carcinogenic process(es).

#### Proposed Course:

Continue the course outlined under Objectives and Major Findings.

#### Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05380-01 LEC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Analysis of Gene Expression During Transformation

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

PI:	Peter J. Wirth	Expert	LEC, NCI
Others:	Timothy Benjamin	Chemist	LEC, NCI
	Dolores M. Schwartz	Biologist	LEC, NCI
	Snorri S. Thorgeirsson	Chief	LEC, NCI
	Joseph A. DiPaolo	Chief	LB, NCI
	Jay Doniger	Expert	LB, NCI

## COOPERATING UNITS (If any)

None

## LAB/BRANCH

Laboratory of Experimental Carcinogenesis

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.4

## OTHER:

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 project was initiated to study those changes in gene expression which may be critical to neoplastic and malignant transformation using the hamster embryo cell model in combination with quantitative two-dimensional electrophoresis. Results obtained to date include: (1) although bisulfite has been shown to be a potent transformation agent in vitro, acute bisulfite treatment (15 minutes, 10 micrograms/milliliter) at neutral pH had no qualitative effect on gene expression; (2) similarly gene expression during "aging" (time in culture) was relatively stable from early passages (2nd) to later passages (10th); and (3) computer-assisted analysis of polypeptide differences between normal control cells (5th passage embryo cells) and various bisulfite transformed clones revealed both qualitative (6 proteins) and quantitative polypeptide differences between untreated control cells and each of the individual transformed clones. Following transformation three polypeptides (pI 5.9, MW 55 kDa; pI 5.4/53 kDa; and pI 5.4/32 kDa) were either not expressed or appeared as a charge-shift variant in each of the transformed lines. In addition to the apparent loss of expression or charge-shift two new polypeptides (pI 6.55/45 kDa and pI 5.90/26 kDa) were expressed in all clones and one polypeptide (pI 5.62/45 kDa) was expressed in all clones except clone A following transformation with either bisulfite or benzo[a]pyrene (BP). One polypeptide (pI 6.36/28 kDa) was expressed only in the BP transformed clone.

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

Peter J. Wirth	Expert	LEC	NCI
Timothy Benjamin	Chemist	LEC	NCI
Dolores M. Schwartz	Biologist	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Joseph A. DiPaolo	Chief	LB	NCI
Jay Doniger	Expert	LB	NCI

Objectives:

The main objectives of this project are to define, mainly through the use of two-dimensional electrophoresis, those changes in gene expression which may be critical to neoplastic and malignant transformation. Initial work will utilize the Syrian hamster embryo transformation model with particular emphasis on (1) analysis of cellular polypeptide changes that characterize the malignant state from the normal untransformed cell; (2) study of the time course of polypeptide changes during chemical transformation in vitro; and (3) analysis of polypeptide patterns from cells transformed by various unrelated chemicals such as the polycyclic hydrocarbons, the acetylarylamines, and various chemotherapeutic agents (e.g., cis-platinum) with various nonchemically transformed lines such as virally (SA-7) or radiation (UV and X-ray) transformed.

Methods Employed:

The principal methods employed are (1) tissue culture techniques, (2) histo-chemical staining, (3) differential centrifugation and chromatographic techniques, (4) autoradiography and fluorography, (5) two-dimensional electrophoresis, and (6) computer-assisted quantitation of autoradiograms and silver-stained gels.

Major Findings:

1. Hamster embryo cells (HEC) were maintained in culture and initial studies were directed towards (a) the study of the effect(s) of acute bisulfite treatment on gene expression in HEC, and (b) the effect(s) of "aging" (time in culture) on gene expression. Cells from the 2nd, 5th, and 10th passages were treated with bisulfite at neutral pH (10  $\mu\text{g}/\text{ml}$ ) for 15 minutes and then labeled immediately (0t) or 48 hours (48t) after bisulfite with [ $^{14}\text{C}$ ] amino acids and total cellular polypeptides were analyzed by two-dimensional electrophoresis. Qualitatively, no spots either appeared or disappeared following bisulfite treatment when compared to untreated control cells (2nd passage HEC) either at 0 time (0t) or 48 hours post-bisulfite treatment (48t). Similarly no qualitative spot differences were observed among the polypeptides from cells at different times in culture (e.g. cells from 2nd, 5th, or 10th passage). In addition "older" cells (e.g., 10th versus 2nd passage cells) appeared to be no more or less sensitive to bisulfite treatment than were younger cells. Although no qualitative polypeptide changes were observed, numerous quantitative protein changes

were readily detected among the various treated and untreated cells. Quantitative changes were analyzed using the 2nd passage untreated HEC for comparison. Roughly 5% of the total number of polypeptides (500) compared exhibited quantitative changes greater than four-fold either increasing or decreasing following bisulfite treatment or during time in culture.

2. Polypeptide changes were analyzed in seven different clones (A,D,E,F,G,H, and 9.2) of bisulfite transformed and one aromatic hydrocarbon (benzo[a]pyrene) transformed HEC line, all of which had previously been characterized with respect to various biochemical parameters (e.g., karyotype, plating efficiency in soft agar, malignancy, surface properties, etc.). Comparison of 5th passage untreated HEC with each of the eight transformed clones revealed both qualitative and quantitative polypeptide differences. Following transformation three polypeptides (a,b,c) (pI 5.9, MW 55 kDa; pI 5.4/53 kDa; and pI 5.4/32 kDa, respectively) were either not expressed in the transformed clones or appeared as charge-shifted variants. Polypeptide (a) was expressed only in nontransformed cells whereas polypeptide (b) appeared as a charge-shift variant in clones A,D,E,G, and 9.2 and the benzo[a]pyrene transformed clone. Polypeptide (c) appeared as a chargeshift variant in clones A, 9.2 and benzo[a]pyrene transformed HEC. In addition to the apparent loss of expression or charge-shift in expression of these polypeptides during chemical transformation, two new polypeptides (pI 6.55/45 kDa and pI 5.90/26 kDa) were expressed in all clones and one polypeptide (pI 5.62/45 kDa) was expressed in all clones except clone A following transformation with either bisulfite or benzo[a]pyrene. Comparison of the benzo[a]pyrene transformed clone with each of the bisulfite transformed clones revealed only one protein difference. Polypeptide (d) (pI 6.36/28 kDa) was expressed only in the benzo[a]pyrene clone. Quantitative comparisons were also performed between untreated control HEC (5th passage cells) and each of the chemically transformed clones and between the benzo[a]pyrene transformed clone and each of the bisulfite transformed clones. Similar to that seen in either the acute bisulfite treatment or "aging" experiment roughly 5% of the total number of polypeptides (600-900) compared exhibited quantitative changes greater than four-fold. Current analysis involves the correlation of any of these qualitative and quantitative polypeptide changes with differences in the various biological characteristics of each of the clones.

3. In addition to the bisulfite transformed HEC clones we have recently extended our studies to include other chemically and nonchemically transformed HEC lines. These included the SA-7 viral transformed, the ultraviolet light (UV-79) transformed, three chemically transformed (7,12-dimethylbenz[a]anthracene, N-acetoxy-2-acetylaminofluorene and cis-platinum) and two additional bisulfite transformed lines, one tumor-derived and another soft agar-derived. Results from these studies hopefully will allow us to define those changes in gene expression that are critical to the transformation process.

#### Significance to Biomedical Research and the Program of the Institute:

The Syrian hamster embryo system offers a very attractive model for the in vitro study of transformation (both neoplastic and malignant) by a wide variety of chemical and nonchemical agents. It is hoped that analysis of polypeptide changes which accompany transformation induced by various transforming agents (e.g., chemicals, virus, radiation, etc.) may allow one to define those gene products critically involved in both neoplastic and malignant transformation.



Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

None



ANNUAL REPORT OF  
THE LABORATORY OF EXPERIMENTAL PATHOLOGY  
NATIONAL CANCER INSTITUTE

October 1, 1983 through September 30, 1984

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 combinations; 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:

The main program of investigations in the Laboratory of Experimental Pathology (LEP) focuses on two correlated problems: (1) the comparative pathogenesis of chemically induced neoplastic disease, particularly in lining epithelia, which are the tissues of origin of most human cancers, studied at all levels of biological organization, ranging from human tissues and animal models to organ and cell cultures, and to the biochemical and molecular levels; and (2) the interactions resulting from concurrent effects of different carcinogens, promoters and cofactors in multifactorial carcinogenesis mechanisms, including the role of oncogenes and of selective growth factors.

Sequential series of biological models linking molecular, cellular and organ levels:

There is a fundamental need to relate the process of carcinogenesis to the specific characteristics of the tissues and cells from which the induced tumors originate. Experimental chemical carcinogenesis is the result of chemicobiological interactions characterized by pathologic responses that are typical of the different tissues and cells of origin. Human cancer is characterized by a similarly wide variety of pathologic response patterns. In order to correlate mechanisms of carcinogenesis, investigated at the cellular and molecular levels, with the corresponding events in animal and human tissues and organs, it is important to connect the different levels of observation.

The approach developed to pursue this goal consists of the study of the effects of carcinogens in a series of biological systems of increasing biological complexity, but closely related to each other in a step-by-step sequence. Such systems include molecular targets in defined microenvironments, cultured cell systems, organized tissues in culture and in vivo, and finally organs and whole organisms, including not only models of animal pathology but also human pathology. Such an approach requires the development of a range of biological models related to selected epithelial target cells and ultimately to human cancer pathology. A great deal of progress has occurred in this direction in the past two decades

through major advances in experimental pathology, cell biology, molecular biology and biochemistry, to which previous work in the LEP has substantially contributed. The current LEP program represents a logical sequence to these advances.

The series of interrelated systems, which was partly developed in previous and current LEP programs, is focused on carcinogenesis models for the cells of origin of major forms of human cancer, e.g., bronchogenic carcinoma, skin cancer, bladder cancer and prostate cancer, and includes the following components: (1) human pathology studies of histopathogenesis and cell differentiation in epithelial carcinogenesis; (2) in vivo animal systems for short-term and long-term studies on target epithelial tissues and organs, including animal models for carcinogenesis closely comparable to their human pathology counterparts (e.g., the hamster model for respiratory carcinogenesis); (3) organ explant culture systems for target epithelia and outgrowth cultures of epithelial cells from animal and human sources (e.g., respiratory, urothelial and epidermal epithelia); (4) mammalian epithelial cell culture systems for growth, differentiation, and neoplastic transformation; (5) mammalian cell systems for neoplastic transformation of embryo or newborn cells or cell lines; (6) mammalian cell mutagenesis systems; (7) mammalian cell systems for the analysis of carcinogen binding, DNA damage and repair, and other biochemical effects; (8) systems for the analysis of specific molecular targets, such as specific proteins, protein kinases, and membrane receptors; (9) systems for the study of bioenergetic changes induced in the process of neoplastic transformation; and (10) systems for the identification of genes and gene products involved in the transformation process, particularly DNA transfection systems for mammalian cell transformation, as well as gene characterization, cloning and sequencing.

#### Emphasis on epithelial systems:

In the decade of the 1970s, major emphasis was given in the LEP to the study of human epithelial tissues and cells in culture (in the Human Tissues Section) and to mouse epidermal culture systems (in the In Vitro Pathogenesis Section). Since its relocation at the Frederick Cancer Research Facility in 1981, the LEP has engaged in further methodological cell culture developments for rodent and human epithelial cell systems related to human cancer pathology and well-known for their response to carcinogens, such as the hamster respiratory tract, the mouse skin and the human bladder. Emphasis is given to the use of serum-free, possibly chemically defined media.

The following main epithelial systems are currently used in the LEP for studies on the mechanisms of carcinogenesis:

(A) Respiratory epithelia - The hamster respiratory carcinogenesis model (Saffiotti, U., et al., Cancer Res. 28: 104-124, 1968) has become well established as closely resembling the pathogenesis of the human bronchogenic carcinoma, and continues to be used for in vivo studies of cell differentiation and tumor induction by various carcinogens and cofactors. Organ culture systems were established for hamster respiratory epithelia, and cell culture systems in serum-free media were developed and used for studies of differentiation and transformation. Work in other laboratories has recently led to the development of cell culture and transformation systems for rat respiratory epithelial cells, which are now also investigated in the LEP.

(B) Epidermal keratinocytes - The mouse epidermal carcinogenesis model has been widely studied *in vivo* for decades for its response to full carcinogens and/or promoting agents, such as 12-O-tetradecanoylphorbol-13-acetate (TPA). Previous work in the LEP led to the development of primary culture methods for mouse keratinocytes in conventional media and the study of their differentiation and transformation (Yuspa, S., et al., *Transplant. Proc.* 12: suppl. 1, 114-122, 1980). Mouse epidermal cell lines transformable by promoting agents (JB-6 and clonal sublines) were also established (Colburn, N. H., et al., *Teratogen. Carcinogen. Mutagen.* 1: 87, 1980). Currently, serum-free and nearly chemically defined media have been developed for primary and secondary cultures of mouse keratinocytes and for their transformation by carcinogens. The cellular response to hormones and growth factors has been quantitatively determined in clonal assays. Induction of terminal differentiation was obtained not only by high calcium concentrations, as previously seen with serum-containing media, but also by the addition of serum to serum-free media. Transformation studies are under way.

Studies in collaboration with the Cell Biology Section, Laboratory of Viral Carcinogenesis, on the JB-6 clonal sublines that are either sensitive or resistant to promoter-induced transformation, showed that promoter-sensitivity can be transmitted by DNA transfection from the sensitive to the resistant clones. Two genes (termed pro) responsible for the susceptibility to promoter-induced transformation in this epithelial system were cloned and are currently being sequenced and studied for their function in the JB-6 system and also in the primary keratinocyte culture system developed in the LEP.

(C) Urothelia - Human urothelium (bladder and ureter epithelium) is cultured using serum-free media, and the conditions for optimal response to growth and transformation are under investigation. The corresponding animal models for chemically induced bladder carcinogenesis were previously studied in the hamster and rat and may provide useful culture counterparts for *in vivo/in vitro* studies; the carcinogenic activity of several chemical carcinogens on the human bladder has been well established in occupational and environmental studies.

(D) Other epithelia - A normal prostate epithelial cell line and a corresponding prostate carcinoma cell line, established previously, were used in collaborative studies on the characterization of the metastasizing variants and for studies on oncogene-mediated transformation. A new study has been planned on the identification of transforming genes present in the carcinoma line and their role in the transformation of the normal epithelial line. Liver cell lines were used in bioenergetic studies and other liver cell preparations were used for some studies on carcinogen binding.

Development of chemically defined culture conditions for studies on mechanisms of differentiation and transformation in epithelial systems:

Methods for chemically induced neoplastic transformation of epithelial cells in culture started to develop in the last decade, but these methods need to be further extended and more rigorously defined from a quantitative point of view. As new and better defined culture conditions are established for target epithelial cell systems, their response to carcinogens needs to be correlated with the mechanisms of neoplastic transformation investigated at the molecular level. The major epithelial systems described above have comparable patterns of response to specific

treatments and culture conditions that can lead, on the one hand, to terminal differentiation, senescence and cell death and, on the other hand, to progressive cell growth, anchorage independence and neoplastic transformation. An important condition for studies on these mechanisms is the ability to grow the target epithelia in serum-free, possibly chemically defined culture media, replacing serum with selected additions of hormones and growth factors at optimal concentrations. Factors that control either continuous cell growth or the induction of senescence and terminal differentiation--and the escape from senescence of transformed cell populations--are under investigation.

In addition to the epithelial models, an established mouse embryo fibroblastic cell line, BALB/3T3 clone A31-1-1, was characterized for its mutagenic and transformation responses to several carcinogens and for mechanism studies. This cell line was also selected for the development of culture media in which serum addition is lowered or eventually replaced by the addition of chemically defined factors.

Elimination of serum additions from the culture media in all of these systems will not only dispose of a source of uncontrolled biological variables from batch to batch, but also makes it possible to analyze the specific role of individual growth factors in the control of cell growth and transformation.

#### Mechanisms of concurrent or sequential effects of different carcinogens and cofactors in multifactorial carcinogenesis:

Mechanisms of chemical carcinogenesis resulting from concurrent exposure to different carcinogens have so far received relatively little attention in the field of carcinogenesis studies and yet multiple exposures to different carcinogens represent the common realistic condition of human contact with carcinogens. Studies of the combined effects of carcinogens in the past were mostly based on in vivo animal experiments, usually limited to two carcinogens at a time. Several examples of marked synergism were reported.

Combinations of carcinogenic factors or cofactors active in multifactorial carcinogenesis include: (1) carcinogens of the same chemical class; (2) carcinogens of different chemical classes; (3) complete and incomplete carcinogens (initiators, promoters and co-carcinogens); (4) carcinogens and modifiers acting on their tissue distribution, retention and response (e.g., particulate materials in the respiratory tract), and on their metabolic activation and detoxification; (5) carcinogens and factors acting on the cellular expression of neoplastic transformation; and (6) carcinogens and altered genes (activated, derepressed, mutated, amplified or translocated) which produce permissive conditions for neoplastic transformation.

Studies on combined exposures were conducted in the hamster respiratory tract model in vivo. Marked effects on epithelial proliferation, mostly of secretory cells, were demonstrated with microtrauma and with instillation of saline or of particulate suspensions. Their role in combination with treatments with topical and systemic carcinogens is under study. The role of chemicals with selective toxicity for certain cell types in the respiratory epithelium is also being investigated in vivo.

The development of cellular models has made it possible to study the combined effects of carcinogens and/or cofactors in relevant in vitro short-term systems for mutagenesis and cell transformation. Methods in molecular biology and biochemistry are used to extend these studies to investigate the mechanisms involved

in the induction of neoplastic transformation by chemical carcinogens singly or in combinations.

Concurrent induction of toxicity, DNA damage and repair, mutagenicity and transformation was used to study the quantitative response of the mouse embryo fibroblastic cell line, BALB/3T3 clone A31-1-1. Dissociation patterns were demonstrated between these different end points in their response to the following test conditions: (1) single- and split-dose treatments, (2) exposure during different phases of the cell cycle, and (3) effects of different exposure durations to alkylating agents. Studies on combined exposures to different carcinogens showed some evidence of synergism in the BALB/3T3 system.

Molecular studies of genes involved in the control of neoplastic transformation are currently addressed to testing the hypothesis that different genes may be activated by different carcinogens and/or cofactors, (e.g., promoting agents), and that transformation may result from the combined effects of these different genes. Transforming DNAs have been obtained from 7 out of 16 chemically transformed BALB/3T3 cell lines. At least three different patterns of sensitivity to a battery of restriction endonucleases were demonstrated for the transforming DNA of three lines, all transformed by the same carcinogen, benzo[a]pyrene. Demonstration of a mutated allele of the Ha-ras oncogene was possible after digestion with MspI but not with other tested restriction enzymes. Genes, termed pro, were identified from DNA of promoter-sensitive mouse epidermal JB-6 cell lines and found to be responsible for susceptibility to promoting agents when transfected to promoter-resistant cell lines. Two pro genes were cloned and one was completely sequenced. Their characterization is under way.

The establishment of culture systems for normal epithelial cells and their transformed neoplastic counterparts has made it possible to approach the identification of transforming genes by DNA transfection from the neoplastic to the normal cells of the same type and by comparative study of growth control requirements.

In summary, the focus of LEP research is addressed to the pathogenesis of epithelial neoplasia, through the elucidation of possibly complementary mechanisms that would induce neoplastic transformation when concurrently or sequentially activated to their respective permissive conditions, studied in cellular systems biologically correlated with the main epithelial tissues of origin of human cancers *in vivo* and in corresponding cell culture models, maintained by optimally defined culture conditions.

#### OFFICE OF THE CHIEF

(1) Provides overall scientific direction and administrative coordination to the Laboratory's intramural research program and its supporting resources; (2) participates in research projects in all components of the Laboratory and provides collaborative research coordination of staff activities and resources; (3) conducts bibliographic research and data analysis; and (4) conducts research on carcinogenesis mechanisms and quantitative studies on the interactive effects of combined exposures to different carcinogens and cofactors, using *in vivo* and *in vitro* systems established in the Laboratory.

Investigators assigned to this Office conduct basic research on mechanisms of neoplastic transformation of cells in culture, on biochemical mechanisms and on the molecular characterization of oncogenes involved in chemical carcinogenesis and tumor promotion.

Projects are under way in the following areas:

(A) - Quantitative studies on the concurrent induction of cytotoxicity, DNA damage and repair, mutation and transformation with the mouse embryo cell line, BALB/3T3 clone A31-1-1. In previous studies this cell line, originally obtained from T. Kakunaga (Laboratory of Molecular Carcinogenesis, NCI), was found to be susceptible to neoplastic transformation induced by a number of carcinogens with different metabolic pathways, including polycyclic aromatic hydrocarbons, aflatoxin, aromatic amines, nitrosamides and arsenic. A mutation assay for ouabain resistance was also established for this versatile cell line. Alkaline elution was used to study DNA damage and repair. Studies on O<sup>6</sup>-alkylguanine repair are planned.

Dissociation patterns were demonstrated between different end points in their response to carcinogen treatment in the following test conditions: (1) Synchronization of cells in the exponential growth phase, and treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 30 min at various points during the cell cycle, showed a dissociation of mutation from transformation in their cell cycle dependence. (2) Split-dose protocols, with varying time intervals between equal split doses of MNNG, compared with single-dose treatments, showed no evidence for recovery from sublethal damage in split-dose tests with MNNG; this response was confirmed for X-rays. DNA damage and repair, as measured by alkaline elution, was significantly demonstrated by split-dose treatment, and appeared dissociated from cytotoxicity, mutation and transformation, which were not affected by dose splitting. (3) MNNG exposures maintained for different periods of time, after different initial exposure concentrations, showed that the exposure periods required for maximal induction differed for DNA damage measured by alkaline elution (30 min), for ouabain-resistant mutations (30-60 min), for cytotoxicity (about 120 min) and for neoplastic transformation frequencies (120-240 min). The half-life of MNNG under the test conditions was determined spectrophotometrically to be about 68 min. Similar studies with ethylnitrosourea (ENU), with a half-life of about 12 min, showed the same dissociation phenomenon, but shifted to shorter exposure times (<5 min for DNA damage and mutation, >45 min for cytotoxicity and for transformation). The observed temporal dissociation in the exposure times required for maximal induction suggests different targets in the mechanisms of mutation and transformation in this system. Current studies are investigating the basis for the events responsible for continued induction of transformation after the inducibility of mutations has been saturated.

(B) - Carcinogen metabolism and binding. Studies on the retention, distribution, metabolism and binding of carcinogens in target tissues and cells have been extended using both in vivo and in vitro model systems. Carcinogens that have been studied so far include MNNG, ENU, and benzo[a]pyrene (BP) in relation to DNA binding sites, cellular models of transformation and BP metabolism in respiratory carcinogenesis and in the presence of cofactors. These studies are reported in the context of their respective projects.

(C) - Bioenergetic pathways in transformed epithelial cells. Bioenergetic studies, initiated by Dr. Ann E. Kaplan in another laboratory of the DCE, were continued after the transfer of her unit to the LEP at the beginning of FY 1984. This line of research will be further integrated into the general research program of the LEP through a developing collaboration within the Tissue Culture Section and other LEP units.



Modifications of glycolytic and oxidative pathways were studied in a chemically transformed rat liver cell line (NMU-3) in comparison with non-transformed control liver cells (TRL). Lactate dehydrogenase (LDH) in the transformed cells showed different kinetic properties from LDH in control cells, indicative of increased LDH production. This finding was confirmed by glucose analysis (75% of the glucose is converted to lactic acid in the transformed line and only 8% is converted in the control line). Analysis of the isoenzymes, LDH-4 and LDH-5, showed that the isoelectric point was acidic for LDH-4 and basic for LDH-5; the distribution showed that LDH-4 dominates in the control and LDH-5 in the transformed cells. The biochemical pathway that converts LDH-4 to LDH-5 is under investigation. LDH characterization was also studied in chick embryo fibroblasts (NIL) and in the corresponding polyoma virus transformed cell line (NIL-py), in collaboration with Dr. Harold Amos, Harvard Medical School. The isoelectric point of LDH in NIL cells was 6.8, but that of NIL-py was 7.2. In the liver cell line, this increase was greater, from 6.0 in TRL cells to 8.8 in NMU-3 cells.

A new method was developed to determine intracellular pH and ion transport by using monolayers of liver cells grown on Leighton-tube slides, in which the indicator dye 6-carboxyfluorescein was introduced into the cytoplasm; the slides are then inserted in a spectrophotometer for direct measurements of absorbance changes. The intracellular pH decreases from 7.3 in TRL cells to 6.8 in NMU-3 cells. However, diffusion remains the main mode of export for lactate and hydrogen ions in both cell lines.

Cytoskeletal proteins in hepatic cells are being studied by electron microscopy and immunofluorescence. Results thus far indicate markedly diminished aggregation of cytokeratin in NMU-3, although the aggregation of tubulin and actin is unaltered.

(D) - Biochemical and molecular mechanisms related to tumor promotion. Molecular mechanisms in multistage carcinogenesis are investigated in the mouse epidermal JB-6 cell line with its clonal variants which, when exposed to tumor-promoting agents, such as TPA, show a positive or negative transformation response ( $P^+$  or  $P^-$ ) or a positive or negative mitogenic response ( $M^+$  or  $M^-$ ). Studies on the 2-deoxyglucose uptake response in these cell lines showed that uptake is required for the mitogenic response to TPA, but not for promotion of transformation. Studies on the phospholipid- and calcium-dependent protein kinase activity (PK-C), associated with the TPA receptor, showed that PK-C is present in equal amounts in derivatives of JB-6 cells of all phenotypes as well as in related mouse epidermal cell lines. By a sensitive technique, over 16 substrates were found for PK-C in JB-6 cell lines. This is more than all previously described substrates for PK-C in whole cell lysates of several different cell types regardless of phenotype. It appears that the preneoplastic phenotype is not determined at the level of substrates for PK-C.

A phosphoprotein species (pp80), which changes in response to TPA was identified in JB-6 clonal sublines. pp80 is a heat shock protein similar or identical to hsp80. This phosphoprotein was characterized and found to represent approximately 2% of the total phosphoprotein in whole JB-6 cell lysates. Transformed JB-6 cell lines were found to lack pp80 and they do not produce it in response to TPA. The role of this phosphoprotein and the heat shock response mechanism in the maintenance of the non-transformed state is being investigated.

Distinct roles for different species of reactive oxygen free radicals were found in the induction phase of promotion by TPA and in the expression of the transformed

phenotype. Superoxide dismutase (SOD) effectively blocked induction of transformation if added to cells within four hours of exposure to TPA. Of seven different types of eliminators of free radicals tested, those which enzymatically catabolized the superoxide anion inhibited TPA promotion most effectively. Inhibitors of the lipoxygenase pathway of the arachidonic acid cascade, also inhibited TPA promotion, while an inhibitor of the cyclooxygenase pathway did not. TPA treatment was found to reduce the levels of endogenous SOD activity by 50% in promotion-competent subclones of JB-6 cells, but only by 17% in promotion-incompetent subclones. This difference in suppression of SOD with TPA treatment appears to mark the promotion-competent phenotype among JB-6 cell lines. Treatment with benzoyl peroxide effectively promoted transformation of JB-6 cell lines. Concentrations of retinoic acid which inhibited promotion by TPA failed to inhibit promotion by benzoyl peroxide.

(E) - Molecular biology studies of oncogenes activated in chemically transformed cells or involved in tumor promotion. DNA-mediated gene transfer, molecular hybridization, gene-cloning and gene-sequencing methods were used to investigate the identity and multiplicity of transforming genes activated by different carcinogens or promoting agents. By transfection in NIH/3T3 cells, transforming activity was detected in DNA from 7 out of 16 BALB/3T3 cell lines transformed by chemical carcinogens or by U.V. light. When the DNAs from three of these lines, all transformed by benzo[a]pyrene, were digested with a battery of four restriction endonucleases (EcoRI, HindIII, BamHI and XbaI), distinct sensitivity patterns were obtained for each line, suggesting that three different transforming sequences were activated by the same carcinogen. Southern blot hybridization with Harvey and Kirsten *ras* oncogenes did not show any evidence of activation of these genes in DNAs digested with these enzymes. When the restriction endonuclease, MspI, was used, however, an additional resistant allele was detected with a c-Ha-*ras* probe. Further studies are under way to investigate the mechanisms that resulted in the detection of different sensitivities in DNAs from cells transformed by the same carcinogen.

Two novel genes termed pro, were characterized. They are required for neoplastic transformation of promoter-sensitive JB-6 mouse epidermal cell lines, and were shown to carry the sensitivity trait when transfected in promoter-resistant cell lines. Two pro genes, p26 and p40, were identified and cloned. Sequencing was completed for the p26 gene and the sequence is now analyzed by a computer aided program. This gene was found to be unrelated to any of the tested viral oncogenes.

In addition, investigators in this Office collaborated on projects in the Respiratory Carcinogenesis Section and in the Tissue Culture Section.

#### RESPIRATORY CARCINOGENESIS SECTION

(1) Conducts research on the pathogenesis of cancers in the respiratory tract and on their induction by carcinogens, alone or in combinations, using animal models closely related to human pathology and corresponding in vitro systems; (2) investigates the carcinogenic effects of chemical and physical agents on the respiratory tract, their quantitative aspects and their pathogenetic mechanisms; (3) studies mechanisms of cell differentiation and carcinogenesis in respiratory and related epithelia; and (4) provides pathology expertise, resources and collaboration to other components of the Laboratory in the study of epithelial carcinogenesis.

The research activities developed in this Section are devoted to the characterization of respiratory carcinogenesis model systems in vivo and in vitro and to the elucidation of mechanisms of epithelial carcinogenesis by chemical and physical factors, alone or in combinations. This Section also provides expertise in pathology research for in vivo animal carcinogenesis studies as well as in the role(s) of inorganic substances in carcinogenesis. The programs are closely correlated with those of the other LEP components.

The hamster respiratory carcinogenesis model (Saffiotti, U., et al., Cancer Res. 28: 104-124, 1968) is used for further studies on the combined effects of different factors and for the development of the corresponding epithelial organ culture and cell culture models for different segments of the respiratory tract in collaboration with the Tissue Culture Section. This hamster model was previously extensively studied and shown to be closely similar to its human counterpart in its differentiation and pathogenesis; it represents, therefore, a model of choice for studies on mechanisms of induction of bronchogenic carcinoma.

Two recently established colonies of Syrian golden hamsters (inbred strain 15:16/EHS:CR and non-inbred strain Syrian/CG.FOD) are used. Studies are continuing on cellular characterization in the different segments of the respiratory tract following intratracheal instillations of inorganic particulates and administration of different topical or systemic carcinogens, alone or in combinations. Histologic, histochemical, ultrastructural, immunochemical and autoradiographic techniques are used for the characterization of the segmental response of the respiratory epithelium to different carcinogens and their combinations. Light and electron microscopic characterization of cellular differentiation in the hamster respiratory tract at various ages (in collaboration with H. M. Schüller, LETM, DCT, NCI) showed large numbers of neuroendocrine cells of the APUD type at early ages (highest frequency at one day) but not in adults.

Long-term animal experiments are mostly completed and their pathology is under examination to evaluate age differences in the response to diethylnitrosamines in hamsters. Early postnatal exposures resulted in a higher proportion of malignant neoplasms than later exposures, especially in the nasal mucosa where malignancies appeared to arise from submucosal glands rather than from the olfactory epithelium.

Extensive studies in the hamster model are continuing on the role of microtrauma (intratracheal or intralaryngeal cannulation) and/or instillation of particulate suspensions or of saline alone; long-term experiments are under way combining these factors with known carcinogens. This study, in collaboration with E. M. McDowell and K. P. Keenan of the Department of Pathology, University of Maryland Medical School, has given extensive evidence, by histological, histochemical, E.M., and autoradiographic methods, that the processes of epithelial proliferation and repair occurring in the respiratory epithelia in response to physical injury are largely due to proliferation of the secretory cells.

Chemicals, that are capable of inducing selective toxic or proliferative effects in different segments of the respiratory tract, are investigated singly and in combinations. The compounds, 3-methylindole and 2-methylnaphthalene, present in tobacco smoke, and selectively toxic for the non-ciliated bronchiolar lining cells (Clara cells) and alveolar type I cells in other species, were confirmed to be similarly toxic in the hamster model, in which their toxic effects were characterized as being more severe in females than in males. Their long-term effects in

respiratory carcinogenesis, alone or in combinations with known carcinogens, are under study.

Arsenic was selected for studies on the interaction of organic and inorganic carcinogens, in view of the positive human findings and the previously negative animal tests. Metabolic species differences are under investigation, as is the possible role of arsenic as a cofactor in carcinogenesis. Trivalent arsenic was found to be more cytotoxic than pentavalent arsenic for cultures of primary BALB/c mouse epidermal cells and of the BALB/3T3 clone A31-1-1 cell line; in the latter system, trivalent arsenic was found to induce neoplastic transformation, while pentavalent arsenic appeared negative. The role of arsenic in epithelial carcinogenesis is under investigation in studies of combined exposures with other carcinogens.

Culture methods for the hamster respiratory epithelium have been developed in collaboration with the Tissue Culture Section (see below). Methods have also been developed for the study of benzo[a]pyrene binding to respiratory epithelia of different segments, both in vivo and in serum-free cell cultures.

### TISSUE CULTURE SECTION

(1) Conducts research on cell culture systems for the characterization and quantitative study of neoplastic transformation induced by chemical and physical carcinogens; (2) develops and characterizes organ and cell culture systems for carcinogenesis studies, especially those derived from epithelia known for their susceptibility to carcinogens in vivo, such as the respiratory tract epithelium; (3) conducts research on mutagenesis, neoplastic transformation, differentiation, and on their expression mechanisms and relationships; and (4) provides expertise, resources and collaboration on tissue culture methods for the entire Laboratory.

The objective of the research program of the Tissue Culture Section is to understand how carcinogenic agents alter the control of growth and differentiation at both the cellular and molecular levels and how cells progress to neoplasia through a sequence of genomic and/or epigenetic alterations. Emphasis is placed on studies of epithelial cells derived from tissues representing major organ sites relevant to human cancer. Cell systems currently under study include mouse epidermal keratinocytes, hamster and rat respiratory epithelial cells, human urothelial cells (bladder and ureter), and human prostatic epithelial cells.

Additional studies on chemically induced mutation and transformation and on DNA-mediated gene transfer make use, respectively, of the mouse embryo fibroblast cell lines, BALB/3T3 and NIH/3T3. Work on the relationships and dissociations between cytotoxicity, DNA damage and repair, mutagenicity and transformation induced by carcinogens in BALB/3T3 cells, is reported under the Office of the Chief.

Work in this Section is particularly concerned with epithelial cell systems in which both the non-transformed and corresponding transformed cells can be replicated in culture. In such systems, the DNA of the transformed cells can be transfected into the corresponding non-transformed cells and analyzed for its ability to induce neoplastic or preneoplastic properties. As reported under the Office of the Chief, this approach was successful for the mouse keratinocyte cell line, JB-6, in providing a system for the identification of the promotion-related pro genes, which are now being investigated for their role in normal primary mouse

keratinocytes and in other cell types. The culture of normal epithelial cells in serum-free (and possibly in totally chemically defined media) provides a methodological approach for the identification of growth and transformation factors in a controlled cellular environment.

A new serum-free medium (LEP-1) was developed for mouse keratinocytes. It consists of Ca<sup>2+</sup>-free Eagle's MEM with nonessential amino acids and seven added factors (hydrocortisone, 5x10<sup>-7</sup> M; insulin, 5 µg/ml; phosphoethanolamine (PEA) and ethanolamine, each 5x10<sup>-5</sup> M; transferrin, 5 µg/ml; epidermal growth factor, 5 ng/ml; bovine pituitary extract (BPE), 180 µg of protein/ml). This medium was found to have a Ca<sup>2+</sup> concentration of 0.03 mM by atomic absorption. It supported at least 25 population doublings (PD) with an exponential growth rate of 0.8 PD/day. A clonal growth assay was established and used to define optimal levels of each growth factor and to investigate the effects of whole serum and serum components on growth and differentiation. Experiments in which individual supplements of LEP-1 were deleted singly showed that all (except PEA) stimulated growth, and that BPE was essential. Addition of >2.5% whole fetal bovine serum (wFBS) or >0.1 mM Ca<sup>2+</sup> induced terminal differentiation while inhibiting growth by more than 90%. Fetuin inhibited growth even at the lowest tested level (50 µg/ml). Addition of a crude platelet extract (commercial PDGF) completely inhibited growth. In contrast, bovine serum albumin stimulated growth in a dose-dependent fashion. This demonstration of inhibitory as well as stimulatory factors in FBS explains earlier problems in culturing mouse epidermal cells with serum-containing medium.

Explant cultures were developed from segments of the tracheobronchial tract of inbred Syrian golden hamsters. Out of several tested media, serum-free F-12 and and CMRL-1066 media with the seven added factors were found to be most suitable to support outgrowth and proliferation of epithelial cells. Culture methods for enzymatically dissociated epithelial cells from the hamster tracheal epithelium were also developed in analogy with the mouse keratinocyte system. Good growth and subculture were obtained using serum-free F-12 medium with the seven factors.

The role of serum-derived factors, defined growth factors and hormones as well as autocrine factors produced by normal and neoplastic cells in culture is under study in the above-mentioned epithelial systems. The major remaining undefined supplement is (BPE). This extract was found to be essential for the growth of mouse keratinocytes in this system and beneficial for both hamster tracheal epithelium and human urothelium. Efforts are being made to replace this extract with factors of defined composition.

The serum-free media systems, now available for several normal epithelial cell systems under study in the Section, will be used to develop selection media for transformed cells. Transformed fibroblasts have long been known to have a reduced serum "requirement." In the case of at least some epithelial cells this situation is reversed; normal epidermal cells, tracheal epithelium and urothelium terminally differentiate in the presence of serum, whereas the growth of their transformed tumorigenic counterparts is stimulated. Chemically-transformed mouse keratinocytes have been grown in serum-supplemented media (Yuspa, S., et al., Cancer Res. 40: 4694-4703, 1980). Thus, following exposure to a carcinogen and initial growth under serum-free conditions, the cells could be switched to serum to select for serum-resistant variants. Similarly, since normal MK cells were found to require BPE, a BPE-free medium could be used for selection of variants. This type of change in growth control could be one of the earliest manifestations of the

multistage neoplastic process. This screening process could be used to identify other critical factors that distinguish normal from transformed cells.

Experiments are under way to induce transformation in cultured rodent epithelia (mouse keratinocytes and hamster tracheal epithelia) by treatment with organic and inorganic carcinogens, and to define the growth characteristics of the transformed cells. These cell systems are also used in preliminary investigations of their susceptibility to transformation by DNA-mediated gene transfer techniques (transfection) using either known oncogenes (including the recently cloned, pro genes) or DNAs from the neoplastic epithelial cells obtained by carcinogen-induced in vitro transformation, or by in vivo carcinogenesis in the corresponding epithelial tissues.

The rat tracheal epithelial (RTE) cell culture system, developed at the Laboratory of Pulmonary Function and Toxicology, NIEHS, has been used to characterize carcinogen-induced foci of epithelial cells with preneoplastic or neoplastic properties, termed EG variants (Thomassen, D., et al., Cancer Res. 43: 5956-5963, 1983). Studies initiated by Dr. Thomassen when he joined the LEP in April, 1984, are directed to the following objectives: (1) to investigate the ability of RTE cell lines with different stages of preneoplastic potential to acquire fully neoplastic properties after treatment with carcinogens or after transfection with transforming DNAs; (2) to develop an assay using preneoplastic RTE cells to detect genes capable of transferring the property of tumorigenicity; (3) to identify the genes that determine the tumorigenic phenotype from the DNA of transformed neoplastic RTE cells; and (4) to clone and characterize these genes.

Transformation of human cells in culture has proven to be much more difficult than was at first anticipated. The reasons for this problem are unknown but may be related to a number of "steps" required for the full expression of neoplasia in human cells, derived as they are from a species with a long life span and a long latent period for cancer in vivo. Thus, in order to compress a 20-30 year process in vivo into a short time frame in culture it may be necessary to "hit" the cells with a sequence of carcinogens and growth factors and to activate specific oncogenes. A normal human prostatic epithelial cell line (NP-2s), and its neoplastic counterpart (PC-3) derived from a bone metastasis of prostatic adenocarcinoma, were previously established (Kaighn, M. E., et al., Invest. Urol. 17: 16-23, 1979). The prostate is an important organ site for cancer in man, and prostatic cancer is the third leading cause of cancer deaths in males in the United States. Collaborative studies were initiated with J. H. Lechner of the Laboratory of Human Carcinogenesis to investigate the role of oncogenes in the human prostatic epithelial line, NP-2s, that had been "transformed" by SV40 virus (NP-2s/T2) but acquired neither the capacity for unlimited growth nor tumorigenicity in nude mice. In an effort to induce the cells to progress toward neoplasia, they were superinfected with Kirsten sarcoma virus or transfected with a plasmid vector carrying the EJ-ras oncogene. Both treatments resulted in new cell lines with extended life spans which contain the ras oncogene and secrete a transforming growth factor (TGF) for NRK cells. Experiments are in progress to test the tumorigenicity of these lines as well as their response to growth factors. A new study in collaboration with the Office of the Chief seeks to identify oncogenes in the established neoplastic cell line, PC-3. DNA from PC-3 will be transfected into the normal prostatic epithelial cells (NP-2s). Since NP-2s cells have a limited life span, escape from "senescence" or from growth limitation in culture will be used to assay for and isolate oncogene-altered lines. In serum-free medium, the growth of this line is population-dependent, indicating the secretion

of an autocrine growth factor. An effort will be made to isolate and purify this factor from PC-3 cells in collaboration with Dr. David Sirbasku of the University of Texas Medical School, Houston. It is not known whether this autocrine activity has TGF activity. The standard TGF will be assayed with PC-3, NP-2s and NRK cells in soft agar assays. Induction of a TGF by PC-3 DNA in normal cells would provide an important model for the genetic control of neoplastic growth by a specific gene product. In another study (with Dr. J. M. Kozlowski, LBI/FCRF), the incidence of metastasis in nude mice of the variant cell lines, will be studied in this system, using the neoplastic and non-transformed human prostate cell lines.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE04491-08 LEP

PERIOD COVERED

October 1, 1983 through September 30, 1984

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

Others: M. Bignami Guest Researcher LEP NCI  
 M. E. Kaighn Expert LEP NCI  
 C. Ficorella Visiting Fellow LEP NCI  
 R. L. Norman Staff Fellow LEP NCI

COOPERATING UNITS (if any)

Laboratory of Toxicology, Istituto Superiore di Sanita', Rome Italy  
 (M. Bignami, E. Dogliotti).

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.5

OTHER:

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.)

The mouse embryo cell line, BALB/3T3 clone A31-1-1, was used for quantitative studies on cytotoxicity, DNA damage and repair, mutagenicity and neoplastic transformation induced by different carcinogens. A ouabain resistance (oua-r) mutational assay was established for this cell line with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Alkaline elution was used to study DNA damage and repair. Split-dose treatments with MNNG showed marked DNA repair during the intervals, but cytotoxicity, mutation and transformation frequencies were not significantly different after single or split doses of MNNG, regardless of the time interval between doses. In synchronized cell populations, although MNNG-induced mutation frequencies varied with cell cycle phases (maximum in S), transformation frequencies did not. Such dissociations in the cellular responses suggest different underlying mechanisms.

The new phenomenon of temporal dissociation between exposure times required for maximal induction of mutation and transformation, previously observed in studies with MNNG at different initial concentrations, was confirmed and extended to ethylnitrosourea (ENU). The half-life of MNNG in the cultures was about 68 min. MNNG exposures required for maximal induction of DNA damage and mutation were only 30-60 min, but they were 120 min for cytotoxicity and 120-240 min for transformation; the ratio of transformation to mutation frequencies was 3.7 at short exposure times but increased to over 20 after 240 min or longer. DNA repair, as measured by alkaline elution, was active after 30-min exposures, but total DNA damage remained constant for MNNG exposures extended from 30 to 120 min. Equitoxic doses of ENU (half-life of about 12 min) induced maximal mutation in 5 min and transformation in 45-60 min. This temporal dissociation supports the hypothesis that transformation is dependent on factors other than a single gene mutation and offers a useful model for investigating the molecular events that occur during this differential time of exposure.



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

U. Saffiotti	Chief	LEP	NCI
M. Bignami	Guest Researcher	LEP	NCI
M. E. Kaighn	Expert	LEP	NCI
C. Ficorella	Visiting Fellow	LEP	NCI
R. L. Norman	Staff Fellow	LEP	NCI

Objectives:

To study mammalian cell culture systems for concurrent induction of cytotoxicity, DNA damage and repair, mutagenicity and neoplastic transformation in order to define the interrelationships of these end points in response to multiple factors. To determine conditions under which carcinogenic effects can result from concurrent exposure to different carcinogens and/or co-factors, and to analyze the mechanisms of such interactive effects in multifactorial carcinogenesis.

Methods Employed:

The BALB/3T3 clone, A31-1-1, mouse embryo cell line was used under the test conditions previously standardized in this laboratory for transformation assays with different carcinogens. Optimal conditions were determined for ouabain resistance mutation assays in this cell line using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). DNA damage and repair were determined by alkaline elution analysis. Split-dose treatments were used to determine repair during dose-intervals. Cell synchronization was obtained by plating cells in the logarithmic growth phase in serum-free medium (MCDB 402 with three added factors) for three days (which induces mitotic block), and then feeding them with complete medium. Tritiated thymidine (<sup>3</sup>H-TdR) incorporation was measured after pulse-labeling at different times following release from the mitotic block; the labeling index was determined autoradiographically. The halflife of MNNG was calculated from spectrophotometric determinations made under the conditions of test, as well as in serum-free and cell-free media. Duration of exposure to alkylating agents was studied in time-course experiments for toxicity, DNA damage, mutation and transformation.

Major Findings:

Dissociation of measured end points was found under the tested experimental conditions. Methods were established for concurrent induction of ouabain resistant (oua<sup>r</sup>) mutations and neoplastic transformation, as well as cytotoxicity, in the BALB/3T3 clone A31-1-1. Split-dose treatments, with varying intervals between two equal split doses, were compared with corresponding single doses. Comparison of cytotoxicity induced by single-versus split-dose treatment with MNNG showed no recovery from sublethal damage; this effect was confirmed with X-rays. Repair of DNA damage (single-strand breaks and alkali-labile lesions) induced by MNNG was rapid during the first 4-5 hr and slower afterwards. DNA repair was significantly demonstrated by split-dose treatment with intervals from 1 to 5 hr. In contrast, no significant differences were detected in the frequencies of oua<sup>r</sup>

mutation or neoplastic transformation with single or split doses of MNNG, regardless of whether the second dose was given during or after the period of rapid DNA repair, suggesting marked differences in the response of this cell line to chemical versus physical agents. These studies show a dissociation of DNA damage and repair, as detected by alkaline elution, from the induction of cytotoxicity, mutation and transformation.

Treatment of this cell line with MNNG for 30 min at various points during the cell cycle showed maximal induction of *oua*<sup>r</sup> mutations throughout the S phase and low induction in the G<sub>1</sub> phase, but a constant level of transformation frequencies was found in G<sub>1</sub>, early S and late S phases. These results show a dissociation of mutation from transformation in their cell cycle dependence in this cell line, which differs in this respect from the C3H 10T1/2 line.

The effects of varying the duration of exposure to MNNG were investigated for periods ranging from 15 min to 72 hr, after starting concentrations of 0.5 and 2  $\mu$ g/ml in complete medium; the results showed that the plateau for maximal induction of *oua*<sup>r</sup> mutations was reached early (30-60 min), the plateau for cytotoxicity was reached later (about 120 min) and the plateau for neoplastic transformation was reached still later (120-240 min). The half-life for MNNG in the cultures was about 68 min, and appeared to be independent of the number of cells present, under the conditions of test. The marked dissociation observed in the exposure times required for MNNG to induce maximal levels of mutation and transformation supports the hypothesis that neoplastic transformation is dependent on factors other than a single gene mutational event. The ratios of transformation to mutation frequencies were within the same order of magnitude for short treatment times (<60 min), while they increased to more than a 20-fold difference for exposure times of 240 min or longer. Detection of DNA damage by alkaline elution showed maximal levels by 30 min of exposure. Exposures of 30 min followed by post-treatment incubation periods of 30 to 90 min showed increasing rejoining of single-strand breaks, but the total DNA damage remained constant for MNNG exposures extended from 30 to 120 min. Similar studies were performed with another alkylating agent, ethylnitrosourea (ENU). The same pattern of temporal dissociation was found, but shifted to much earlier times. DNA damage and mutation reached their maxima after 5-min exposures, while cytotoxicity and transformation reached their maxima only after 45 min, with a transformation/mutation ratio of 51-fold. (Additional studies with this cell system are reported in the LEP Projects, Z01CE05265-03, Z01CE05275-03 and Z01CE05276-02.)

#### Significance to Biomedical Research and the Program of the Institute:

These studies are part of a long-term project designed to investigate the quantitative response of cellular systems to the concurrent induction of mutation and neoplastic transformation, as a basis for quantitative studies on the combined effects of different carcinogens and cofactors. The present findings support the hypothesis that different molecular mechanisms are involved in the induction of mutation, of transformation, and of DNA damage, as measured by selected methods. Different cell systems currently used for mutation and transformation studies show different characteristics in their responses to chemical and physical agents in relation to certain biologic parameters, (e.g., cell synchronization is required to demonstrate transformation by MNNG in C3H 10T1/2, but not in BALB/3T3 clone A31-1-1). The temporal dissociation of exposure times required for maximal

induction by MNNG of mutation and transformation provides a new biological phenomenon for investigating the differences between mutation and transformation mechanisms, especially in relation to molecular target mechanisms.

Proposed Course:

Analysis of O<sup>6</sup>-alkyl-guanine persistence in this system; analysis of methotrexate-resistant mutants in this system; analysis of the factors involved in the continuing induction of transformation during exposure periods when the mutagenic response is already saturated, particularly by the use of treatment-conditioned media and of selective inhibitors of DNA repair enzymes.

Publications:

Bignami, M., Ficorella, C., Dogliotti, E., Norman, R. L., Kaighn, M. E. and Saffiotti, U.: Temporal dissociation in the exposure times required for maximal induction of cytotoxicity, mutation and transformation by N-methyl-N'-nitro-N-nitrosoguanidine in the BALB/3T3 Cl A31-1-1 cell line. Cancer Res. 44: 2452-2457, 1984.

Saffiotti, U.: Comparability of in vitro and in vivo systems for carcinogenesis evaluations in different species, tissues and cells. In Vouk, V., Butler, G. C., Hoel, D. G., and Peakall, D. B. (Eds.): Methods for Estimating Risks of Chemical Injuries: Human and Non-human Biota Ecosystems. Sussex, England, John Wiley & Sons, 1984. (In Press)

Saffiotti, U., Bignami, M., Bertolero, F., Cortesi, E., Ficorella, C. and Kaighn, M. E.: Studies on chemically induced neoplastic transformation and mutation in the BALB/3T3 Cl A31-1-1 cell line, in relation to the quantitative evaluation of carcinogens. Toxicol. Pathol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE04493-06 LEP

PERIOD COVERED

October 1, 1984 to September 30, 1984

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

Bioenergetic Pathways in Chemically-Transformed Epithelial Cells

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

PI: A. E. Kaplan Research Chemist LEP NCI

COOPERATING UNITS (if any)

Laboratory of Applied Studies, Division of Computer Research and Technology, NIH, Bethesda, MD (B. Bunow); Department of Microbiology, Harvard Medical School, Boston, MA (H. Amos); Program Resources, Inc., Frederick, MD (R. L. Brown).

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

1.8

PROFESSIONAL:

1.0

OTHER:

0.8

CHECK APPROPRIATE BOX(ES)

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

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

Bioenergetic studies include the glycolytic and oxidative pathways which convert metabolites into chemical energy and the conversion of this energy for cellular functions. Studies of bioenergetic modifications in neoplastic cells identify alterations in the glycolytic pathway and in ultrastructure, including mitochondria. Lactate dehydrogenase is modified in a chemically-transformed neoplastic cell line from rat liver compared with its non-transformed control, showing a higher overall rate of reaction. The results support the increased synthesis of lactic acid found in these and other neoplastic cells. The change in kinetic behavior is accompanied by modifications in the proportions of LDH-4 and -5, isozymes associated with the liver, and with a marked increase in LDH with an isoelectric point of 8.8. The mitochondria show decreased numbers and pleomorphic structures in the neoplastic cell line. They are displaced in the cytosol, along with other organelles. One energy utilization pathway seems unchanged, the transport of protons. This was determined by a spectrophotometric change in the intracellular dye, 6-carboxyfluorescein, relative to pH. These experiments were carried out with monolayers of anchorage-dependent cells grown on Leighton tube slides and examined by split beam/dual wavelength spectrophotometric techniques. In transformed cells, both lactate and hydrogen ions diffuse from the cytosol into the medium and also are exported to a small extent by active transport processes as in the control cells. These studies show that the pH of the cytosol is more acidic in the transformed cells, for these cells convert ten times as much glucose to lactic acid as the control cells.

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

A. E. Kaplan                                  Research Chemist                                  LEP NCI

Objectives:

An increased rate of synthesis of lactic acid characterizes most neoplastic cells. This sign of imbalance in the glycolytic pathway is but one aspect of the modified production and utilization of energy found in malignant cells. This project focuses on the identification of the molecular modifications in the glycolytic and oxidative pathways of energy production in chemically-transformed neoplastic cells of epithelial origin and in the altered pathways of utilization of this chemical energy. The specific areas of investigation include:

1. Identification of kinetic and molecular modifications in the enzyme, lactate dehydrogenase (LDH, E.C.1.1.1.27), which supports the increased rate of synthesis of lactic acid in neoplastic cells. Investigation of the sensitivity of the LDH isozymes from control cells to alkaline (serine) phosphatase relative to its kinetic and molecular properties.
2. Ultrastructural and cytoskeletal modifications in neoplastic cells relating to defective mitochondria with respect to reduced numbers, increased fragility, altered intracellular distribution and defective structure.
3. Enzymatic breakdown at the point of introduction of pyruvate from the glycolytic pathway into the citric acid cycle for oxidative phosphorylation.
4. Identification of defective transduction pathways in the distribution of chemical energy produced in the oxidative cycle. Normal cells use energy for differentiated synthetic reactions, and for heat production and ion exchange. In neoplastic cells, differentiated reactions decrease and heat production and ion exchange increases, suggesting that proteins of the energy transduction system are modified or lost with malignant transformation.

Methods Employed:

TRL 12-13 cells, established from 10-day-old rat liver, are used as control cells for limited (22) passage numbers. TRL cells exposed in vitro to nitrosomethylurea (NMU) underwent neoplastic transformation resulting in the NMU-3 line. In vivo, the NMU-3 cells produce carcinomas which appear in rats in 10-20 weeks. In vitro, by light microscopy, the appearance of NMU-3 cells does not differ from that of TRL 12-13 cells, with both lines growing as anchorage-dependent monolayers. By electron microscopy, the ultrastructure of the NMU-3 cell is distinctly different from the TRL 12-13 cell in its organelles, cytoplasm and plasma membrane, and is useful for studies of mitochondria in neoplastic cells.

For studies of LDH in both cell lines, kinetic analyses are carried out by stopped-flow methods; the results are calculated by differential analysis using

the MLAB On-Line Modeling System resident in the DECsystem 10. This method was developed with Dr. B. Bunow, Division of Computer Research and Training, because Michaelis-Menten kinetics cannot be used for analyses of the tetrameric LDH enzyme. Comparison of the LDH isozymes, -4 and -5, found in liver cells *in vivo*, is carried out by gel electrophoresis on agarose and polyacrylamide gel, and by isoelectric focusing methods. Gels are stained for LDH activity with lactate, NAD<sup>+</sup> and Tetrazolium Blue, and for protein by the silver stain method which is 100 times more sensitive than Coomassie Blue. The enzyme is separated from extraneous proteins by column chromatography and high performance liquid chromatography.

In collaboration with Dr. H. Amos, Harvard Medical School, comparative studies are carried out with LDH from NIL and NIL-py cells. NIL is a chick embryo cell line. NIL-py is transformed from NIL with polyoma virus and produces sarcomas *in vivo*.

Cytoskeletal proteins of the TRL 12-13 and NMU-3 cell lines are under investigation with Dr. R. L. Brown, Program Resources Incorporated, using fluorescent antibodies against tubulin, actin and intermediary fibers to correlate aggregation patterns with overall cell dimensions and with the organization of organelles, such as mitochondria.

Enzymatic reactions of mitochondria will also be studied by difference spectrophotometry which permits data analysis after subtraction of the light scattering effect of cells and organelles. The entry of pyruvate into the citric acid cycle will be analyzed by such methods.

In studying energy utilization in the neoplastic cell, lactate and proton ion exports is measured as an indicator of membrane transport. A new spectrophotometric method was developed to determine intracellular pH through absorbance changes in the indicator dye, 6-carboxyfluorescein, introduced into the cytoplasm of anchorage-dependent monolayers of control or transformed liver cell lines. Uptake of glucose from the medium is also correlated with lactate ion export for the evaluation of the differences in glucose utilization with neoplastic transformation.

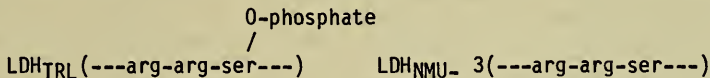
#### Major Findings:

Kinetic and Molecular Modification of LDH: LDH from chemically-transformed neoplastic cells shows significant differences in both kinetic and molecular properties relative to control cells. In kinetic analysis, LDH shows a high, initial rate of reaction which decelerates to a lower steady state rate. This discontinuity is believed to be due to an inhibitory complex which arises from the interaction of the enzyme with its substrate, pyruvate, and its reduction product, NAD<sup>+</sup>. The concentration required for inhibition varies with LDH preparations from different sources. Thus, initial and final rates as well as the extent to which the initial high rate can continue, or the amount of NADH oxidized can be used to compare different enzyme preparations. Data were analyzed by the MLAB On-Line Modeling System resident in the DECsystem 10 in collaboration with

Dr. B. Bunow, using the parametric function for OD,  $A \exp BT + C + DT$ . In LDH from NMU-3 versus TRL 12-13 cells, both parameters in the first term representing the initial, rapid velocity, are doubled. It should be noted that B is a negative exponential term and shows an increased rate of oxidation of NADH at more than double the rate of LDH from TRL 12-13 cells.

A five-fold increase in the term representing the steady state rate shows that this portion of the reaction also increases. The MLAB analysis provides numerical values which give further insight into the altered reaction properties of LDH from NMU-3 cells. Kinetic modifications in this enzyme support an increased production of LDH on the part of the neoplastic cell line. This was further confirmed by glucose analysis which shows that during growth to confluence, NMU-3 cells convert 75% of the sugar taken up to lactic acid compared to 8% in TRL 12-13 cells. Investigation into molecular differences shows that LDH preparations from both cell lines have LDH-4 and -5, two types found in liver. However, whereas LDH-4 dominates in TRL cells, LDH-5 dominates in NMU-3 cells. Each isoenzyme is characterized by its own isoelectric point (pI), acidic for LDH-4 and basic for LDH-5.

Treatment of LDH from TRL cells with alkaline phosphatase, which reacts with serine-0-phosphate to remove phosphate, alters most of the LDH-4 in TRL cells so that this isozyme now migrates to LDH-5. The resulting isozyme distribution has the appearance of LDH from NMU-3 cells. This suggests that the untreated LDH from TRL cells has a serine-0-phosphate group which is lost in NMU-3 cells. The differences in isozyme distribution in the control and in the neoplastic cells are also shown in the properties of LDH with acidic and basic pI values, the latter making up about 80% of the enzyme in NMU-3 cells and only 10% or less in TRL cells. In other enzyme pairs which have serine-0-phosphate groups, arginine dimers are adjacent to the serine derivatives. This suggests that, in the case of LDH, the following reaction occurs:



and yields LDH with a very alkaline pI. This hypothesis is being tested experimentally.

Using SDS gel electrophoresis, the molecular weight of the LDH monomers is between 30,000 and 40,000. Further analyses using other methods are under way to obtain a more precise molecular weight.

In collaboration with Dr. H. Amos, LDH in NIL versus NIL-py cells has been examined. These are fibroblast lines and show the typical LDH-3 of this type of cell. Transformation with polyoma virus does not alter the LDH isozyme although it does cause an increase in enzyme content as in the NMU-3 versus TRL cells. However, isoelectric focusing separations show an increased alkalinity in LDH from NIL-py cells. The difference here is only about 0.5 pH units, far less than in the hepatocyte lines mentioned above, suggesting that the fibroblast enzymes differ from the epithelial enzymes in both control and transformed isozyme structures.

Studies of cytoskeletal proteins extend earlier work on the ultrastructure of the hepatic cell line. Using fluorescent antibodies, in collaboration with Dr. R. L. Brown, Program Resources, Inc., it was found that tubulin aggregation is similar in both TRL and NMU-3 cells. This observation supports those by light and electron microscopy which show that both cell types are similar in shape and size. Further studies are under way for identification of intermediary fibers and actin in these cell lines. The former are altered in virally-transformed fibroblasts and are believed to relate to alterations in mitochondrial structure characteristic of the NMU-3 cell line and other neoplastic cell lines. Results thus far are in agreement with the previous report, showing that cytokeratin assembly is markedly diminished in NMU-3 cells, whereas actin is unchanged.

#### Significance to Biomedical Research and the Program of the Institute:

The studies undertaken in the areas of cell bioenergetics provide further biochemical definition of the metabolic differences acquired by neoplastic cells in comparison with their non-transformed counterparts. In the case of NMU-3 cells, ten times as much glucose is converted to lactate as in the control TRL cells, giving a numerical example of the difference in only one aspect of the bioenergetic cycle. This difference is supported by kinetic and molecular modifications in LDH, which lead to the increased synthesis of lactic acid, and make use of an unaltered membrane transport system for export of the acid from the cell. The marked differences in the capability of the neoplastic cell for effective energy production and energy utilization ultimately relate to cachexia and death in the cancer patient, the relatively smaller number of neoplastic cells resulting in imbalances in the metabolism of their host organism.

#### Proposed Course:

This project will be moved to new space in the LEP at the end of this fiscal year. The present studies will be continued and new collaborative studies with the Tissue Culture Section, LEP, are planned. Plans for extending the present studies include purification of the LDH isozymes from the hepatocyte lines, comparison of protein structure, clarification of post-translational modifications of LDH, and identification of the new forms of LDH synthesized in the neoplastic cell. The role of  $Ca^{++}$  uptake versus lactic acid export will be investigated.

With the NIL cell pairs (in collaboration with Dr. H. Amos, Harvard Medical School) the kinetic analysis of LDH will be completed, and further protein studies carried out.

Studies with cytoskeletal proteins will be completed to see if the intermediary fibers relate spatially to the mitochondria in the two cell types from rat liver. While the ras oncogene is considered to control changes in morphology and anchorage-dependence in neoplastic cells, not all modifications are observed in NMU-3 cells. The incomplete change in morphology suggests step-wise rather than all-or-none changes. This question will be investigated.

With a new recording spectrophotometer DW-2C, mitochondrial studies will be initiated to see if phosphoenol-pyruvate can serve as a substrate for citrate condensation.



Methods to analyze energy utilization in single epithelial cells will be developed to study the changes induced by chemical carcinogens.

With the Tissue Culture Section, LEP, methods developed with the rat liver cells for LDH analysis and for the study of LDH transport will be extended to epidermal and other epithelial cells. Efforts will be made to develop more sensitive methods of identifying early stages of transformation, for example by lactate analysis. New studies will be directed to the fractionation and purification of epithelial growth factors from conditioned medium.

Publications:

Kaplan, A. E. and Bunow, M. R.: Spectrophotometric determination of intracellular pH with epithelial cells growing as anchorage-dependent monolayers. Anal. Biochem. (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05265-03 LEP

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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:	M. I. Lerman	Visiting Scientist	LEP	NCI
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Others:	M. Bignami	Guest Researcher	LEP	NCI
	R. L. Norman	Staff Fellow	LEP	NCI
	U. Saffiotti	Chief	LEP	NCI
	M. E. Kaighn	Expert	LEP	NCI
	N. H. Colburn	Expert	LVC	NCI
	E. H. Westin	Expert	LTCB	NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Experimental Pathology

## SECTION

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

2.4

## PROFESSIONAL:

1.9

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

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

This project seeks to determine specific changes in the genome induced by chemical carcinogens and their relevance to the initiation and development of neoplasia. (1) To explore if different transforming DNA sequences are activated by different carcinogens, DNA from BALB/3T3 cells transformed by several chemical carcinogens and by U.V. light was transfected in NIH/3T3 cells. Three positive benzo[a]pyrene (BP)-transformed lines were analyzed for sensitivity to the restriction endonucleases, EcoRI, HindIII, BamHI and XbaI, and showed different patterns of sensitivity with no evidence of rearrangement or amplification of ras oncogenes. However, further analysis with MspI revealed a polymorphism in the v-Ha-ras homologous sequences. Since a MspI polymorphism at the 12th codon of the human and the rat c-Ha-ras gene distinguishes the malignant from the normal alleles, these results suggest that the transforming gene may be a point mutated allele of c-Ha-ras. Further work is under way to investigate the mechanisms responsible for different restriction enzyme sensitivity patterns of transforming DNAs induced by BP. (2) The molecular basis for the frequent activation of c-Ha-ras genes by point mutations is being explored. The binding of BP to hamster liver DNA reached a maximum shortly after injection of BP into the portal vein. A major portion of this BP binding occurred in DNAase I hypersensitive regions which are correlated with putative regulatory regions of actively expressed genes. These results suggest that persistent adducts may be located within the coding region and therefore may be relevant to gene activation by point mutation. (3) To establish the molecular events underlying the mechanisms of tumor promotion, two apparently new transforming genes, required for tumor promotion in the JB-6 mouse epidermal cell lines, were cloned from promoter-sensitive preneoplastic cells. Studies are under way on their structure and DNA sequence and on their presence and function in normal mouse keratinocytes. Human homologs of these genes are being isolated from a genomic library and their structure and function are being studied.

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

M. I. Lerman	Visiting Scientist	LEP	NCI
M. Bignami	Guest Researcher	LEP	NCI
R. L. Norman	Staff Fellow	LEP	NCI
U. Saffiotti	Chief	LEP	NCI
M. E. Kaighn	Expert	LEP	NCI
N. H. Colburn	Expert	LVC	NCI
E. H. Westin	Expert	LTCB	NCI

Objectives:

Two fundamental biological traits of cancer cells are considered, namely: (1) the ability to escape constraints over cell growth and differentiation, and (2) the acquisition of genomic instability. Both properties are likely to be determined by specific molecular changes in the genome. These changes are believed to occur in steps in a pattern that may be characteristic for all cancers. Furthermore it is likely that (1) the activation of cellular oncogenes is responsible for uncontrolled growth; (2) since the number of these oncogenes may be an order of magnitude lower than the number of known cancer types, cooperation or complementation between oncogenes must occur during progression; and (3) the higher susceptibility to mutations may result from the inactivation of suppressor gene(s) leading to the activation of a specific transposable element(s), which may, in turn, initiate a process of genomic instability and provide a molecular basis for the activation of oncogenes by rearrangements and translocations known to occur in many cancers.

This project seeks to identify these molecular changes by selecting biological systems appropriate for the identification of transforming DNA activities and by subsequent cloning and molecular characterization of oncogenes involved in the different stages of carcinogenesis. Special emphasis is given to identifying the changes initiated by chemical carcinogens in epithelial cell systems.

The specific aims are to characterize and clone the following types of genes:

(A) Oncogenes activated in chemically transformed animal and human cells:

DNA is generally considered to be the major target for the initiation of carcinogenesis with most known chemicals. The knowledge of oncogenes suggests that they may be the specific target genes of carcinogens and that the chromosomal structure of specific oncogenes in a given cell, as well as the nature of the chemical agent, may determine the molecular mechanism by which the genes become activated.

Two approaches are pursued:

(1) Transformation is induced with different chemical carcinogens in normal animal and human cells (primary cultures) and in immortalized cells (cell lines).

The DNA of the transformed cells is analyzed for transforming activity by transfection back into the nontransformed parent cells and used as a source for the cloning of activated oncogenes by established and novel approaches. The molecular lesions leading to activation will be investigated by comparing the alleles from transformed and nontransformed cells at the DNA sequence level.

Transformation will also be induced in human cells from cancer-prone gene carriers. Human cells in culture are extremely resistant to chemical transformation. However, evidence has accumulated from human cancer genetics that the predisposition to specific types of cancer is specified by dominantly transmitted genes. In the case of Wilm's tumor and retinoblastoma these genes seem to represent altered suppressor genes, whose normal form suppresses the activity of specific oncogenes. Therefore, cell lines established from cancer-prone humans may carry altered suppressor genes which will permit the expression of human oncogenes and consequently their identification and isolation as described above.

(2) Brief treatment of nuclei with low concentrations of DNAase I has been 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. Similar studies employing carcinogen treatment in vivo or in vitro followed by DNAase I treatment of the nuclei should determine whether or not carcinogens preferentially bind to DNAase I hypersensitive regions. Labeled probes of known oncogenes will be hybridized to blots of genomic DNA to study the effects of carcinogen and DNAase I treatments at the gene level.

(B) Genes involved in tumor promotion:

The biology of tumor promotion is well defined especially in mouse skin; the genes responsible for the response to promoters are under investigation. Isolation of clonal preneoplastic cell lines responding to tumor promoters with an irreversible induction of the transformed phenotype indicated that tumor promoters directly affect the initiated cell and suggested that specific genes may be involved. Direct DNA-mediated gene transfer from promoter-sensitive to promoter-resistant clones of the JB-6 cells demonstrated the presence of such genes. In a joint effort with the Cell Biology Section, LVC (see Project #Z01CE05382-01 LVC), these genes, termed pro, were cloned and they are currently being analyzed for their structure and function at the DNA sequence level. The mechanisms of their activation by chemicals are also under study. Human homologs are being isolated and investigated. The characteristics of the corresponding normal proto-pro genes are being investigated using primary cultures of mouse keratinocytes developed in Project #Z01CE05276-02 LEP.

Methods Employed:

(A) Transformation of selected cell types by chemical carcinogens and characterization of transformed cell lines.

(B) Preparation of high quality DNA, RNA (total, poly A<sup>+</sup>) and nuclei by standard techniques.

(C) DNA-mediated gene transfer by the calcium phosphate precipitation technique, with or without digestion with selected restriction endonucleases.

(D) Southern transfer analysis for detecting genes and analyzing their chromosomal organization.

(C) Northern transfer analysis to analyze the transcriptional activity of the genes.

(F) Gene cloning by techniques developed in the last three years using either "search" or "rescue" strategies: sizing the gene, establishing sub-genomic libraries in suitable vectors, selecting for the gene by "tracking" it in a biological assay (sib-selection) or screening the library with available probes.

(G) Gene sequencing by the chemical degradation method of Maxam and Gilbert and analysis with computer-aided programs for its structure and function.

(H) Treatment of nuclei with DNAase I to establish the location of DNA-carcinogen adducts.

#### Major Findings:

(A) (1) DNA was extracted from a series of 16 BALB/3T3 cell lines obtained from foci transformed by different chemical carcinogens or by U.V. light. Transfection into NIH/3T3 cells showed transforming activity for 7 of the 16 lines. In order to investigate whether the same or different transforming sequences were induced by the same carcinogen, the three most active lines transformed by benzo[a]pyrene (BP) were studied further after digestion with the restriction endonucleases, EcoRI, HindIII, BamHI and XbaI. Different patterns of sensitivity to endonuclease activity were obtained and confirmed for each of the three tested lines. This shows that different patterns of sensitivity can be induced by the same carcinogen in the same target cells. Hybridization with probes containing Ha-ras and Ki-ras oncogenes, repeated independently twice, failed to show any mutation, rearrangement or amplification of the ras gene. However, since the NIH/3T3 transfection assay is known for its sensitivity to transformation by ras, an additional series of experiments was undertaken. Transforming DNA from the BP-transformed lines was assayed for MspI polymorphism, which detects a point mutation at the 12th codon of the c-Ha-ras oncogene in the human gene. So far, two lines have been tested and both were found to contain an additional allele resistant to MspI (680 bp), suggesting that the transforming gene is a point mutated allele of c-Ha-ras. Additional transformed lines are being investigated.

(2) Preliminary results of in vivo experiments with hamsters indicate that the formation of DNA-BP adducts reaches a maximum in the liver within 30 to 60 minutes after injection of BP into the portal vein. In addition, 30 to 40% of these adducts appear to be in DNAase I hypersensitive regions as determined by counting the high molecular weight DNA isolated from liver nuclei from BP-treated hamsters after treatment of the nuclei in vitro with DNAase I. Further analysis revealed that v-Ha-ras homologous sequences, present in hamster liver, were DNAase I hypersensitive but this hypersensitivity was not affected by

BP-treatment in vivo. This analysis also indicated that no detectable rearrangements or amplification of the v-Ha-ras homologous sequences had occurred in liver DNA following BP treatment. A similar lack of effect of BP and aflatoxin B<sub>1</sub> was observed on v-Ha-ras homologous sequences in rat liver. Additional studies employing other oncogene probes and in vitro labeling systems are planned.

(B) By using TPA-sensitive (P<sup>+</sup>) and -resistant (P<sup>-</sup>) clonal lines of JB6 mouse epidermal cells, the transforming genes required for neoplastic transformation of these cells by the tumor promoter, TPA, were identified (see also Project #Z01CE05382-01 LVC). The cloning of these genes was obtained by: (1) establishment of an enriched genomic plasmid library of P<sup>+</sup> DNA in the BamHI site of the Okayama-Berg expression vector pcD-X; and (2) isolation by Lederberg's sib-selection technique (Cavalli-Sforza, L. L. and Lederberg, J. Genetics 41: 367-381, 1956), of two independently active clones, p26 and p40 (Inserts of 3.4-kb and 12-kb, respectively). Molecular characterization of the cloned genes showed that (1) they have equal specific activity (on a molar basis) compared to the specific activity of total P<sup>+</sup> DNA; (2) restriction mapping and transfection of segments of the inserts located the active genes to 1.1-kb and 3.8-kb segments of p26 and p40, respectively, thus demonstrating that the cloned genes are functionally active and do not need the SV-40 promoter of the pcD-X vector; (3) EM heteroduplex analysis and hybridization between the functional segments failed to show any structural homologies between them, thus indicating that they represent different genes; (4) the active genes, which are termed pro (for promoting genes), exhibit DNAase I hypersensitivity, indicating that they exist in a transcriptionally active configuration in the chromatin of P<sup>+</sup> and transformed cells; (5) the p26 pro gene is not related to any of 11 known viral oncogenes (abl, fes, fms, mos, myc, myb, Ha-ras, Ki-ras, src, sis, erbA, erbB) as shown by Southern blot hybridization using the active segment of p26 as a probe; and (6) sequencing of the p26 gene is now completed and the sequence is being analyzed by a computer-aided program.

In addition, it was found that P<sup>+</sup> cells transformed by TPA contain another active oncogene which is able to transform P<sup>+</sup> cells in the absence of TPA and is distinguishable from pro by restriction enzyme sensitivity.

#### Significance to Biomedical Research and the Program of the Institute:

Elucidation of the molecular lesions leading to the activation of oncogenes will provide in-depth understanding of cancer pathogenesis. Present studies are particularly relevant to the understanding of the mechanisms of chemical carcinogens and promoters in animal models and eventually in human carcinogenesis.

#### Proposed Course:

(A) Completion of current studies on chemically induced DNA changes in BALB/3T3 transformed cells by additional characterization of the transforming activities and cloning of the transforming gene(s).

(B) Identification and cloning of transforming genes and their normal alleles from mouse keratinocytes transformed by different chemicals.

(C) The identification and isolation of human oncogenes and their normal alleles from chemically treated cell lines of cancer-prone humans.

(D) Identification and cloning of transforming genes from established human cancer cell lines, e.g., human prostate carcinoma and bladder carcinoma, and determination of their activity in the corresponding normal epithelial cells in culture (see Project #Z01CE05276-02 LEP).

Publications:

Colburn, N. H., Lerman, M. I., Hegamyer, G. A., Wendel, E. and Gindhart, T. D.: Genetic determinants of tumor promotion: Studies with promoter resistant variants of JB-6 cells. In Bishop, M., Greens, A. and Rowley, Y. (Eds.): Genes and Cancer. Los Angeles, Alan R. Liss, Inc. (In Press)

Colburn, N. H., Lerman, M. I., Srinivas, L., Nakamura, Y. and Gindhart, T. D.: Membrane and genetic events in tumor promotion: Studies with promoter resistant variants of JB6 cells. In Fujiki, H. and Sugimura, T. (Eds.): Cellular Interactions by Environmental Tumor Promoters. Tokyo, Scientific Societies Press (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05273-03 LEP

PERIOD COVERED

October 1, 1983 through September 30, 1984

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

**Molecular Mechanisms in Multistage Carcinogenesis**

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

PI:	T. D. Gindhart	Expert	LEP	NCI
Others:	Y. Nakamura	Guest Researcher	LVC	NCI
	B. M. Smith	Guest Researcher	LVC	NCI
	U. Saffiotti	Chief	LEP	NCI
	N. H. Colburn	Expert	LVC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

0.8

OTHER:

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.)

The objectives of these studies are to analyze the molecular mechanisms underlying specific stage transitions in multistage carcinogenesis, comparing the mechanisms of promoting agents with those of carcinogens, singly or in combinations. This project addresses the primary question of how committed preneoplastic epithelial cells are irreversibly transformed by phosphorylation of specific proteins induced by the phospholipid- and calcium-dependent protein kinase (PK-C) triggered by pharmacologic diacylglycerol. Over 16 substrates for PK-C have been found in JB-6 cell lines. They reversibly associate with the particulate fraction of cells depending on the availability of divalent cations. Some are more readily phosphorylated under cation conditions favoring phosphorylation of more basic proteins (5.0 mM Ca<sup>++</sup>, 7.5 mM Mg<sup>++</sup>), but most are phosphorylated under high salt conditions (5.0 mM Ca<sup>++</sup>, 75 mM Mg<sup>++</sup>). One heat shock protein, pp80, is stimulated by TPA in untransformed but not in transformed derivatives of JB-6 cells. Hyperthermia induces a full heat shock response and blocks TPA promotion. The relationship between this defect in heat shock protein regulation and the synergistic anti-tumor effects of interferon and hyperthermia is being pursued. The generation of reactive oxygen, especially the superoxide anion, has been found to be a required event in TPA-induced promotion in JB-6 cells. Secondary free radicals implicated in this activity are OH- and lipid peroxides but hydrogen peroxide is only toxic.



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

T. D. Gindhart	Expert	LEP	NCI
Y. Nakamura	Guest Researcher	LVC	NCI
B. M. Smith	Guest Researcher	LVC	NCI
U. Saffiotti	Chief	LEP	NCI
N. H. Colburn	Expert	LVC	NCI

Objectives:

The objectives of these studies are to analyze the molecular mechanisms underlying specific stage transitions in multistage epithelial carcinogenesis. Malignant transformation of mammalian cells by chemical carcinogens proceeds by progression through stages which can be phenotypically recognized in vivo and in parallel cell culture systems. Biochemical differences between populations of cells at successive stages can initially be utilized as stage-specific molecular markers and then exploited to analyze the underlying mechanisms responsible for progression of cells from one stage to the next. The biological model currently utilized is the mouse epidermal cell line JB-6 with its unselected clones sensitive and resistant to transformation by promoting agents. Recent studies have shown that only one or a few genes determine the late-stage preneoplastic phenotype in these cell lines. Other epithelial cell systems, being characterized in other projects of this laboratory, will be used for further studies to estimate the activity of these genes in other carcinogenesis models, e.g., in respiratory epithelia. Emphasis will be placed upon comparison of the mechanisms of tumor-promoting agents with those of carcinogens administered singly and in combinations.

This project includes two specific parts:

Part A: Determination of the roles of free radicals, especially those of reactive oxygen, in the transformation response to tumor promoters in mouse epidermal cell lines; and

Part B: Evaluation of changes in protein kinase (PK) activities as epithelial cells pass through defined stages of preneoplastic progression.

Methods Employed:

Part A: Clonal sublines of the JB-6 cell line were used to study the roles of free radicals in preneoplastic progression. The effects of eliminators of free radicals on the promotion of transformation of JB-6 cell lines by TPA have been determined. The effect of TPA on endogenous levels of superoxide dismutase was measured. An oxidant, NaIO<sub>4</sub>, and an industrially common free radical generator, benzoyl peroxide, were tested for their ability to promote transformation of JB-6 cell lines.

Part B: The same battery of JB-6 clonal variants and their transformed counterparts were metabolically labeled with  $^{32}\text{P}$ -orthophosphate and the phosphoprotein patterns of whole cell lysates and subcellular fractions were analyzed by one- and two-dimensional gel electrophoresis. Possible differences between promotion-sensitive and -resistant JB-6 cell lines in the calcium- and phospholipid-dependent protein kinase-C (PK-C) were sought by varying the conditions of the PK-C assay. Possible differences between these cell lines in substrates for this enzyme were sought in parallel by gel electrophoresis of in vitro reaction products.

Tumor-promoting phorbol esters are known to directly activate PK-C, a recently described PK present in all cells. The opportunity to identify TPA-induced phosphoprotein changes relevant to transformation should be maximized by analyzing induced changes in cell lines which differ by only one or a few genes in their capacity to be transformed by TPA. This is the case for the JB-6 cell lines.

#### Major Findings:

Part A: Of seven different types of eliminators of free radicals tested those which enzymatically catabolized the superoxide anion inhibited TPA promotion most effectively. Superoxide dismutase (SOD) and the lipophilic copper coordination compound, copper (II), (3,5-diisopropylsalicylic acid)<sub>2</sub>, with SOD-mimetic activity inhibited TPA promotion of JB-6 cell lines up to 95% with no effect on expression of the transformed phenotype. Eliminators of hydrogen peroxide, catalase and glutathione peroxidase, enhanced expression of the transformed phenotype by 200-500%. One scavenger of hydroxyl radicals, D-mannitol, moderately inhibited TPA promotion of transformation as did two antioxidants, n-propyl gallate and butylated hydroxyanisole. Two inhibitors of the lipoxygenase pathway of the arachidonic cascade, nordihydroguaiaretic acid and quercetin, also inhibited TPA promotion while an inhibitor of the cyclooxygenase pathway, indomethacin, did not. Inhibition by SOD required either pretreatment of the cells or addition of SOD within four hours of the start of exposure to TPA.

TPA treatment was found to reduce the levels of endogenous SOD activity by 50% in promotion-competent subclones of JB-6 cells but only by 17% in promotion-incompetent subclones. This difference in suppression of SOD with TPA treatment appears to mark the promotion-competent phenotype among JB-6 cell lines.

Oxidizing treatment with  $\text{NaIO}_4$  effectively promoted transformation of JB-6 cell lines as did treatment with benzoyl peroxide. Concentrations of retinoic acid which inhibited promotion by TPA failed to inhibit promotion by benzoyl peroxide.

Part B: A heat shock protein (pp80) increases in response to TPA in untransformed JB-6 cell lines but is deficient in tumor cell lines. It has the following properties: molecular weight 80,000, isoelectric point 4.5, and localization to the membrane fraction from which it is readily solubilized by 1% triton X-100. It was found that pp80 is a major cellular phosphoprotein in JB-6 cells representing approximately 2% of the total phosphoprotein in whole cell lysates. TPA treatment (10 ng/ml) increases the amount of  $^{32}\text{P}$ -labelled pp80 by 2-3 times in non-transformed JB-6 cells. This enhancement is first detectable within 30 min, peaks at 1 hr and returns to baseline levels within 24 hrs. Variant cell

lines resistant to promotion to anchorage-independence by TPA increased pp80 more than sensitive cell lines. pp80 is increased by TPA but not by EGF. Dibutyl cAMP at  $10^{-3}$  molar inhibits the effect of TPA on pp80 when administered concomitantly. Heat stress also increased pp80 and several other phosphoproteins. Transformed JB-6 cell lines lack pp80 and fail to produce it in response to TPA. Loss of pp80 and of the pp80 response to TPA was linked to the transformed phenotype in JB-6 cells while the presence of pp80 and the ability to increase it in response to TPA were associated with the maintenance of the untransformed state. Heat stress itself inhibited TPA promotion in a dose-dependent fashion at 38.5°C and 41°C.

PK-C enzyme activity is similar in JB-6 cell lines of different phenotypes including tumor cell lines. Over 16 substrates for PK-C have been found with no absolute differences between promotion-competent and promotion-incompetent cell lines. The procedure used detects PK-C substrates representing as little as 0.1% of the total cellular protein. The 16 substrates can be categorized as histone H<sub>1</sub>-like or histone H<sub>2</sub>-like according to cation conditions favoring their phosphorylation. Most of them reversibly associate with the particulate fraction of cells depending on the availability of divalent cations. The TPA-sensitive heat shock protein, pp80, is not a PK-C substrate.

#### Significance to Biomedical Research and the Program of the Institute:

Identification of specific molecular mechanisms by which epithelial cells progress from one stage to the next in multistage carcinogenesis should contribute to a more complete understanding of the pathogenesis of epithelial cancers.

Identification of the phorbol ester receptor's PK substrates should lead to the characterization of the physiologic system which is the cellular target of tumor-promoting agents in post-initiated cells. This physiologic system is perturbed by many agents capable of triggering the later steps of preneoplastic progression including the chemical carcinogen, dioctylphthalate. How non-phorbol ester promoters such as benzoyl peroxide affect the physiologic system regulated by PK-C can now be analyzed in terms of the 16 substrates for PK-C.

The heat shock protein defect found in the tumor cells suggests dysregulation of this stress resistance mechanism in malignant transformation. Identification of a change in substrate for the PK-C activated by TPA, associated either with susceptibility to TPA induction of the transformed phenotype in epithelial cells or with its subsequent TPA-independent maintenance, should lead to a more detailed description of the biochemical reactions underlying these events.

Identification of superoxide anion generation as a critical event in preneoplastic progression suggests that chemopreventative efforts could be specifically aimed at that species of reactive oxygen. Tumor induction by complete carcinogens and promotion by benzoyl peroxide have important common properties not shared by phorbol ester promotion: resistance to inhibition by retinoids and induction of a high ratio of carcinomas to papillomas. As an apparently more universal tumor-promoting agent more closely related to complete carcinogens in its mode of action, benzoyl peroxide warrants further analysis as a tumor promoter.

Lipid peroxidation is a likely common pathway for the tumor-promoting effects of the superoxide anion and benzoyl peroxide.

Proposed Course:

(a) Identify the promotion-relevant substrates for PK-C especially as related to the cloned genes determining the promotion response found in these cells, superoxide anion generation and non-phorbol ester promoters.

(b) Identify the promotion-relevant targets for free radicals.

(c) Attempt to exploit specific defects in the heat shock response of tumor cells in hyperthermia treatment of cancer.

Publications:

Colburn, N. H., Lerman, M. I., Hegamyer, G. A., Wendel, E. and Gindhart, T. D.: Genetic determinants of tumor promotion: Studies with promoter resistant variants of JB-6 cells. J. Cell. Biochem. Suppl. (In Press)

Colburn, N. H., Lerman, M. I., Srinivas, L., Nakamura, Y. and Gindhart, T. D.: Membrane and genetic events in tumor promotion: Studies with promoter resistant variants of JB-6 cells. In Fujiki, H. and Sugimura, T. (Eds.): Cellular Interactions by Environmental Tumor Promoters, Tokyo, Scientific Society Press. (In Press)

Colburn, N. H., Srinivas, L., Hegamyer, G. A., Dion, L. D., Wendel, E. J., Cohen, M. and Gindhart, T. D.: The role of specific membrane and gene-level changes in the mechanism of tumor promotion: Studies with promoter resistant variants. In Borzsonyi, M., Yamasaki, H. and Hecker, E. (Eds.): The Role of CoCarcinogens and the Promoters in Human Experimental Carcinogenesis. Lyon, France, International Agency for Research on Cancer Scientific Publications. (In Press)

Gindhart, T. D., Lerman, M. I., Nakamura, Y., Hegamyer, G. A., Wendel, E. and Colburn, N. H.: Genes and signal transduction in tumor promotion: Studies with promoter resistant variants of JB-6 cells. In Mass, M. (Ed.): Tumor Promotion and Enhancement in the Etiology of Human and Experimental Respiratory Tract Carcinogenesis. New York, Raven Press (In Press)

Gindhart, T. D., Stevens, L. and Copley, M. P.: Transformation and tumor promoter sensitive phosphoproteins in JB-6 mouse epidermal cells: One is also sensitive to heat stress. Carcinogenesis. (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05274-03 LEP

## PERIOD COVERED

October 1, 1983 through September 30, 1984

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

Respiratory Carcinogenesis by Chemical and Physical Factors in the Hamster Model

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

PI: U. Saffiotti Chief LEP NCI

Others: S. F. Stinson Biologist LEP NCI  
 R. L. Norman Staff Fellow LEP NCI  
 H. M. Schdller Visiting Scientist LETM 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

Respiratory Carcinogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

3.6

## PROFESSIONAL:

1.4

## OTHER:

2.2

## CHECK APPROPRIATE BOX(ES)

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

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

Studies on the mechanisms of bronchogenic carcinoma induction by chemical and physical factors, alone or in various combinations, are pursued in the hamster respiratory carcinogenesis model. Two recently established Syrian golden hamster colonies (inbred 15:16/EHS:CR and outbred Syrian/CG.FOD) are used for intratracheal instillation of carcinogens adsorbed on particulate carriers alone or in combinations, systemic versus topical treatments, and combined treatments, with carcinogens and cofactors. Respiratory epithelial tissue responses are characterized by histological, ultrastructural, histochemical and biochemical methods, and by their study in organ and cell culture conditions.

Light and electron microscopy studies showed that respiratory epithelia of very young hamsters contain a large population of neuroendocrine cells of the APUD type, most frequent in one-day-old animals and declining thereafter. Sensitivity to diethylnitrosamine carcinogenesis was higher in newborn hamsters, with high incidences of nasal cavity neoplasms. Submucosal glands in the ethmoid region appear as the site of origin of the malignancies.

The regionally selective toxic agents, 3-methylindole and 2-methylnaphthalene, (two components of cigarette smoke with selective toxicity for Clara cells and alveolar type I cells in certain species) have been studied in the hamster model: acute and chronic toxicity levels were determined and found to be different for males and females. Long-term carcinogenicity studies are under way for these agents, also in combinations with other carcinogens.

The effects of microtrauma induced by cannulation of the trachea or larynx are studied with or without instillation of saline, particulates and carcinogens; mitotic index measurements show marked epithelial proliferation, mostly of secretory cells.

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

U. Saffiotti	Chief	LEP	NCI
S. F. Stinson	Biologist	LEP	NCI
R. L. Norman	Staff Fellow	LEP	NCI
H. M. Schüller	Visiting Scientist	LETM	NCI

Objectives:

The main objective of this project is the elucidation of the mechanisms by which bronchogenic carcinoma, a major form of human cancer, is induced by chemical and physical factors, alone or in various combinations.

The main biological model selected for these studies is the hamster respiratory carcinogenesis model, originally developed by intratracheal administration of saline suspensions of carcinogens carried by fine inorganic particles (Saffiotti, U., et al., Cancer Res. 28: 104-124, 1968). This model was subsequently extensively studied. Differentiation markers were characterized for basal cells, intermediate cells, mucous granule cells and keratin-containing cells. The cellular responses and the types of tumors induced by carcinogens in the hamster model are closely similar to their human counterparts. The role of different carcinogens acting synergistically was identified in this system, especially for polycyclic hydrocarbons in combinations with N-nitroso compounds. The special role of particulate carrier materials was identified in a series of studies with particulates having different physical characteristics; their pathogenetic mechanisms are being further investigated. An adequate induction model remains to be defined for the pathogenesis of small cell undifferentiated carcinomas.

Pathogenetic studies of this model received new emphasis, in this and other laboratories, with the development of organ culture and cell culture methods for respiratory epithelia and with their use in investigating the interaction of carcinogens with target cells. (See Project #Z01CE05277-02 LEP.)

Specific objectives of this project include the following studies:

(1) In vivo studies: Establishment and pathological characterization of specific pathogen-free colonies of inbred and outbred hamsters. Establishment of optimal treatment procedures including intratracheal instillations. Study of the response to particulates alone or as carriers of carcinogens adsorbed on their surface, singly or in combinations. Study of the role of fibrogenic dusts. Study of the mechanisms involved in the effects of particulates on the retention, distribution and metabolism of carcinogens on epithelial cell proliferation and on the induction of neoplastic transformation. Study of the effect of the age of the hamsters on the populations of susceptible cells in the respiratory tract and the type of respiratory neoplasms induced. Study of the conditions and mechanisms of the synergistic effects of carcinogens in the respiratory tract. Comparative study of chemicals with selective target effects in different regions of the respiratory

tract (such as polycyclic aromatic hydrocarbons with various carriers and certain N-nitroso compounds). Study of the effects in hamsters of two components of cigarette smoke, 3-methylindole (3MI) and 2-methylnaphthalene (2MN), which are known to induce selective damage to nonciliated bronchiolar lining cells (Clara cells) and alveolar type I cells in other animal species. Study of the effects of these agents on tumor formation in the lung and respiratory tract when administered simultaneously with particulate-bound benzo[a]pyrene (BP). Binding studies of [<sup>3</sup>H] benzo[a]pyrene to tissue macromolecules in vivo, particularly to DNA, from the trachea, bronchus and lung, including studies of the effects of multiple treatments with particulate-bound BP or concurrent treatment with 3MI or 2MN on binding.

(2) In vitro studies: Determination of the optimal conditions for the explant and organ culture of tracheobronchial hamster epithelium and for the establishment of primary epithelial cell cultures, from adult and/or newborn hamsters. Determination of the conditions for correlating in vivo and in vitro studies on the effects of carcinogens. These in vitro studies are pursued jointly with Project #Z01CE05277-02 LEP. Determination of optimal conditions for BP metabolism by tracheal and bronchial epithelia in culture and measurement of BP metabolism and binding in respiratory epithelia derived from animals receiving single or multiple treatments with particulate-bound BP, 3MI, or combinations of the two.

#### Methods Employed:

Controlled breeding at the FCRF Animal Production Area, under specific pathogen-free conditions, of two colonies of Syrian golden hamsters, inbred 15:16/EHS:CR and outbred Syrian/CG.FOD. Establishment of lifetime and serially sacrificed colony control and treatment groups with general histopathological study and special investigation of respiratory tract cell differentiation and carcinogenesis. Intratracheal instillations of solutions and of particulate suspensions. Segmental treatments of the respiratory tract using a special cannula (Schreiber, H., et al., J. Natl. Can. Instit. 54: 187-197, 1975), designed to expose only a localized region of the trachea to specific soluble carcinogens. Systemic treatments. Study of respiratory epithelia by histologic, histochemical, autoradiographic and immunochemical methods and by scanning and transmission electron microscopy. Epithelial tissue isolation, fractionation and use for biochemical analysis of carcinogen localization, metabolism, and binding.

Carcinogens currently studied include polycyclic aromatic hydrocarbons and N-nitroso compounds; studies with aflatoxin and arsenic are planned (see Project #Z01CE05275-03 LEP). Particulate materials under consideration include the oxides of iron, magnesium, titanium and aluminum, as well as carbon, talc and silica (quartz, cristobalite, tridymite) and others to be selected.

Two cigarette smoke components, 3MI and 2MN, are being evaluated in the hamster; the time course, affected cell types and extent of damage following single or multiple injections are studied by light and electron microscopy. The effects of concurrent exposures to 3MI or 2MN and polycyclic aromatic hydrocarbons, such as would occur during smoking, are being investigated in the hamster respiratory carcinogenesis model.

Tracheal and bronchial epithelial cells are cultured in vitro with [<sup>3</sup>H] benzo[a]pyrene and the metabolites are separated into ethylacetate-soluble, water-soluble, and -bound fractions. The ethylacetate-soluble metabolites are resolved by HPLC, collected and measured in a scintillation counter. DNA is isolated from the bound fraction by enzyme digestion and the total binding of [<sup>3</sup>H] benzo[a]pyrene to DNA is determined.

#### Major Findings:

(1) Morphological cell characterization: Light and electron microscopy were used to characterize the cellular composition of the respiratory tract epithelium in hamsters at different ages. Preliminary results indicated that the airways of very young hamsters contain large numbers of a primitive type of cell which is not found in older animals; further characterization of these cells showed that they are neuroendocrine cells of the APUD type. Their concentration is greatest in one-day-old animals and decreases thereafter. They appear mostly localized in the segmental bronchi near the points of bifurcation and also in alveolar ducts near the entrance of the bronchioles.

(2) Differential susceptibility to respiratory carcinogens: Three major long-term studies were started in collaboration with Dr. H. M. Schüller to examine any differences in type and incidence of respiratory neoplasms induced by 12 weekly subcutaneous injections of diethylnitrosamine (DEN) in hamsters which were one day, four weeks and eight weeks old at initiation. Animals have been killed at 4-week intervals, and a large group is being held for lifetime observations. Proliferative changes are characterized by light and electron microscopy.

Most of the animal experiments are completed and their results are being evaluated. Preliminary findings indicate that one-day-old hamsters are more sensitive to carcinogenesis with DEN than the older animals, with respiratory neoplasms appearing at earlier times and in greater numbers. A higher proportion of malignant neoplasms, especially in the nasal cavities, was found in the hamsters given DEN at the earlier age. Pathogenetic study of malignant neoplasms of the ethmoid regions of the nasal cavities suggests that they arise in the submucosal glands rather than from the olfactory epithelium as reported by previous investigators.

(3) Effects of regionally selective toxic agents on the respiratory tract and lungs of hamsters: Pilot studies were conducted to determine acute toxicity of single systemic treatments of hamsters with varying doses of 3MI and 2MN. 3MI was shown to be toxic to hamsters, with females being more sensitive than males; the LD<sub>50</sub> for a single i.p. injection was found to be approximately 500 mg/kg in males and 250 mg/kg in females. The highest acceptable dose for repeated administrations was found to be 25 mg/kg in males and 10 mg/kg in females. Systemic exposure to single doses of 3MI results in acute toxic changes of the terminal bronchiolar epithelium which are completely resolved in survivors after one week. Studies of the effects of chronic administration are near completion and studies of the time course of 3MI effects await evaluation at the electron microscopic level.

(4) Respiratory carcinogenesis with benzo[a]pyrene in inbred hamsters: Production of inbred hamsters reached the level needed for long-term carcinogenesis studies. Several large groups of male and female inbred hamsters are being treated intratracheally with BP-ferric oxide. These animals will be used for long-term incidence studies as well as for correlated studies on tumor cell differentiation and



culture properties. In vitro and combined in vivo/in vitro techniques are used for the study of BP metabolism in different segments of the respiratory tract.

(5) Combined effects of topical and systemic factors on respiratory carcinogenesis:

a) N-methyl-N-nitrosourea (MNU): A series of pilot studies was started to test whether various topically applied insults to the respiratory tract can enhance the respiratory carcinogenic effect of MNU given systemically. MNU alone was not found to induce respiratory tumors when injected systemically in hamsters, but it is a potent respiratory carcinogen by topical administration. Initial long-term studies, now nearing completion, include treatments with MNU given intratracheally combined with Fe<sub>2</sub>O<sub>3</sub> given intratracheally or with 3MI given systemically.

b) 3-Methylindole (3MI): Long-term studies have been undertaken on the effect of multiple concurrent treatments with BP given intratracheally and 3MI given intraperitoneally.

c) Combined studies on physical factors (microtrauma, saline and/or particulate instillation) with BP and/or MNU: Studies of cell injury and response have been undertaken with a variety of protocols, ranging from the simple microtrauma of intratracheal or intralaryngeal cannulation to the instillation of saline or of saline with Fe<sub>2</sub>O<sub>3</sub> particles, with or without the addition of carcinogens. The cellular response is evaluated by histochemical and electron microscopic methods and by determining the mitotic index of various cell types. Long-term experiments reproducing these combined treatment protocols are under way. This project is conducted in collaboration with the Department of Pathology, University of Maryland under intramural support contract #N01-CP-25605 (see below for contract report).

(6) [<sup>3</sup>H] Benzo[a]pyrene metabolism in vitro by tracheal and bronchial explants: Mechanical methods were developed for obtaining adequate quantities of bronchial tissue for incubation. Since defined media were used to maintain tracheal and bronchial explants in culture in the absence of serum, a suitable system for maintaining BP in solution in such media had to be devised. Incorporation of BP into phosphatidyl choline liposomes resulted in minimal losses over the 24 hr incubation period and was adopted. Preliminary results from the incubation of explants from untreated hamsters with 1.4 to 7 M of BP indicate a linear increase in ethylacetate-soluble metabolites and DNA binding with increasing BP concentration for both the trachea and bronchus. Tracheal explants incubated 20 hr with 5 M of BP, the concentration selected for further studies, were judged to be viable on the basis of the presence of beating cilia, the exclusion of trypan blue from the epithelium, and the ability of epithelial cells to grow out of the explants when cultured after the incubation. Control procedures were developed using tissues treated with ethanol prior to incubation.

(7) [<sup>3</sup>H] Benzo[a]pyrene binding in vivo to tissue macromolecules from the trachea, bronchi and lung: A protocol for BP administration similar to that used to produce respiratory tract tumors in hamsters, was adopted. This treatment required administration of >5x10<sup>8</sup> dpm/animal. Extraction procedures were worked out to reduce background and total binding was determined after dissolving exhaustively extracted samples with proteinase K. DNA was subsequently isolated by enzyme

digestion and ethanol precipitation and the binding to DNA was determined. Preliminary results from a study of BP binding as a function of time (0.5, 4, 10, 24 hr) after a single dose suggests that significant binding occurs only in the bronchus and that the highest levels are observed at four hours after a single treatment.

(8) Data reviews: Literature data and original results were analyzed and critically reviewed to establish a background for the design of new experimental plans. Reviews were completed on the effects of polycyclic aromatic hydrocarbons on experimental respiratory carcinogenesis, on factors involved in laryngeal carcinogenesis, on the pathology and carcinogenesis of nasal cavity, nasopharynx and upper respiratory tract tumors, on respiratory cancer pathogenesis and on the role of enhancing and promoting factors.

Significance to Biomedical Research and the Program of the Institute:

This project is addressed to the elucidation of the pathogenetic mechanisms of one of the major forms of human cancer; it is expected to contribute new knowledge on the conditions of concurrent or synergistic effects of different agents in respiratory cancer induction, a topic highly relevant to the understanding of human susceptibility to the multiple exposures that concur in lung cancer causation. This project is also expected to contribute basic knowledge to the poorly explored field of the mechanisms of epithelial carcinogenesis by providing an experimental pathology basis correlated with model studies on growth and neoplastic transformation of epithelial cells and on the molecular changes controlling their neoplastic transformation.

Proposed Course:

This project is designed to include long-term studies of several years' duration as well as shorter-term studies. It is closely interrelated to other projects in the laboratory on carcinogen metabolism and synergism, on epithelial cell culture and transformation, and on the identification of transforming genes in epithelial cells and in the experimentally induced tumors of the respiratory epithelia treated with different carcinogens.

Publications:

Reznik, G. and Stinson, S. F. (Eds.): Nasal Tumors in Animals and Man. Boca Raton, CRC Press, 1983, Vol. I, 274 pp.

Reznik, G. and Stinson, S. F. (Eds.): Nasal Tumors in Animals and Man. Boca Raton, CRC Press, 1983, Vol. II, 265 pp.

Reznik, G. and Stinson, S. F. (Eds.): Nasal Tumors in Animals and Man. Boca Raton, CRC Press, 1983, Vol. III, 264 pp.

Saffiotti, U.: Pathogenesis of bronchopulmonary cancer: Experimental models in vivo and in vitro. In Lung Cancer, Epidemiology and Prevention. Padua, Italy, University of Padua, 1983, pp. 5-35. (In Italian)

Saffiotti, U., Stinson, S. F., Keenan, K. P. and McDowell, E. M.: Tumor enhancement factors and mechanisms in the hamster respiratory tract carcinogenesis model. In Mass, M. (Ed.): Tumor Promotion and Enhancement in the Etiology of Human and Experimental Respiratory Tract Carcinogenesis. New York, Raven Press (In Press)

Stinson, S. F.: Nasal cavity cancer in laboratory animal bioassays of environmental compounds. In Reznik, G. and Stinson, S. F. (Eds.): Nasal Tumors in Animals and Man. Boca Raton, CRC Press, 1983, pp. 157-170.

Stinson, S. F. and Reznik, G.: Conclusions on comparative nasal carcinogenesis. In Reznik, G. and Stinson, S. F. (Eds.): Nasal Tumors in Animals and Man. Boca Raton, CRC Press, 1983, pp. 251-252.

Stinson, S. F. and Reznik, G.: Adenocarcinoma of the upper respiratory epithelium in the rat. In Jones, T. C., Mohr, U. and Hunt R. D. (Eds.): Pathology of Laboratory Animals. Washington, D.C., I.L.S.I. (In Press)

Stinson, S. F. and Reznik-Schuller, H. M.: Neoplasms of the mucosa of the ethmoid turbinates in the rat. In Jones, T. C., Mohr, U. and Hunt R. D. (Eds.): Pathology of Laboratory Animals. Washington, D.C., I.L.S.I. (In Press)

Stinson, S. F. and Saffiotti, U.: Experimental laryngeal carcinogenesis. In Ferlito, A. (Ed.): Cancer of the Larynx. Boca Raton, CRC Press (In Press)

## CONTRACT IN SUPPORT OF THIS PROJECT

UNIVERSITY OF MARYLAND (N01-CP-25605)Title: Hamster Respiratory Carcinogenesis Resource for In Vivo/In Vitro Correlation StudiesCurrent Annual Level: \$393,240Man Years: 6.9Objectives: To conduct in vivo respiratory carcinogenesis studies in the Syrian golden hamster and to provide in vitro techniques for the culture of respiratory epithelium to complement the in vivo studies.

Major Contribution: In vivo studies: Short-term studies on the effect of mechanical and chemical insults on the mitotic rates in various segments of the respiratory tract were completed. Hamsters were catheterized intralaryngeally or intratracheally to the level of the carina with a 19 gauge cannula. Simultaneously, other groups of hamsters were instilled with saline, or ferric oxide suspended in saline. Intratracheal cannulation, with or without the instillations, focally denuded the tracheal epithelium and markedly increased its mitotic rate, while intralaryngeal cannulation with or without instillations had no detectable effect on the trachea. Instillation of saline or ferric oxide-saline intratracheally or intralaryngeally increased the mitotic rate in the epithelia of the intrapulmonary bronchi and bronchioles. Most of the mitotic figures in the tracheal and bronchial epithelia were seen in secretory rather than basal cells. These findings demonstrate that physical or chemical trauma commonly associated with treatments in respiratory carcinogenesis studies can effect cell turnover throughout the respiratory tract and may influence the carcinogenic response. Long-term studies have been undertaken to elucidate the role of these physical factors in respiratory carcinogenesis. In vitro studies: Techniques for in vitro cultivation, transportation and long-term storage of respiratory cells and tissues were optimized. Cells and explants cultured for one week in CMRL 1066 with 1% fetal bovine serum, were placed on ice for the 1 to 2 hr transit from the University of Maryland to the LEP at FCRF; they survived well and continued their growth and replication at the LEP. The same was true of cells and explants frozen in liquid nitrogen prior to transport. Further, tracheal rings frozen and stored in liquid nitrogen for up to 60 days were shown to maintain their viability in culture.

The ability of different regions of the respiratory tract to activate diethylnitrosamine (DEN) was examined in vitro, determining the mutation frequency of Chinese hamster V79 cells co-cultured with Syrian hamster tracheal, bronchial or bronchiolar tissues in the presence of DEN. Evaluation of these pilot studies indicated that bronchial and bronchiolar cells may generate enough active metabolites of DEN to induce mutations in V79 cells, but the techniques require further refinement.

Proposed Course: The series of long-term in vivo respiratory carcinogenesis studies now under way will be completed and analyzed, to determine the effects

on carcinogenesis in different regions of the respiratory tract of a direct-acting carcinogen (N-methyl-N'-nitrosourea), of a carcinogen requiring metabolic activation (benzo[a]pyrene), and of physical microtrauma (cannulation, saline and ferric oxide instillations), alone and in different combinations. Tumor tissue from these studies will be used for various in vitro studies. In vitro techniques for studying carcinogen metabolism in different regions of the respiratory tract are being further refined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05275-03 LEP

PERIOD COVERED

October 1, 1983 through September 30, 1984

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

Arsenic Metabolism and Carcinogenesis Studies

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

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	F. Bertolero	Visiting Associate	LEP	NCI
	M. E. Kaighn	Expert	LEP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Respiratory Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.3

OTHER:

0.2

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.)

Studies are aimed at the development of in vitro and in vivo models for investigating the role of arsenic (As) and other metals in carcinogenesis, particularly in epithelial tissues. Defined cell culture models were selected to study metal compounds for their metabolism, toxicity, carcinogenic activity and possible interaction with other xenobiotics. Initial studies were conducted using the mouse embryo cell line, BALB/3T3 clone A31-1-1, and primary cultures of epidermal cells also obtained from BALB/c mice. Induction of neoplastic transformation by As was obtained in BALB/3T3 clone A31-1-1 cultures; trivalent As was found to have higher toxicity than pentavalent As; trivalent As induced transformation foci at concentrations of 10E-5 and 10E-5.5 M, while pentavalent As was negative.

The effects on mouse epidermal cells were studied after serum-free culture conditions were successfully optimized. In this system inorganic and direct-acting organic carcinogens were tested in a clonal assay to determine toxic dose-responses. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) showed a much lower effective toxic dose in this serum-free system than in equivalent serum-containing systems. The relative colony-forming efficiency was reduced to 25% by 10E-5 M trivalent As whereas pentavalent As did not inhibit colony-forming efficiency even at a ten-fold higher molarity. In the same epidermal cell system, hexavalent chromium (Cr) induced a high toxic response at doses from 3 x 10E-6 M to 3 x 10E-7 M whereas trivalent Cr was not toxic at doses as high as 10E-4 M. Transformation studies are under way and conditions will be defined for a qualitative and quantitative transformation assay for As and other metals in serum-free cultures of primary epidermal cells. The effects of As compounds on the respiratory tract epithelia will be investigated in the hamster model both in vivo and in vitro.

PROJECT DESCRIPTIONNames, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

U. Saffiotti	Chief	LEP	NCI
F. Bertolero	Visiting Associate	LEP	NCI
M. E. Kaighn	Expert	LEP	NCI

Objectives:

These studies are aimed at the development of in vitro and in vivo models for investigating the role of arsenic and other metals in carcinogenesis, particularly in epithelial tissues.

The study of metal carcinogenesis is important because of the ubiquity of the exposure and the persistence of metals in the environment. Environmental exposure to metals generally occurs concomitantly with other complex exposures to xenobiotics, therefore particular attention will be given to studies on the potential interactions of metals in combination with other carcinogens or cocarcinogens.

Conclusive epidemiological evidence exists for an increased risk for lung cancer and for non-melanotic skin cancer following exposure to inorganic arsenic (As). Animal experiments, however, have so far mainly resulted in negative findings. Two main hypotheses are investigated to explain this discrepancy: (a) species-specific metabolic differences between human subjects and laboratory rodents used so far in carcinogenesis tests (mostly rats and mice); and (b) requirements for combined exposures with other agents in a cocarcinogenic interaction. Recent results from a study in hamsters by Pershagen et al. (Environ. Res., in press) suggest a cocarcinogenic effect of As on the induction of adenomatous tumors in the hamster lung. Further in vivo carcinogenesis studies will be designed on the basis of metabolism and cellular studies. The increase in cancer risk observed in epidemiological occupational studies has been attributed mainly to the presence of inorganic trivalent As, whereas the pentavalent compound was generally regarded as less harmful; however, inorganic pentavalent As can be reduced in vivo to the more toxic trivalent form.

Defined cell culture models were selected to study As compounds for their metabolism, toxicity, carcinogenic activity and possible interaction with other xenobiotics. Initial studies were conducted using the mouse embryo cell line, BALB/3T3 clone A31-1-1, and primary cultures of epidermal cells also obtained from BALB/c mice. These mouse cell systems were selected because As metabolism in this species was reported to be qualitatively similar to that observed in humans. The BALB/3T3 clone A31-1-1 cell line was characterized for transformation studies in this Laboratory (see Project #Z01CE04491-08 LEP). A mouse epidermal primary serum-free cell culture method was recently developed in this laboratory (Bertolero et al., Exp. Cell Res., in press) (see Project #Z01CE05276-02 LEP). This system was selected for studies on As for the following reasons: (a) Primary keratinocytes grown in serum-containing media can be chemically transformed in vitro, as shown by S. H. Yuspa and coworkers in a previous project

from this Laboratory (see Project #Z01CE04504-09 LEP, 1980-81); (b) As and several other metals are accumulated in vivo in keratinizing tissues, and As is known to induce hyperkeratosis. (c) The induction of tumors by As in humans occurs in tissues that are capable of undergoing keratinization, i.e., epidermis and respiratory epithelium.

#### Methods Employed:

(1) Analytical methods. (a) separation of As binding sites and metabolites obtained in vivo and from tissue culture systems, by conventional preparative biochemistry techniques, i.e., tissue homogenization, differential ultracentrifugation, gel chromatography, gel electrophoresis and membrane filtration; (b) speciation of the diffusible metabolites, performed by two methods. Ion-exchange chromatography on AG50 resin allows the separation of trivalent and pentavalent inorganic arsenic from the monomethylated and dimethylated metabolites. HPLC separation on a C18 reverse-phase column improves speciation by permitting the separation of all four As metabolites with a single elution (Brinckman, F. E., et al., *J. Chromatogr.* 191: 31-46, 1980); and (c) detection and quantification of As metabolites by gamma counting, employing As-74- and As-73-labelled compounds in all metabolic studies.

(2) Cell culture methods. (a) The BALB/3T3 clone A31-1-1 mouse embryo cell line is used to study toxicity and neoplastic transformation according to methods currently used in this laboratory (see Project #Z01CE04491-08 LEP); (b) mouse epidermal keratinocytes are isolated from newborn BALB/c mice and are cultured directly into a serum-free medium supplemented with growth factors according to methods developed in this laboratory (see Project #Z01CE05276-02 LEP).

#### Major Findings:

(1) Induction of neoplastic transformation by As was obtained in BALB/3T3 clone A31-1-1 cultures; As<sup>+3</sup> was found to have higher toxicity than As<sup>+5</sup>; As<sup>+3</sup> induced transformed foci at concentrations of 10<sup>-5</sup> and 10<sup>-5.5</sup> M, while As<sup>+5</sup> was negative; the transformed foci were isolated and found positive in soft agar assays.

(2) Studies on primary cultures of mouse epidermal cells, to determine optimal culture conditions, showed that effective growth was successfully established for these cultures by replacing serum with seven defined factors in low calcium basal medium; epithelial cell identity was verified morphologically and histochemically. Inorganic and direct-acting organic carcinogens were tested in the clonal assay in mouse keratinocytes in order to measure a toxic dose-response. The direct-acting carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), showed a much lower effective toxic dose in this serum-free system compared to equivalent serum-containing systems. In this system, As<sup>+3</sup> was more toxic than As<sup>+5</sup>. The relative colony-forming efficiency (CFE) was reduced to 25% by 10<sup>-5</sup> M As<sup>+3</sup>, whereas As<sup>+5</sup> did not inhibit CFE or cell growth even at a ten-fold higher molarity. In the same cell system, Cr<sup>+6</sup> induced a high toxic response at doses from 3 x 10<sup>-7</sup> to 3 x 10<sup>-6</sup> M, whereas Cr<sup>+3</sup> was not toxic at doses as high as 10<sup>-4</sup> M. Transformation studies are under way.

#### Significance to Biomedical Research and the Program of the Institute:

Arsenic carcinogenesis has long been considered to be a unique example of a discrepancy between positive human findings and negative animal tests. The reasons



for this apparent divergence need to be found. The elucidation of arsenic mechanisms in carcinogenesis can provide a basis for the understanding of metal carcinogenesis in general and for risk evaluation and prevention. Positive results obtained in transformation studies on cells in culture are expected to provide a lead for investigating the mechanisms of As carcinogenesis.

Proposed Course:

(1) To define the conditions for a qualitative and quantitative transformation assay for As and other metals in serum-free cultures of primary mouse epidermal cells and in BALB/3T3 cells; (2) To determine the conditions responsible for the effect of As compounds on respiratory tract carcinogenesis in the hamster model, both in vivo and in cultures of hamster respiratory epithelia; and 3) To investigate the direct and/or cocarcinogenic mechanisms responsible for the transforming activity of As salts, and the possible gene changes associated with transformation by As.

Publications:

Saffiotti, U., Bignami, M., Bertolero, F., Cortesi, E., Ficorella, C. and Kaighn, M. E.: Studies on chemically induced neoplastic transformation and mutation in the BALB/3T3 Cl A31-1-1 cell line, in relation to the quantitative evaluation of carcinogens. Toxicol. Pathol. (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05276-03 LEP

## PERIOD COVERED

October 1, 1983 through September 30, 1984

## 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:	M. E. Kaighn	Expert	LEP	NCI
Others:	U. Saffiotti	Chief	LEP	NCI
	F. Bertolero	Visiting Associate	LEP	NCI
	R. T. Jones	Senior Scientist (IPA)	LEP	NCI
	M. I. Lerman	Visiting Scientist	LEP	NCI
	J. F. Lechner	Senior Staff Fellow	LHC	NCI

## COOPERATING UNITS (if any)

University of Texas Medical School, Houston, TX (D. A. Sirbasku); Cancer Metastasis and Treatment Laboratory, Litton Bionetics, Inc., Frederick, MD (J. M. Kozlowski).

## LAB/BRANCH

Laboratory of Experimental Pathology

## SECTION

Tissue Culture Section

## INSTITUTE AND LOCATION-

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

0.8

## PROFESSIONAL:

0.6

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

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

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

A serum-free medium (LEP-1) was developed for mouse epidermal keratinocytes, consisting of Eagle's MEM without calcium, with non-essential amino acids and with seven added supplements (hydrocortisone, 0.5  $\mu$ M; insulin, 5  $\mu$ g/ml; phosphoethanolamine and ethanolamine, 50  $\mu$ M each; transferrin, 5  $\mu$ g/ml; epidermal growth factor, 5 ng/ml; bovine pituitary extract, 180  $\mu$ g of protein/ml). The culture system was dependent for growth on bovine pituitary extract as the only remaining undefined supplement. LEP-1 supports sustained multiplication of mouse keratinocytes for 25 or more population doublings. A clonal growth assay was established and used to investigate the action of growth factors, hormones and other supplements on keratinocytes. When the calcium concentration of the medium was raised to 1.0 mM or when 1% FBS was added, the cells underwent terminal differentiation, confirmed by electron microscopy and by immunostaining with anti-keratin antibody. Serum factors were identified as having either stimulatory (albumin) or inhibitory activity (fetuin, crude platelet extract). This finding may explain why keratinocytes could not be carried beyond primary cultures in serum-supplemented media. This mouse keratinocyte culture system in serum-free medium is highly reproducible and is currently used in studies of chemically-induced transformation and gene activation.

It was found that the life span of a transformed but non-tumorigenic human prostatic epithelial line (NP-2S/T2) was extended by transfection of the EJ-ras oncogene and that the line containing the oncogene produces a transforming growth factor. In another study, the incidence of metastasis in nude mice of the prostatic adenocarcinoma cell line, PC-3, was increased by isolation of variant cell lines, use of a preferred inoculation site (spleen) and administration of beta-estradiol. The role of oncogenes will be studied in this system, using both the neoplastic and non-transformed human prostate cell lines.

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

M. E. Kaighn	Expert	LEP	NCI
U. Saffiotti	Chief	LEP	NCI
F. Bertolero	Visiting Associate	LEP	NCI
R. T. Jones	Senior Scientist (IPA)	LEP	NCI
M. I. Lerman	Visiting Scientist	LEP	NCI
J. F. Lechner	Senior Staff Fellow	LHC	NCI

(Note: This project includes continuation of research previously conducted under Project #Z01CP05278-02 LEP, now terminated).

Objectives:

The overall goal of this project is to identify mechanisms controlling the growth and differentiation of normal epithelial cells and to define the sequence of changes in these mechanisms produced by carcinogenic agents. Rodent and human epithelial cell systems are used in these studies, including mouse epidermal keratinocytes, human urothelium and human prostate epithelium. Hamster respiratory epithelium is studied in a parallel project (see Project #Z01CE05277-03 LEP).

Specific objectives include: (1) development of optimal culture methods for replicative growth and transformation of epithelial cells under experimentally defined conditions, particularly with serum-free media; (2) identification of exogenous factors that regulate growth and differentiation of normal epithelial cells; (3) identification of factors produced by cells that regulate their own growth (autocrine factors); (4) development of selective media for transformed cells; (5) development of optimal assays for the neoplastic transformation of epithelial cells in culture and determination of their susceptibility to different carcinogens singly or in combinations; (6) analysis of carcinogen-treated cells for changes in morphology, culture longevity, response to growth factors, alterations in enzymatic activities or structural proteins, karyotype, anchorage-independent growth, and tumorigenicity in athymic nude mice; (7) DNA-mediated transfer of transforming activity and tumorigenicity by DNA from neoplastic epithelial cells to the corresponding normal cells in culture; (8) identification of oncogenes in transformed cells; and (9) development of an assay for transforming growth factor production by established tumor cell lines and by carcinogen-treated cells.

Methods Employed:

The following methods have been developed:

(A) Mouse keratinocytes: BALB/c newborn mouse epidermal cells are isolated by cold trypsinization and separation of the epidermis from the dermis. The suspended epithelial cells are cultured in the medium, LEP-1, (see Major Findings) at high density ( $3.5 \times 10^6$  cells/60 mm dish) for 5-7 days, then trypsinized and frozen in liquid N<sub>2</sub>. Clonal growth is measured by determining the average

colony size after seven days of incubation. Frozen secondary cells are thawed, suspended in LEP-1 and plated in 60 mm dishes ( $4 \times 10^4$  viable cells/4 ml). After 24 hrs the medium is removed and experimental media are added.

The cultures are incubated for six days, then fixed to determine average colony size (10 colonies per dish, containing five or more cells each, are counted). The clonal growth rate is defined as population doublings/day (PD/d). Cultured cells are fixed and imbedded in situ for transmission electron microscopy (TEM) or on coverslips for scanning electron microscopy (SEM) and for immunofluorescence staining of keratin and vimentin.

For transformation experiments, the cells are exposed to graded concentrations of chemical carcinogens. Toxicity is determined by clonal survival assays. Various selection techniques are being investigated for the identification of transformed colonies, including switching from low to high  $\text{Ca}^{2+}$  concentrations in the medium or the addition of serum, to induce terminal differentiation in normal but not in transformed cells. The appearance of altered growth properties, ultrastructure (using both SEM and TEM) and karyological changes are studied as a function of time after application and of dose level. Growth in soft agar and tumorigenicity in nude mice will be used to demonstrate acquired neoplastic properties.

(B) Human urothelial cells: Human urothelial tissues (ureter and bladder) are obtained from surgical or autopsy specimens. The urothelium is dissected from the stroma and cut into 0.5 cm square pieces for explant cultures or separated from the stroma by the cold trypsin flotation method, as used for mouse keratinocytes. Cell suspensions or explants are cultured directly in serum-free medium (MCDB-152 with the  $\text{Ca}^{2+}$  level at 0.1 nM; EGF, 5 ng/ml; ethanolamine and phosphoethanolamine, 0.1 nM; bovine pituitary extract, 120-150  $\mu\text{g}/\text{ml}$  of protein). Cultures are characterized and used for transformation experiments as described for mouse keratinocytes.

(C) Human prostate epithelial cells: A normal human prostatic epithelial cell line (NP-2s) and a prostatic adenocarcinoma line (PC-3) are available as frozen stocks. Autocrine and transforming growth factors (TGFs) are isolated from serum-free medium conditioned by the cells. These factors are further purified by established biochemical methods (in collaboration with Dr. D. Sirbasku) and assayed for TGF activity in soft agar using NP-2s and NRK cells as indicators. The transforming activity of DNA isolated from PC-3 or other lines, e.g., carcinogen-treated NP-2s, are transfected into NP-2s and NIH/3T3 cells by the protoplast fusion technique (in collaboration with J. F. Lechner). Known oncogenes are also used for comparison. Putative oncogenes are isolated from PC-3 cells by established methodology (see Project #Z01CE05265-03 LEP). The metastatic activity of PC-3 cells is determined by inoculation of cells into nude mice (in collaboration with J. M. Kozlowski).

#### Major Findings:

(A) Mouse keratinocytes: Since attempts to culture mouse keratinocytes (MK) in serum-supplemented media were successful only in primary cultures, serum was replaced by a mixture of hormones and growth factors. A new medium formulation

(called LEP-1) was developed consisting of  $\text{Ca}^{2+}$ -free Eagle's MEM with nonessential amino acids and seven added factors (hydrocortisone,  $5 \times 10^{-7}$  M; insulin, 5  $\mu\text{g}/\text{ml}$ ; phosphoethanolamine (PEA) and ethanolamine, each  $5 \times 10^{-5}$  M; transferrin, 5  $\mu\text{g}/\text{ml}$ ; epidermal growth factor, 5  $\text{ng}/\text{ml}$ ; bovine pituitary extract (BPE), 180  $\mu\text{g}$  of protein/ml). This medium was found to have a  $\text{Ca}^{2+}$  concentration of 0.03 mM by atomic absorption. It supported at least 25 population doublings (PD) with an exponential growth rate of 0.8 PD/day. The colony-forming efficiency of secondary cultures, although low (0.3%), was highly reproducible. The clonal growth assay was used in dose response experiments to define optimal levels of each growth factor and to investigate the effects of whole serum and serum components on growth and differentiation. Addition of either 1.0 mM  $\text{Ca}^{2+}$  or 2.5% fetal bovine serum induced terminal differentiation. Experiments in which the individual supplements of LEP-1 were deleted singly showed that all (except PEA) stimulated growth, and that BPE was essential. The optimal concentration of each factor is that adopted in the formulation of the medium, LEP-1.

The serum-free medium, LEP-1, was used to investigate the control of growth and differentiation by serum factors. Addition of >2.5% whole fetal bovine serum (wFBS) or  $\text{Ca}^{2+}$  (>0.1 mM) induced terminal differentiation while inhibiting growth by more than 90%. Removal of  $\text{Ca}^{2+}$  from wFBS by a chelating resin reduced the inhibitory activity by approximately 50%. Fetuin inhibited growth even at the lowest tested level (50  $\mu\text{g}/\text{ml}$ ). Addition of a crude platelet extract (commercial PDGF) completely inhibited growth. In contrast, bovine serum albumin stimulated growth in a dose-dependent fashion. Maximal stimulation was three-fold at 500  $\mu\text{g}$  protein/ml and 1.6-fold at 50  $\mu\text{g}/\text{ml}$ , the lowest tested dose. This demonstration of inhibitory as well as stimulatory factors in FBS explains earlier problems in culturing MK cells with serum-containing medium. The morphology of primary and secondary cells in LEP-1 was studied by light and electron microscopy. They showed typical features of basal epidermal cells, polygonal in shape, small in size and with wide intercellular spaces. There was no evidence of terminal differentiation, such as cell enlargement or stratification. Normal epidermal cells are known to undergo terminal differentiation in conventional serum-supplemented media (1-2 mM  $\text{Ca}^{2+}$ ). This response was also observed when cells grown in media with low  $\text{Ca}^{2+}$  (with chelexed serum) are switched to a high  $\text{Ca}^{2+}$  level (Hennings, H., *Cell*, 19: 245-254, 1980). In the present serum-free LEP-1 medium, when the  $\text{Ca}^{2+}$  concentration was increased to 1.0 mM, the terminal differentiation response was also clearly observed. Within 48 hrs after the switch to high  $\text{Ca}^{2+}$  the cells became enlarged, tightly apposed, and began to stratify. After seven to 14 days in high  $\text{Ca}^{2+}$  LEP-1, the cells had completely differentiated and most had detached. When examined by SEM 48 hrs after the switch to high  $\text{Ca}^{2+}$ , the cells had become flattened, enlarged, and the spaces between them were reduced so that adjacent cells were in tight apposition. Microvilli persisted on the cell surface although pseudopodia had disappeared. Detaching terminally-differentiated cells had a wrinkled cell surface devoid of microvilli. As seen by TEM, the keratinocytes growing in low  $\text{Ca}^{2+}$  LEP-1 had numerous 10 nm intermediate filaments mainly localized in the perinuclear region. After the switch to high  $\text{Ca}^{2+}$ , the filament bundles were more abundant, thicker, and more highly oriented in differentiating cells, while the cells became multilayered and joined together by numerous desmosomes. Immunological staining with anti-keratin serum confirmed the epithelial nature of these cells and demonstrated the identity of the 10 nm filaments as keratin fibers, mainly localized in the perinuclear region. When switched to high calcium, the cells enlarged, flattened and became multilayered.

Diffuse keratin filaments radiated out from the nucleus with equal intensity into the margins of the cells so that no intercellular space could be recognized. No fluorescence was observed when the epidermal cells were stained with anti-vimentin serum, confirming their epithelial nature. Quantitative experiments with chemical carcinogens are underway to investigate the induction of altered colonies that are resistant to the differentiation stimulating activity of 1.0 mM  $\text{Ca}^{2+}$  and/or serum and to study their expression of neoplastic transformation. Exposure of keratinocytes to MNNG or sodium arsenite yielded calcium-resistant colonies in this serum-free system.

(B) Human urothelial cells: Dispersed primary cell cultures were obtained with two bladder specimens using the cold trypsin flotation technique employed for mouse keratinocytes. These cells underwent limited multiplication in serum-free medium. Several attempts to transform dispersed cultures and explants derived from bladder were unsuccessful to date, possibly because of the age of the tissue donors (60 yrs or older). A more suitable source of tissue is being sought.

(C) Human prostatic epithelial cells: (1) The difficulty in transforming normal human cells in culture is well known. This problem has been approached using normal prostatic epithelial cells as a model in collaboration with Dr. John F. Lechner, (see Project #Z01CE05130-04 LHC). A line of normal human prostatic epithelial cells (NP-2s) had previously been transformed by SV-40 (NP-2s/T2). Although this line had altered properties, including aneuploidy, anchorage independence, reduced serum requirement for clonal growth, and increased culture longevity, it has never acquired the capacity for either unlimited growth or tumorigenicity in athymic nude mice. Thirty-five population doublings post-SV-40 virus infection, NP-2s/T2 cells were either infected with Kirsten sarcoma virus or transfected via protoplast fusion with a derivative of plasmid pSV2gpt<sup>+</sup> carrying the EJ-ras oncogene. The control and both experimental cultures became quiescent within ten days of initiating the experiment. However, after 20 and 30 additional days of incubation, colonies of small mitotic cells appeared in both the Kirsten virus-infected and EJ-ras-transfected cultures, respectively; no colonies developed in the control cultures after 60 days of incubation. Both the Kirsten virus-infected and EJ-ras-transfected cells have continued to multiply (>15 population doublings to date) and have the ras oncogene incorporated into their nuclear DNA. In addition, both of these ras-oncogene-containing cultures elaborate a transforming growth factor demonstrated by the induction of growth in soft agar of normal rat kidney (NRK) cells. Experiments are in progress to determine whether other changes in growth behavior have occurred as a result of acquiring a ras oncogene and to assess whether either of these ras-oncogene-containing cultures has become tumorigenic. These findings have underscored the role of oncogenes in extending the life span of prostatic epithelial cells and may provide a means to analyze the stages leading to tumorigenicity. For this reason, a new line of research is developing, within this LEP project, to identify a transforming gene(s) in the prostate cell system (see Proposed Course). (2) The metastatic capability of the prostatic adenocarcinoma line (PC-3) in nude mice has been investigated in collaboration with Dr. James M. Kozlowski. The incidence of metastasis was increased by the isolation of variant sublines from secondary tumor deposits, by the use of an advantageous inoculation site and by prolonged administration of  $\beta$ -estradiol to suppress natural killer cell activity. Intra-splenic injection resulted in the most dramatic expression of metastasis, giving large and frequent metastases to the liver and lungs as cell as to mesenteric,

omental and mediastinal lymph nodes. For example, a PC-3 variant (PC-3-M) isolated from a lung metastasis produced 13 times the incidence of pulmonary metastasis as did the parental line after i.v. injection. Direct intrasplenic injection of PC-3 gave an incidence of metastasis of 16/20 in the lung, 20/20 in the liver and 13/20 in the lymph nodes. The basis of this enhancement of metastatic capability in variant lines is unknown. The fact that alternate metastatic phenotypes can be selected may be correlated with different responses to growth factors, substrates and even activated or amplified oncogenes.

#### Significance to Biomedical Research and the Program of the Institute:

Since epithelial cells give rise to the vast majority of human cancers, an understanding of their mechanisms of carcinogenesis is therefore of critical importance. Both bladder and prostatic cancers are important forms of human cancer. The normal human bladder and prostatic epithelial culture systems, that have been developed, offer an opportunity to investigate etiologic factors and mechanisms of carcinogenesis in these human epithelial cell types. The mouse keratinocyte culture system is a counterpart to the well-established in vivo model of carcinogenesis, and offers the opportunity of analyzing the action of carcinogens in epithelial cells that exhibit terminal differentiation responses of general significance.

#### Proposed Course:

Following the development of selected culture conditions for human and rodent epithelial cell systems, attention is now primarily directed toward the investigation of sequential changes produced by carcinogenic treatments. In the mouse keratinocyte system, optimal conditions for initial transformation and progression to neoplasia will be investigated using treatments with chemical carcinogens, promoters and known oncogenes, singly and in combinations. In an effort to identify stages in the neoplastic process, alterations in cellular responses to growth factors will be assessed, and used as markers to develop selective media for carcinogen- or oncogene-altered cells. For example, since mouse keratinocytes require BPE for growth in LEP-1, it will be determined whether BPE-independent lines will develop after treatment with carcinogens. Since the nature of the growth-promoting activity in BPE is unknown, an effort is under way to identify the factor(s) involved.

A new line of investigation is being developed in an effort to identify the stages required to transform human diploid prostatic epithelial cells. Normal prostatic epithelial cells will be treated with chemical carcinogens and/or selected oncogenes. Changes in culture life span and response to growth factors will be monitored. An attempt will be made to transform the normal cells (NP-2s) by transfection of DNA from the prostatic carcinoma cells (PC-3). NIH/3T3 cells will also be transfected for comparison. The presence and identity of known or possibly new oncogenes in PC-3 cells will be investigated. Because PC-3 cell growth is population-dependent in defined medium (Kaighn, M. E., et al., Proc. Natl. Acad. Sci. USA 78: 5673-5676, 1981), it produces an autocrine factor. This factor(s) will be isolated in collaboration with Dr. David Sirbasku. Since the PC-3 cell line is anchorage independent, growth in soft agarose will be used to determine whether this line produces another transforming growth factor, using NRK and NP-2s cells as targets.

Publications:

Bertolero, F., Kaighn, M. E. and Saffiotti, U.: Mouse epidermal keratinocytes: Clonal proliferation and response to hormones and growth factors in serum-free medium. Exp. Cell Res. (In Press)

Kozlowski, J. M., Fidler, I. J., Campbell, D., Xu, Z., Kaighn, M. E. and Hart, I. R.: Metastatic behavior of human tumor cell lines grown in the nude mouse. Cancer Res. (In Press)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05277-03 LEP

PERIOD COVERED

October 1, 1983 through September 30, 1984

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

Hamster Respiratory Epithelial Culture Carcinogenesis Model

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

PI:	M. E. Kaighn	Expert	LEP	NCI
Others:	R. T. Jones	Senior Scientist (IPA)	LEP	NCI
	F. Bertolero	Visiting Associate	LEP	NCI
	U. Saffiotti	Chief	LEP	NCI
	S. F. Stinson	Biologist	LEP	NCI

COOPERATING UNITS (If any)

Department of Pathology, University of Maryland Medical School, Baltimore, MD  
 (R. T. Jones)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Culture Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:

1.8

PROFESSIONAL:

1.4

OTHER:

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.)

The Syrian golden hamster is the classic animal model for the in vivo study of respiratory carcinogenesis. Explant cultures of respiratory epithelia have been used in the past to study in vitro carcinogenesis, usually with serum-containing media. Because of the advantages of using serum-free media, the efficiency of various formulations for the explant culture of hamster respiratory epithelia was explored. The following media were tested: (1) Ham's F-12, (2) CMRL-1066, (3) Eagle's MEM, (4) MCDB 152, (5) Eagle's MEM without calcium with nonessential amino acids, (6) CMRL-1066 and Eagle's MEM without calcium with nonessential amino acids (1:1), and (7) Eagle's MEM with citrulline substituted for arginine. Each medium was tested either with 1% heat inactivated fetal bovine serum or with seven growth factors (insulin, hydrocortisone, ethanolamine, phosphoethanolamine, transferrin, epidermal growth factor, and bovine pituitary extract). The addition of serum resulted in a massive overgrowth of fibroblasts. Serum-free Ham's F-12 and CMRL-1066 supported the outgrowth of differentiated epithelial cells and retarded the growth of fibroblasts. In the case of tracheal epithelial cell suspensions isolated by protease digestion, F-12 supplemented with the above seven factors supported both growth and subculture. Coating of Petri dishes with a mixture of collagen, fibronectin and bovine serum albumin enhanced attachment of the isolated tracheal cells. Studies are under way to characterize these cells by morphological and biochemical techniques and to use them in chemically-induced transformation studies.

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

M. E. Kaighn	Expert	LEP	NCI
R. T. Jones	Senior Scientist (IPA)	LEP	NCI
F. Bertolero	Visiting Associate	LEP	NCI
U. Saffiotti	Chief	LEP	NCI
S. F. Stinson	Biologist	LEP	NCI

Objectives:

The main objective of this project is to develop optimal culture systems for hamster respiratory epithelia and then to investigate the mechanisms responsible for growth control, differentiation and neoplastic transformation in these cells. The cellular responses in vitro will be compared with their known counterparts in vivo, with emphasis on the segmental differences in susceptibility to carcinogens which induce tumor types with different degrees of malignancy (e.g., papilloma versus carcinoma) and differentiation (e.g., epidermoid, glandular, anaplastic).

The following specific goals are pursued: (1) optimization of a serum-free medium to support the replicative culture of respiratory tract epithelium from inbred Syrian hamsters, (2) determination of the control of growth and differentiation of normal respiratory epithelial cells by growth factors, (3) development of optimal conditions for chemically-induced transformation, (4) investigation of the effect of in vivo pretreatment with various carcinogens on both explant and cell cultures, and (5) comparison of the response of epithelial cells derived from different segments of the laryngo-tracheo-bronchial tract to carcinogens.

Methods Employed:

(1) Explant Culture - Segments of the tracheo-bronchial tract from inbred 15:16/EHS:CR Syrian hamsters are cut into rings while in cold L-15 medium and then covered with 2 ml of test medium. Media are changed 3 times a week, rings are transferred to new dishes at 7-day intervals and the residual outgrowths are fixed and stained. The following media were tested: (a) Ham's F-12, (b) CMRL-1066, (c) Eagle's MEM, (d) MCDB 152, (e) Eagle's MEM without calcium and with nonessential amino acids, (f) CMRL-1066 and Eagle's MEM without calcium with nonessential amino acids (1:1), and (g) Eagle's MEM with citrulline substituted for arginine. Each medium was tested either with 1% fetal bovine serum or with seven growth factors (bovine insulin, hydrocortisone, ethanolamine, phosphoethanolamine, human transferrin, epidermal growth factor, and bovine pituitary extract).

(2) Cell Dissociation and Culture - Tracheas are dissected from adult Syrian hamsters and are cannulated with PE 160 tubing. The lumens are then flushed with phosphate buffered saline (PBS), filled with protease solution, sealed and incubated in L-15 medium at 4°C for 24 hours. Following incubation, the lumens are flushed with medium and the enzymatically dissociated cells are collected

by centrifugation, resuspended in test media and plated on fibronectin coated plastic dishes at  $10^4$  cells/cm<sup>2</sup>.

(3) Identification of Cell Types - Explant cultures as well as isolated cells and cell cultures are examined daily by phase-contrast microscopy. For transmission electron microscopy, they are fixed in a phosphate-buffered solution containing 4% formaldehyde and 1% glutaraldehyde, then postfixed for 30 minutes in cold 1.33% osmium tetroxide buffered with *s*-collidine (pH 7.4), dehydrated in a graded series of ethanol and infiltrated with 1:3, 1:1, and 3:1 mixtures of Epon-hydroxypropyl-methacrylate (30 minutes each). Thick (1  $\mu$ m) sections are examined by light microscopy; areas are selected for thin-sectioning. Thin (600 Å) sections are placed on copper mesh grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 100CX transmission electron microscope.

Special stains are used on cytocentrifuge preparations to identify products of the cultured cells: the stains are alcian blue-periodic acid Schiff (PAS) for acidic and neutral mucopolysaccharides (mucus) and glycogen, orange G-alcian blue for prekeratin and keratin, and oil red O for lipids.

The triple-layer peroxidase-antiperoxidase (PAP) method is used to detect keratin. Cells are cultured on glass coverslips, rinsed with PBS, fixed with cold acetone, and dry-stored at -70°C. The coverslips are stained by the PAP method using antibodies raised in rabbits against prekeratin proteins isolated from the stratum corneum. To detect keratin, the specimens are reacted with the primary rabbit antibody, then with goat anti-rabbit IgG, and finally with rabbit PAP. Then 3,3'-diaminobenzidine tetrachloride (DAB, 0.6 mg/ml) containing 1  $\mu$ l/ml of 30% hydrogen peroxide in PBS (pH 7.6) is added to the specimen for 8 minutes; oxidation of DAB by horseradish peroxidase reveals keratin as a brown staining reaction.

(4) Experimental Treatments - Experimental variables include the media and supplements (hormones, growth factors) indicated above. In addition, treatments include the carcinogens N-methyl-N'-nitro-N-nitrosoguanidine and benzo[a]pyrene, for studies on metabolism, binding and transformation. DNA is extracted from normal and transformed cells for molecular biology studies (see Project #Z01CE05265-03 LEP).

#### Major Findings:

Initial studies using explant cultures of tracheal rings were undertaken to select the most suitable media formulation that would support a selective outgrowth of epithelial versus mesenchymal cells and a sustained growth of the explant as measured by the size of the outgrowth. Outgrowth of fibroblasts was first constrained by reducing from 10% to 1% the amount of serum supplement added to the various basal media. The problem of fibroblastic overgrowth was eliminated when seven growth supplements were used to replace serum, essentially following a similar approach as that successfully developed for mouse keratinocyte cultures (see Project #Z01CE05276-02 LEP). Ham's F12 medium with seven factors (insulin, hydrocortisone, ethanolamine, phosphoethanolamine, epidermal growth factor, transferrin and bovine pituitary extract) yielded the best results.

In subsequent studies methods were developed for hamster tracheal epithelial cell culture. These cells were effectively dissociated by digestion with purified bacterial protease, yielding  $5 \times 10^6$  dispersed cells/trachea. Although these cells do attach to untreated plastic dishes, fibronectin-collagen coating of the dishes enhances cell attachment. Under these conditions 50% of the cells have attached by 24 hrs. Primary cultures proliferate to confluence and monolayers can be maintained for up to 4-5 weeks. Alternatively, the cells can be passed up to three times. Cells have also been successfully cryopreserved. Histochemical, electron microscopy and immunohistochemical studies have demonstrated the epithelial nature of the cells when isolated and also in primary culture. Carcinogen-treated cultures are now under study.

#### Significance to Biomedical Research and the Program of the Institute:

Lung cancer, a leading cause of death, continues to increase in incidence. The hamster respiratory carcinogenesis model, which reflects the histopathology of human lung cancer development, is used as a source of tissues for these studies. Establishment of an organ culture system, which preserves tissue relationships and the development of replicative epithelial cell cultures, complete the battery of correlated biological models. Experimental questions can now be addressed to progressive levels of biological organization in the same animal cell system. This approach fosters a more comprehensive investigation of carcinogenic mechanisms in the respiratory tract and provides a relevant model for human lung cancer pathogenesis.

#### Proposed Course:

Now that suitable conditions for the selective dissociation of hamster respiratory epithelium and its serum-free replicative culture have been developed, this system will be used for several studies. Efforts will be made to separate different specific cell types known to be present in the respiratory epithelium. Carcinogenesis experiments employing different types of carcinogens will be pursued. Changes in growth factor response and differentiation of the carcinogen-treated cells will be investigated. Comparisons will be made between tracheal and bronchial epithelial cells in their in vitro response to carcinogens that are known to produce segment-specific responses in the respiratory epithelia in vivo.

#### Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05381-01 LEP

## PERIOD COVERED

March 4, 1984 through September 30, 1984

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

Cellular/Molecular Stages of Carcinogenesis in Respiratory Epithelia

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

PI:	D. G. Thomassen	Senior Staff Fellow	LEP	NCI
Others:	U. Saffiotti	Chief	LEP	NCI
	M. E. Kaighn	Expert	LEP	NCI
	M. I. Lerman	Visiting Scientist	LEP	NCI

## COOPERATING UNITS (if any)

Laboratory of Pulmonary Function and Toxicology, NIEHS, NIH, Research Triangle Park, NC (P. Nettesheim, J. C. Barrett, T. Gilmer)

## LAB/BRANCH

Laboratory of Experimental Pathology

## SECTION

Tissue Culture Section

## INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

## TOTAL MAN-YEARS:

0.8

## PROFESSIONAL:

0.7

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

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

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

The objective of this project is to identify and characterize changes at the cellular and molecular levels responsible for the development of preneoplastic and neoplastic variants of rat tracheal epithelial (RTE) cells. To identify cells having critical preneoplastic changes, comparative transformation experiments are described in which independent populations of preneoplastic RTE cells are treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or with cloned oncogene-containing DNAs from Harvey murine sarcoma virus (HaMSV, ras), Rous sarcoma virus (RSV, src), polyoma virus, and MC29 virus (myc). Differential responses of the various cell lines to these treatments will be used as evidence of differences in their preneoplastic potential. Using the hypothesis that differential responses of "preneoplastic" cell lines to carcinogens or oncogenic DNAs have a genetic basis, efforts will be directed towards the molecular cloning of the genes responsible for the conversion of preneoplastic to neoplastic cells. These studies require the development of assays suitable for the detection of these genes. The use of preneoplastic RTE cells for identification of RTE cell tumor genes is described. This assay for tumor genes will provide an intraspecific system in which the genes will be identified in cells that are genetically related to the cells in which the gene was originally active. Finally, strategies are planned for cloning RTE cell tumor genes.

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

D. G. Thomassen	Senior Staff Fellow	LEP NCI
U. Saffiotti	Chief	LEP NCI
M. E. Kaighn	Expert	LEP NCI
M. I. Lerman	Visiting Scientist	LEP NCI

Objectives

This is a new project, initiated in April, 1984. The specific short-term objectives are: (1) comparison of preneoplastic rat tracheal epithelial (RTE) cell lines, which differ in their ability to progress to neoplasia, for the inducibility of neoplastic potential by treatment with the carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or by transfection with oncogenic DNAs; (2) to develop an assay using preneoplastic RTE cells to detect genes transferring the tumorigenicity phenotype; (3) to transfer the tumorigenicity phenotype by transfection using DNA from RTE tumor cells; and (4) to begin cloning a gene from an RTE tumor cell which encodes the tumorigenicity phenotype. The long-term objective of this project is to provide evidence on multistage carcinogenesis by identifying critical preneoplastic intermediates using the RTE cell system.

Methods Employed:

Neoplastic RTE cells will be identified using the athymic nude mouse tumor assay. BALB/c AnNCr nude mice will be injected subcutaneously with cell suspensions and monitored for tumor formation. Treatment of RTE cells with MNNG will be done using a 4-hour treatment in HEPEs buffered F12 medium without serum. DNA transfections using cloned oncogenes or high molecular weight DNAs will be done using the calcium-phosphate DNA precipitation technique. Clones of oncogene-transfected RTE cells will be isolated following cotransfection of oncogene and pSV2-neo DNA and selecting for antibiotic G418-resistant clones. Carcinogen- or DNA-treated cells will be assayed in nude mice for the induction of neoplastic potential. Molecular analyses of oncogene-transfected RTE cells will be done using DNA and RNA isolation, RNA dot blotting, enzyme restriction of DNA, separation of DNA on agarose gels, Southern blotting, and Western blotting for some oncogene-encoded proteins. Techniques to be used for molecular cloning include those listed above in addition to separation of DNA fragments on sucrose gradients and construction and screening of partial genomic libraries.

Major Findings:

This project is just being initiated and, therefore, there are no major findings at this time.

Significance to Biomedical Research and the Program of the Institute:

Epithelial cells, especially respiratory epithelial cells, represent a major target for carcinogens and for the development of neoplasia in vivo. The

development of the rat tracheal epithelial cell culture system provides the opportunity to: (a) simultaneously study normal, preneoplastic, and neoplastic cells; (b) determine the effects of chemical carcinogens; (c) examine the role of oncogenes; and (d) identify and isolate genes involved in the development of neoplasia. The opportunity to study all stages of neoplastic development in one cell type at both the cellular and molecular levels should provide new insights into the mechanisms of carcinogenesis in a major epithelial tissue.

#### Proposed Course:

Although the development of neoplasia is assumed to be a progressive multistage process, little is known about the nature of specific cellular changes involved or the number of stages which may be required for the development of neoplasia. Certain changes in normal cells have been described as "preneoplastic" in both *in vivo* and culture systems. However, it is difficult to assess which changes are essential for the development of neoplasia. It should be possible to identify critical intermediates by using a cell culture system.

Clonal lines of different presumed preneoplastic RTE cells will be isolated and the relative probabilities of each of these progressing to neoplasia will be determined. By repeating this process with different clonally related cell lines, cellular phenotypes essential to the development of neoplasia can be identified. RTE cell lines having different neoplasia-associated phenotypes should identify a set of changes "essential" for the progression of these cells to neoplasia.

When cellular changes essential to the development of neoplasia have been identified and characterized phenotypically, it will become important to characterize them at the genetic level by isolating and characterizing the specific DNA sequences which encode information for these changes. This type of analysis is similar to that outlined in LEP Project #Z01CE05265-03 which describes the use of DNA-mediated gene transfer (transfection) to identify cellular transforming genes. This approach will be used to analyze sequences (genes) which have been shown to be involved in all identifiable stages of progression to neoplasia. This analysis also requires that suitable assays for the detection of the various genes be developed. Ideally, detection systems for "transforming genes" should utilize target cells and cells which serve as DNA donors from the same or closely related genetic backgrounds. In addition, this method, in principle, permits the identification and isolation of genes involved in the conversion of both preneoplastic to neoplastic cells and also normal to preneoplastic cells. Efforts will be made to develop detection systems for both early and late stages of progression to neoplasia. In order to facilitate comparison of our results with those of others using heterologous target cells, NIH/3T3 cells will also be used to identify transforming genes.

To accommodate the widest range of studies on the development of neoplasia, cellular systems should be chosen: (a) which utilize cells from tissues that are targets for carcinogens; (b) in which preneoplastic and neoplastic variants can be induced by carcinogens, identified, and isolated; and (c) in which normal cells are readily available for treatment with carcinogens, for biochemical and molecular analyses, and for comparison with preneoplastic and neoplastic variants.

There are currently three cell culture models available in LEP in which such studies can be performed or which will soon become available. An RTE cell culture model developed by D. G. Thomassen et al. at the NIEHS (Thomassen, D. G., et al., Cancer Res., 43: 5956-5963, 1983) is now in use. Primary RTE cells can be isolated, propagated in culture, and treated with carcinogens to induce preneoplastic variants which progress to neoplasia using established methodology. The studies described in this project description will initially be performed using the RTE cell model. In addition, a reproducible procedure for the replication of mouse keratinocytes (see Project #Z01CE05276-03 LEP) has recently been developed (Bertolero, F., et al., Exp. Cell Res., in press). Development of a similar system with the Syrian hamster trachea (see Project #Z01CE05277-03 LEP) is nearing completion. Both of these new culture models will be employed in collaborative carcinogenesis studies.

Publications:

None



ANNUAL REPORT OF  
THE LABORATORY OF HUMAN CARCINOGENESIS  
NATIONAL CANCER INSTITUTE

October 1, 1983 to September 30, 1984

The Laboratory of Human Carcinogenesis conducts investigations to assess (1) mechanisms of carcinogenesis in epithelial cells from humans and experimental animals, (2) experimental approaches in biological systems for the extrapolation of carcinogenesis data and mechanisms from experimental animals to the human situation, and (3) host factors that determine differences in carcinogenic susceptibility among individuals.

The scientific and managerial strategy of the Laboratory is reflected in its organization into three sections, i.e., In Vitro Carcinogenesis Section (IVCS), Carcinogen Macromolecular Interaction Section (CMIS), and Biochemical Epidemiology Section (BES). Scientifically, the emphasis is on the role of inherited or acquired host factors as important determinants in an individual's susceptibility to environmental or endogenous carcinogens and cocarcinogens. Our investigations of host factors involve interspecies studies among experimental animals and humans, cover the spectrum of biological organization ranging from molecules to the intact human organism, and are multidisciplinary, including molecular and cellular biology, pathology, epidemiology, and clinical investigations. Two sections (IVCS and CMIS) devote their major efforts to more fundamental and mechanistic studies. The scientific findings, techniques, and concepts developed by these two sections and, of course, the scientific community at large, are utilized by the BES in selected and more applied studies of carcinogenesis and cancer prevention. The laboratory-epidemiology studies in this section require the expertise found in the IVCS and CMIS and in the NCI Epidemiology Program. Resources needed by the Laboratory are unique and complex. For example, collection of viable normal as well as neoplastic epithelial tissues and cells--well characterized by morphological and biochemical methods from donors with an epidemiological profile--requires the continued cooperation among donors and their families, primary care physicians (internists, surgeons, house staff), surgical pathologists, nurses, epidemiologists, and laboratory scientists.

#### CULTURE OF HUMAN TISSUES AND CELLS

Remarkable progress has been made during the last few years by this and other laboratories in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. We have developed chemically defined media for long-term culture of human bronchus, colon, esophagus, and pancreatic duct. Primary cell cultures of human epithelial outgrowths have been obtained from many different types of human tissues. Isolated epithelial cells from human bronchus and esophagus can be transferred three or more times and can undergo more than 30 cell divisions. Human bronchial and esophageal epithelial cells can also be grown in serum-free culture medium. Morphological, biochemical, and immunological cell markers have been used to identify these cells as unequivocally of epithelial origin. Clonal growth of normal human pleural mesothelial cells in a low serum culture medium has also

been achieved so that the *in vitro* transformation by asbestos of these cells can be studied.

The availability of nontumorous epithelial tissues and cells that can be maintained in a controlled experimental setting offers an opportunity for the study of many important problems in biomedical research, including carcinogenesis. For example, the response of human bronchial epithelial cells (enhanced growth or differentiation) after either exposure to carcinogens and/or tumor promoters or DNA transfection by oncogenes is being actively investigated. Parallel investigations using epithelial tissues and cells from experimental animals allow investigators to study interspecies differences in response to carcinogens, cocarcinogens, and anticarcinogens.

#### CELLULAR GROWTH AND DIFFERENTIATION

Our operational definitions of normal, premalignant, and malignant cells are biological, e.g., differentiated state, growth, altered cellular affinities and architecture, and tumorigenicity when injected into the appropriate host. Methods for the culture of human epithelial tissues and cells provide an opportunity to investigate the biology and molecular mechanisms of carcinogenesis directly in human target cells and to conduct studies comparing carcinogenesis in cells from experimental animals and humans.

We have focused our primary attention on two sites of human cancer, i.e., bronchus and esophagus. As noted above, our initial effort was devoted to developing methods to culture and unequivocally identify human epithelial cells. We are now studying the factors controlling growth and differentiation of these normal cells and their malignant counterparts; the ability to culture these normal and malignant cells in chemically defined media is essential for such studies.

Terminal squamous differentiation in the normal bronchial epithelial cells can be induced by blood-derived serum, platelet lysates, suspension in semisolid medium, confluence culture conditions or calcium ions ( $> 1 \text{ mM}$ ) and small amounts of serum. However, these inducers of differentiation do not have the same effects in either carcinoma cells or, as to be described later, oncogene-transformed cells which continue to grow and, in some cases, grow at a faster rate. These observations are consistent with the hypothesis that preneoplastic and neoplastic cells are resistant to endogenous and exogenous inducers of terminal differentiation and thus have a selective survival-growth advantage. We are currently identifying these inducers of differentiation of normal human bronchial epithelial (NHBE) cells and studying their mechanisms of action.

For example, interleukin I at low concentrations induces growth of NHBE cells and at high concentrations induces terminal squamous differentiation. Interleukin I can be detected in NHBE cells by immunocytochemistry. We propose that interleukin I is both an autogenous growth factor and a squamous differentiation-inducing factor. This putative autogenous growth factor is detected by measuring growth rate as a function of cell density. This factor is found in NHBE cell conditioned medium. NHBE cell conditioned medium will increase the growth rate of NHBE cells at clonal density incubated in LHC-9 medium without epinephrine. In addition, the conditioned medium stimulates multiplication of normal human bronchial fibroblasts and mouse lymphocytes, under assay conditions that are used to measure interleukin I. At high cell density, i.e., near

confluence, the NHBE cells undergo squamous differentiation and high calcium ion concentration increases the rate. Whereas optimal growth occurred at clonal densities in medium containing 1 mM  $\text{Ca}^{2+}$ , rapid squamous terminal differentiation occurred when the medium of dividing high-density cultures was changed from 0.1 to 1 mM  $\text{Ca}^{2+}$ . These observations suggest that the  $\text{Ca}^{2+}$  concentration influences the activity of an autocrine squamous differentiation-inducing factor. This autocrine differentiation-inducing factor may be interleukin I, because interleukin I is thought to be a calcium ionophore, and excess exogenous interleukin I induces NHBE cells to undergo squamous differentiation.

Because human bronchial carcinomas and fetal NHBE cells frequently produce polypeptide hormones, e.g., alpha and beta human chorionic gonadotropin (HCG) and gastrin-releasing peptide, we have proposed that these polypeptides may have normal growth promoting functions during fetal development and the regulation of these autocrine growth factors is aberrant in carcinoma cells. Gastrin-releasing peptide (GRP) and its amphibian equivalent, bombesin, stimulate clonal growth of NHBE cells. Although neither alpha nor beta HCG alone is a growth factor, the combination is growth stimulatory, which is consistent with the hypothesis that beta HCG binding to its membrane receptor allows access of alpha HCG to its receptor to trigger the subsequent mitogenic stimulus. These observations suggest that studying the regulation of the genes of these polypeptide hormones in fetal versus adult NHBE cells and in bronchial carcinomas may reveal the molecular mechanism for their control and their possible role in carcinogenesis.

Blood-derived serum (BDS) contains both mitogenic and differentiation-inducing factors. Supplementation of the culture medium, LHC-9, with as little as 0.25% fetal bovine BDS results in a decrease in clonal growth rate of NHBE cells; 8% supplementation completely inhibits growth by inducing terminal squamous cell differentiation. Human lung carcinoma lines were also incubated in LHC-9 medium without and with 8% BDS. The results showed that serum toxicity per se is not responsible for the observed inhibition of NHBE cell growth; all 10 carcinoma lines divided significantly more rapidly ( $p < 0.05$ ) in BDS-supplemented medium. Thus, the carcinoma cells have both increased requirements for BDS mitogens and a greatly reduced ability to respond to factors in BDS that induce the normal cells to undergo squamous differentiation. Since cell planar areas and opposition index increase in direct proportion to the concentration of BDS, these characteristics can be used as a quantitative assay for squamous differentiation-inducing activity. Immunoperoxidase staining for involucrin clearly revealed that NHBE cells exposed to BDS are arranged in a multilayered fashion. The overlying cells are large and strongly involucrin positive, whereas the basal cell sheets are involucrin negative. The serum factor that induces squamous differentiation was found by gel filtration to be approximately 50,000 daltons. In addition, the factor is resistant to beta mercaptoethanol, stable at pH 8.0, rather labile at pH 5.0, and soluble when compared with media containing BDS, in low concentrations of salt. There was significantly less inhibition of NHBE cell growth with plasma-derived serum. On the other hand, platelet factors at concentrations that stimulate fibroblastic cell multiplication also inhibit DNA synthesis and stimulate terminal differentiation of NHBE cells.

The information from the above-mentioned studies is being used in the design of in vitro carcinogenesis experiments in which these inducers of terminal differentiation are being used in a strategy to provide selective advantage of preneoplastic and neoplastic cells.

## CARCINOGENESIS STUDIES

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 will be discussed in a latter section, metabolism of carcinogens, DNA damage, and DNA repair are considered to be important factors in these stages of carcinogenesis.

### Tumor Promotion

Selective clonal expansion of preneoplastic ("initiated") cells is a basic tenet of tumor promotion. Examples of mechanisms that could lead to selective clonal expansion of "initiated" cells compared with normal cells include (a) resistance to either exogenous or endogenous inducers of terminal differentiation, (b) resistance of the preneoplastic and neoplastic cells to cytotoxic products of integrated viral genes, (c) enhanced expression in the preneoplastic cells of either cellular or integrated viral genes whose products stimulate cell division, (d) autocrine production of growth factors, (e) increased sensitivity of the initiated cell to growth factors, and (f) cell surface modifications, both antigenic and functional, that could cause aberrant intercellular communication, recognition, and adhesion. In the previous section, examples of endogenous growth factors and squamous differentiation factors were described.

Tumor promoters are examples of exogenous agents that may induce terminal differentiation in one cell type and stimulate another subpopulation to proliferate. Therefore, the effects of tumor promoters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), teleocidin B, and aplysiatoxin on growth and differentiation of NHBE cells have been investigated. Nanomolar quantities of TPA rapidly inhibit the clonal growth rate of NHBE cells and concomitantly induce terminal squamous differentiation, as measured by an increase in cell surface area, progressive stratification of the squamous cells, enhanced plasminogen activator activity, and increased formation of crosslinked envelopes. Teleocidin B, aplysiatoxin, and 2,3,7,8-tetrachlorodibenzodioxin, tumor promoters with markedly different chemical structures from each other and TPA, also cause similar changes. In contrast, ten different human lung carcinoma cell lines were relatively resistant to TPA induction of terminal differentiation. Therefore, TPA may be useful for identifying preneoplastic human cells and for allowing the selective growth of these cells during in vitro carcinogenesis studies.

Acute effects of putative cocarcinogens and tumor promoters were also investigated using cultured human esophageal epithelial cells in serum-free LHC-8 medium and 199 medium containing 10% fetal calf serum. The effects were evaluated on clonal growth rate, cross-linked envelope (CLE) formation, and the enzymatic activities of ornithine decarboxylase (ODC) and plasminogen activator. The major findings included: teleocidin B, capsaicin, and cigarette smoke condensate were inhibitory to clonal growth; in contrast to results found in NHBE cells, none of the compounds induce CLE formation under conditions used; TPA induced ODC in medium 199, but inhibited it in LHC-8 media. Capsaicin induced ODC in both media; and TPA and capsaicin induced plasminogen activator activity in both media. These observations will be utilized in the design of in vitro carcinogenesis studies.

## Oncogenes

Activation and/or modification of cellular oncogenes is likely to be important in carcinogenesis, especially in the latter phases of this multistage process. Because the vast majority of the studies utilized an interspecies assay, i.e., transfection of human tumor DNA into mouse NIH 3T3 cells, we are using human epithelial cells as recipients of oncogene DNA transfected into the cells by a modified protoplast fusion method. Transfection of primary human bronchial cultures with plasmids carrying the vHa-ras oncogenic complementary DNA results in the following changes in the epithelial cells: altered cell growth properties, resistance to inducers of squamous differentiation, immortality, progression to anchorage independent growth, and tumorigenicity. The characterization of one of these recombinant cell lines (TBE-1) has established cells from clonal isolation at various stages of development for carcinogenic complementation studies with (1) other oncogenes and (2) chemical and physical carcinogens. TBE-1 has integrated vHa-ras into its genome and expresses transcripts that hybridize to Ha-specific structural gene and vHa-LTR probe DNA. TBE-1 cells express detectable levels of phosphorylated vHa-ras polypeptide, p21.

TBE-1 cultures were tested to determine growth characteristics, response to TPA, the ability to form colonies of anchorage independent cells in soft agar, and tumorigenicity in athymic nude mice. The results indicate that TBE-1 cells (1) are not induced to squamous terminal differentiation by BDS, confluence, or  $10^{-7}$ M TPA; (2) form colonies in soft agar; and (3) produce an autogenous growth factor since population doublings per day (PD/D) increase by a factor of 5.0 when autogenously conditioned medium is used to supplement cell growth at clonal density. The injection of nude mice with TBE-1 cells before selecting an anchorage independent population leads to the development of nodules less than 0.1 cm in size at a frequency of 2 per 16 animals tested that regress after 14 days. Nodules are removed for histopathologic analysis at intervals up to 14 days postinjection, and many mitotic figures are present in the nodule before regression. Isozyme phenotype analysis for six human isozyme markers following electrophoretic separation and staining of cell extracts indicates that TBE-1 cells are human. The morphology of the injected cell nodules resembles an aneoplastic carcinoma.

TBE-1SA cells were isolated by selection of anchorage independent cells growing in soft agar cultures of TBE-1 cells. The TBE-1SA cells were characterized for tumorigenicity, karyology, isozyme phenotype, and histochemical staining for keratin and beta human chorionic growth hormone (HCG). The progression of this subpopulation is indicated by its ability to form tumors that can grow to at least 0.5-0.8 cm in size and persist longer than 90 days in 13 of 14 nude mice. The TBE-1SA tumors have not regressed at 2 months, and this experiment is still in progress. Positive histochemical staining of TBE-1SA nodules for keratin confirms the epithelial origin of TBE-1SA cells. Since more than 70% of bronchogenic carcinomas contain detectable levels of HCG, TBE-1SA tumors were stained for beta HCG. The histochemical characterization reveals beta HCG product within TBE-1SA tumor cells. The tumorigenicity of TBE-1SA is in contrast with the pretumorigenic phenotype of unselected TBE-1 cell populations that form nodules which regress 14 days after injection. The pretumorigenic TBE-1 cells produced no anchorage independent colonies within the limits of detection ( $10^{-6}$ ) immediately after focus formation and were expressing vHa-ras gene transcripts and p21 gene product during the period of growth and progression to tumorigenicity of the population.

The karyotypic analysis of TBE-1SA cells and isozyme phenotype of TBE-1SA tumor tissue collected 84 days after transplantation indicate that these cells are human, and that the tumor tissue is identifiable as TBE-1SA by the correspondence of isozymes measured from the tumor tissue. The modal distribution of chromosomes is 74-75 for TBE-1SA, with marker chromosomes and extensive chromosomal abnormality. The karyotypic instability of TBE-1 cells was also an early event detectable at the first passage after the isolation of foci, and the karyology of TBE-1SA cells shows the extent of abnormality the bronchial epithelial vHa-ras transfectants have sustained during their progression to tumorigenicity in athymic nude mice. In conclusion, the progression of vHa-ras transfectants to immortality, anchorage independence, and tumorigenicity required expression of the oncogene for approximately 100 population doublings and alteration of many phenotypic properties of the cell, inferring a pleiotropic mechanism for the role of the Ha-ras oncogene in human carcinogenesis.

The oncogene, raf, initially described by investigators in the Laboratory of Viral Carcinogenesis, NCI, has been further studied. The amino acid sequence, predicted from the nucleic acid sequence of v-raf, revealed a close relationship between the members of the tyrosine kinase-oncogene superfamily and v-raf. The predicted protein sequences of v-raf and v-mht, the avian homolog, also demonstrate striking similarities between these sequences and those of v-fms and v-erb B. In contrast to the more recently evolved members of the src family of oncogenes, only the above sequences display features of transmembrane glycoproteins. Since the v-erb B product has been shown to share homologous regions with the epidermal growth factor (EGF) receptor, it is likely that the raf and fms oncogenes also represent growth factor receptors. Consistent with this proposal, raf expression can be found in specific tissues and is inducible upon stimulation with particular biomolecules and probably down regulated in cells which produce the specific growth factor.

To define the raf receptor, a nontransformed cell line which expresses high amounts of raf-specific mRNA has been identified. The plasma membrane fraction from these cells was prepared, and predetermined conditions were employed to release a biologically active receptor. This material is being analyzed for the presence of (1) raf polypeptide (approximately 90 Kda) by immunological methods and (2) receptor by its ability to compete with responsive cells for the growth factor.

The mechanism of activation of the raf sequence has not been ascertained. Determination of the raf-specific mRNA size in the malignant cell revealed no differences relative to normal cells, thus, gross rearrangements and/or truncation of the raf cistron has not occurred. Activation may be the consequence of cellular reprogramming since these carcinomas express other surface antigens (as well as oncogenes--myb and myc) found on nonepithelial cells.

The role of these sequences in human malignancy is not fully understood. Experiments indicate that cells derived from human small cell lung carcinomas express significantly more raf-related RNA than normal bronchial epithelial cells. Small cell carcinomas are especially metastatic. This may be in part the consequence of the cell's expression of a new growth factor receptor (raf) because metastases contain 10-40 times more raf RNA than any of the small cell carcinoma cell lines investigated.

## Physical Carcinogenesis Studies

Epidemiological studies have established that exposure to asbestos fibers is the primary cause of mesothelioma in the industrialized world. Because the latency period for this disease averages 40 years and because there has been a marked increase in the use of asbestos during and since World War II, an epidemic of mesothelioma has been predicted for the latter part of this century. Carcinogenesis studies with animals have shown that mesothelioma can be caused by intrapleural or intraperitoneal injections of asbestos. However, the long-term effects of asbestos fibers on human mesothelial cells in culture have not been reported previously. To study this important problem, methods to culture replicative normal mesothelial cells from adult human donors have been developed. The cells contain keratin and hyaluronic acid-mucin, exhibit long, branched microvilli, and retain the normal human karyotype to senescence. The mesothelial cells are 10 and 100 times more sensitive to the cytotoxicity of asbestos fibers than are bronchial epithelial or fibroblastic cells, respectively, from normal adult humans. Exposure of the mesothelial cells to amosite asbestos causes chromosomal rearrangements, including dicentrics. These aneuploid mesothelial cells have an extended population doubling potential of more than 35 divisions beyond the culture life span (30 doublings) of the control cells. Mesothelial cells have distinct keratin proteins and have a remarkable ability to regulate their cytoskeletal composition; the content of keratin or vimentin in the cytoskeleton reflects the growth conditions. We speculate that this uniquely fluid cytoskeleton may be more easily perturbed by penetrating asbestos fibers than are the cytoskeletons of other cell types and that this leads to an increased risk for chromosomal instability and transformation.

Another important problem in human carcinogenesis concerns the mechanism responsible for the cocarcinogenic effect of asbestos in enhancing the tumorigenicity of tobacco smoke in the bronchial epithelium. We and our coworkers have initiated investigations to define the effects of asbestos on cultured bronchial epithelial tissues and cells. The differential cytotoxic activity of various asbestos and glass fibers was estimated by measuring the inhibition of epithelial cell growth as a function of fiber concentration. The data show that various fiber types have different effects on human bronchial epithelial cells. Chrysotile was extremely toxic; amosite and crocidolite were less toxic; glass fibers were only mildly toxic. For comparison, human bronchial fibroblastic cells were also exposed to fibers and were found to be markedly more resistant (more than ten-fold) than the epithelial cells to all of the types of asbestos tested.

Monolayers of bronchial epithelial cells were used to investigate the effect of amosite asbestos at the cellular level. With scanning electron microscopy and high voltage electron microscopy, amosite fibers ingested by human bronchial epithelial cells can be seen. In contrast to macrophages, the fibers penetrate the surface of epithelial cells without the development of filopodia. While macrophages seem to phagocytose fibers along both the long and short axes, epithelial cells seem to take up fibers along the short axis with only a membrane sleeve surrounding each fiber. Examination by scanning high voltage and transmission electron microscopy with associated energy dispersive X-ray spectra clearly revealed that short fibers (< 12  $\mu\text{m}$ ) are taken up quickly by the cells. Asbestos is present within the cells by 2 hours after exposure, and by 28 hours, many fibers are found in the cytoplasm and occasionally in the nucleus.

Asbestos fibers induce abnormal cell growth. Addition of amosite asbestos (10, 100, or 1,000  $\mu\text{g/ml}$ ) to human respiratory mucosa in explant culture causes numerous focal lesions including squamous metaplasia and dysplasia. When examined by scanning electron microscopy, the epithelial lesions appear as focal elevations of nonciliated cells.

Cytopathological aberrations of the bronchial epithelial cells are manifested by cellular polymorphism and variation in nuclear size. Since it is important to know whether there are asbestos fibers in the cytoplasm of cells involved in the lesions, studies are now being done using X-ray microanalysis in combination with transmission electron microscopy including high voltage electron microscopy. These studies are being extended to determine the progression of these lesions and eventually their malignant potential.

Epidemiological studies have shown that inhalation of nickel compounds enhances the risk for human respiratory cancer. Cultures of normal human bronchial epithelial cells were continuously exposed to a dose (5-20  $\mu\text{g/ml}$ ) of  $\text{Ni}_2\text{SO}_4$  that reduced their colony-forming efficiency 30-80%. After 40 days of incubation, the cultures consist of large, squamous cells; mitotic cells are very rare. The cells are then maintained in growth medium without nickel. After 40-75 total days of incubation, colonies of mitotic cells appear at a rate of 1 colony per 100,000 cells at risk; no colonies appear in control cultures or in cultures exposed to < 5  $\mu\text{g}$  of nickel/ml for 90 days. Twelve cultures isolated from five experiments have been expanded into mass cultures. Most of the cell lines have an increased population doubling potential (> 50 divisions). Some have a reduced response to squamous (terminal) differentiation-inducing signals whereas others have lost growth factor requirements for clonal growth. Aneuploidy and marker chromosomes have also been noted. However, none of these Ni-altered clones of bronchial epithelial cells is anchorage independent and they do not produce tumors upon injection into athymic nude mice. Experiments are in progress to ascertain if these abnormal cells will progress to tumorigenicity after either re-exposure to carcinogens or transfection with oncogenes.

### Methylation

The pattern of 5-methylcytosine residues in mammalian DNA has recently been found to be crucial to the control of genetic expression. Decreases in DNA 5-methylcytosine content are known to alter the level of differentiation of cells in culture. Thus, changes in DNA 5-methylcytosine patterns may be critical to the process of carcinogenesis. Human tumor DNA is being probed for DNA methylation pattern alterations in selective DNA sequences and genes. Since chemical carcinogens have been shown to decrease genomic 5-methylcytosine levels in BALB-3T3 cells, DNA from carcinogen-treated human epithelial cells is also being probed for changes in 5-methylcytosine patterns. New methods to assess 5-methylcytosine content in non-dividing differentiated human cells have been developed. Previously, the determination of genomic 5-methylcytosine levels required the labeling of DNA in dividing cells with 6- $^3\text{H}$ -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  $^{32}\text{P}$ . The labeled nucleotides are then separated by TLC and the ratio of 5-methylcytidine to the total cytidine and 5-methylcytidine determined. This highly sensitive  $^{32}\text{P}$  post-labeling method not only



enables the above-described chemical carcinogenesis studies to be performed on human epithelial cells but also allows for monitoring of genomic as well as specific gene levels of 5-methylcytosine in tumors, tissues, and cell types from human and animal sources. Thus, changes in 5-methylcytosine levels in specific genes, including oncogenes, during differentiation and carcinogenesis and during the normal aging process in vivo can now be followed.

### Somatic Cell Genetics

In this new project, genetic changes related to carcinogenesis are being studied using hybrids of human lung carcinoma cells with NHBE cells. We are interested in determining if the malignant phenotype and immortality of human bronchial epithelial cells are recessive or dominant. For these experiments, two bronchial carcinoma cell lines (ouabain-resistant and HPRT minus) have been produced to be used as universal recipients. One of these cell lines, A1146, was selected because it contains the mutated Ha-ras oncogene. Initial studies suggest that a limited population doubling potential (mortality) is a dominant genetic trait in tumor-normal hybrid cells. Other hybrid cell lines have been isolated and are being characterized for doubling potential, karyotype, and tumorigenicity in athymic nude mice.

### INTERMEDIATE FILAMENTS IN NORMAL AND CARCINOMA CELLS

Cancer of the human esophagus and lung represents a major cause of death in certain populations of people throughout the world. In focusing our efforts on these two organ systems, we have initially evaluated the usefulness of analysis of keratin protein patterns and cross-linked envelopes in the characterization of epithelial neoplasms. Distinctive qualitative and quantitative differences in the spectrum of keratin proteins are found in the carcinomas compared to their nontransformed counterparts. Analysis of keratin protein patterns is a useful adjunct in defining the type of tumor present. Moreover, assessment of cross-linked envelope-forming capabilities serves as a specific marker for squamous differentiation and the extent of envelope formation correlates well with the degree of squamous differentiation in the tumor with more well-differentiated squamous carcinomas forming more cross-linked envelopes. We have established human esophageal and lung carcinoma cell lines in cell culture to evaluate if their properties in vitro faithfully manifest those of the original tumor, thereby representing useful models of carcinogenesis in vitro. Moreover, we have compared the growth and differentiated properties of these carcinoma cells to their nontransformed counterparts. Numerous morphological and biochemical differences are observed between normal and malignant epithelial cells in culture. Significant changes in the array of keratins and in the proportions of cells making cross-linked envelopes were found. The results we obtained paralleled findings with tumor masses indicating that the tumor cells in cell culture continue to maintain a program of gene expression reflective of that of the original tumor.

### METABOLISM OF CHEMICAL CARCINOGENS AND FORMATION OF CARCINOGEN-DNA ADDUCTS

The earliest events in the multistage process of chemical carcinogenesis are considered 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 (1) many environmental carcinogens require metabolic activation to exert their oncogenic effects; (2) the metabolic balance between carcinogen activation and deactivation may, in part, determine a person's oncogenic susceptibility; and (3) knowledge of the comparative metabolism of chemical carcinogens among animal species will aid efforts to extrapolate data on carcinogenesis from experimental animals to humans. We and our coworkers have systematically examined the metabolism of procarcinogens of several chemical classes which are considered to be important in the etiology of human cancer. Procarcinogens of several chemical classes can be activated enzymatically to electrophilic reactants that bind covalently to DNA in cultured human tissues. The studies of activation and deactivation of representative procarcinogens have revealed that the metabolic pathways and the predominant adducts formed with DNA are generally similar between humans and experimental animals. Wide quantitative interindividual differences (50- to 150-fold) are found in humans and other outbred animal species. When the metabolic capabilities of specimens from different levels of biological organization are compared, the profile of benzo[a]pyrene metabolites is similar in cultured tissues and cells, but subcellular fractions, e.g., microsomes, produce a qualitative and quantitative aberrant pattern.

The metabolism of benzo[a]pyrene was studied in both epithelial and fibroblastic cells initiated from the same bronchus specimens. The total metabolism of benzo[a]pyrene and binding of its ultimate carcinogenic metabolite is three-fold higher in the epithelial than in the fibroblast cells. No qualitative differences in the metabolic profile of benzo[a]pyrene between the explant culture and the epithelial cell cultures were observed.

To test the interactive effects of cell types in the metabolic activation of carcinogens and to further assess interindividual differences among people, human tissue- and cell-mediated mutagenesis assays have been developed. The fact that terminally differentiated cells, such as pulmonary alveolar macrophages, can activate benzo[a]pyrene and mediate an increase in frequencies of mutations and sister chromatid exchanges in cocultivated "detector" cell populations, i.e., Chinese hamster V79 cells, suggests that nontarget cells of chemical carcinogens may play an important role in the activation of environmental carcinogens.

Because the specific carcinogenic agents responsible for the etiology of colon cancer are not known, we are systematically studying the metabolic activation of several chemical classes of carcinogens. The following carcinogens are converted by cultured human colon to metabolites that bind to DNA: benzo[a]pyrene; 6-nitrobenzo[a]pyrene; 1-nitropyrene; aflatoxin B<sub>1</sub>; N-nitrosodimethylamine; 1,2-dimethylhydrazine; and 3-amino-1,4-dimethyl-5H-pyridine(4,3-b) indole. The latter compound is formed by pyrolysis of tryptophan.

The extrapolation of data from studies of N-nitrosamine carcinogenesis between experimental animals and humans is a pressing problem. Abundant evidence of N-nitrosamine carcinogenesis from both *in vitro* and *in vivo* studies using experimental animals has accumulated. Although N-nitrosamines are widespread pollutants, the carcinogenicity of these chemicals in humans has been difficult to prove by epidemiological studies. *In vitro* studies comparing pathobiological responses of N-nitrosamines in humans and experimental animals offer an approach to solve this problem at least at the cellular and tissue levels of biological organization. N-nitrosamines can be metabolized by cultured human epithelial

tissues and cells. Quantitative differences in metabolism and alkylation of DNA are found among humans, among various organs within an individual, and among adult versus fetal tissues. Whether these differences are sufficient to influence an individual's cancer risk and organ site is as yet unknown.

### DNA REPAIR

Although DNA repair has been extensively studied in human fibroblasts, lymphoid cells, and neoplastic cells, little information is available concerning DNA repair in normal human epithelial cells. Using the methodology to culture human bronchial epithelial and fibroblastic cells developed in our laboratory, we have initiated studies to investigate DNA damage and repair caused by chemical and physical carcinogens as examined by alkaline elution methodology, BND cellulose chromatography, unscheduled DNA synthesis, and high pressure liquid chromatographic analysis of the formation and removal of carcinogen-DNA adducts. As we reported last year, human bronchial epithelial cells repair single-strand breaks in DNA damaged by X-radiation, UV-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines at rates similar to bronchial fibroblasts.

During metabolic activation, N-nitrosodimethylamine yields equal molar quantities of methyl carbonium ions and formaldehyde. Both of these metabolites can react with nucleophilic sites in cellular macromolecules, carbonium ions by alkylation, and aldehydes via formation of unstable alkyl-ol derivatives preferably with amine groups ( $R-NH-CHOH-R_1$ ). The monomethylol derivatives of formaldehyde can form intermediary labile products that by secondary reactions can yield stable methylene bridges between macromolecules. Although the alkylating metabolites of N-nitrosamines and their cytotoxic, mutagenic, and carcinogenic effects have been extensively studied, the possible contribution of other metabolites, especially aldehydes, has not received much attention. We have investigated the effect of formaldehyde on the repair of X-ray-induced single-strand breaks. Human bronchial cells were exposed to X-rays and then incubated with or without the presence of formaldehyde, and the repair of DNA single-strand breaks was measured. The presence of formaldehyde significantly inhibits the repair of the X-ray-induced single-strand breaks correlating with the potentiation of cytotoxicity in human cells and mutation frequency in Chinese hamster V79 cells by combinations of the agents. Formaldehyde, a common environmental pollutant and metabolite of carcinogenic N-nitrosamines, also inhibits repair of  $O^6$ -methylguanine, decreases  $O^6$ -alkylguanine alkyltransferase activity, is mutagenic at high concentrations ( $> 100 \mu M$ ), and potentiates the cytotoxicity and mutagenicity of the methylating agent, N-methyl-N-nitrosourea, in normal human cells. Exposure to formaldehyde may lead to the dual genotoxic mechanism of both directly damaging DNA, i.e., formation of DNA-protein crosslinks and single-strand DNA breaks, and inhibiting repair of mutagenic and carcinogenic DNA lesions caused by alkylating agents and physical carcinogens.

Formaldehyde is also a ubiquitous environmental contaminant and, in addition to acrolein and acetaldehyde, is present in large quantities in tobacco smoke. We are currently studying the biochemical and mutagenic effects of these tobacco smoke-related aldehydes in human lung cells.

## INTERACTIVE EFFECTS BETWEEN CHEMICAL CARCINOGENS AND HEPATITIS B VIRUS IN LIVER CARCINOGENESIS

Liver cancer incidence is high in areas with both food contamination by carcinogens, such as aflatoxin B<sub>1</sub>, a liver carcinogen in experimental animals, and a high incidence of chronic active viral hepatitis. Due to the insensitivity of epidemiological methods, the role of single agents or combinations of agents is uncertain. Our long-term goal is to study the interactive effects of hepatitis B virus and chemical carcinogens, such as aflatoxin B<sub>1</sub>, in the malignant transformation of cultured human hepatocytes. During this fiscal year, and in collaboration with coworkers at the Cancer Institute, Beijing, People's Republic of China, methods to culture tissue explants and epithelial cells from fetal human liver have been developed. Metabolism of chemical carcinogens has been investigated, and the role of core antigen gene in the cytopathology of hepatitis B virus has been studied.

In order to assess the role of chemical carcinogens, e.g., aflatoxin B<sub>1</sub>, in human liver carcinogenesis, we have initiated several projects with coworkers at the Cancer Institute, Beijing, People's Republic of China. For example, the metabolic activation of aflatoxin B<sub>1</sub> and other carcinogens, such as N-nitrosodimethylamine and benzo[a]pyrene, have been investigated using cultured human fetal liver explants. One major aflatoxin B<sub>1</sub>-DNA adduct is formed by addition of aflatoxin B<sub>1</sub>-2,3-oxide to the 7-position of guanine. This reaction product is unstable, and the imidazole ring of the guanine will open to stabilize the molecule. The major aflatoxin B<sub>1</sub>-DNA adduct is similar to the one formed in fetal human liver explants and in rat liver *in vivo*, an organ susceptible to the carcinogenic action of aflatoxin B<sub>1</sub>.

In a second related project, we collected urine samples in Murang'a district, Kenya, for analysis of aflatoxin B<sub>1</sub>-guanine, a "nucleic acid repair product." It has previously been shown that food samples collected in this district are known to be contaminated with aflatoxin B<sub>1</sub>, and a positive correlation exists between the dietary intake of aflatoxin B<sub>1</sub> and the incidence of liver cancer. The urine samples collected at the outpatient clinic of Murang'a district hospital were concentrated on C<sub>18</sub>Sep-Pak columns, and aflatoxin B<sub>1</sub>-guanine was isolated by high pressure liquid chromatography in two different systems. Eleven of 126 samples had a detectable level of a compound whose synchronous fluorescence spectrum was identical to chemically synthesized aflatoxin B<sub>1</sub>-guanine. The spectrum did not show any bathochromic shift when the pH was made alkaline. These results are an indication of interactions between the ultimate carcinogenic form of aflatoxin B<sub>1</sub> and cellular nucleic acids *in vivo* and further support the hypothesis that aflatoxin B<sub>1</sub> may play an important role in the etiology of human liver cancer.

We are also investigating the cytopathological and oncogenic effects of hepatitis B virus. Using the protoplast fusion method to transfect human cells with pSV2-derived plasmids at frequencies greater than 10<sup>-3</sup>, it is possible to test the biological effect of hepatitis B virus genes independent of the viral structures required for infection. A pSV2gpt<sup>+</sup> plasmid constructed to carry a subgenomic fragment of hepatitis B virus that contained the core antigen gene (Hbc gene) was transfected into human cells. The cytopathologic effects of Hbc gene expression were observed immediately after the transfection. A human epithelial cell line was stably transfected with the Hbc<sup>+</sup> gene by selecting recipient cells for gpt<sup>+</sup> expression. The gpt<sup>+</sup>/Hbc<sup>+</sup> cell line was used to

determine that growth in serum-free medium or 5'-azacytidine treatment stimulates the production of the HBc gene product (HBcAg). These factors were used to test a hepatocellular carcinoma that has carried the entire hepatitis B virus genome since its isolation from a chronically infected patient. This cell line was stimulated to produce the HBc gene product in response to the same factors that stimulated HBcAg production in the gpt<sup>+</sup>/HBc<sup>+</sup> cell line constructed by transfection. The temporal relationship of the cytopathologic response to HBc gene expression was similar for both cell types, indicating a primary role for HBc gene expression in the cytopathology of hepatitis B virus-infected human liver. Such information may be useful for development of therapeutic regimens for chronically infected patients and may provide insight into the biological nature of the virus at the genetic and molecular levels. Methylation of cytosine in 5' flanking DNA sequences of the hepatitis B virus core antigen gene appears to control its expression. Since the core antigen may be responsible for the cytopathologic effects of hepatitis B virus, controlling core antigen gene expression by methylation could give preneoplastic and neoplastic liver cells a selective growth advantage during carcinogenesis. Consistent with this hypothesis is our recent result showing methylation of the promoter-enhancer DNA sequences of the core antigen gene which inhibits its expression in a human hepatocellular carcinoma.

#### 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 (1) high exposure of target cells to carcinogens and/or (2) increased host susceptibility due to inherited or acquired factors. Laboratory methods have been recently developed to be used in combination with analytical epidemiology to identify individuals at high cancer risk. These methods include (1) techniques to assess specific host susceptibility factors; (2) assays that detect carcinogens in human tissues, cells, and fluids; (3) cellular assays to measure pathobiological evidence of exposure to carcinogens; and (4) methods to measure early biochemical and molecular responses to carcinogens.

Currently available techniques exist that would allow the utilization of biochemical and molecular measures to better characterize exposure to carcinogens, to serve as intermediate end points on the path to malignancy, to identify measures which halt or reverse this process, and to investigate the mechanisms of human carcinogenesis. Included in these investigations would be the following: (1) efforts to evaluate body burden of chemical carcinogens in studies of occupational and general environmental cancer risk factors; (2) sophisticated analyses of air, water, and biological specimens for carcinogenic and mutagenic substances in conjunction with specific analytical studies; (3) search for evidence of viral infection including viral segments or oncogenes in the DNA of individuals at high risk of cancers that may be associated with infectious agents or heritable states; (4) evaluation of disturbances in immune function as they may relate to malignancies, particularly those of the hematopoietic system; (5) investigation of the relationship between micronutrients and a variety of epithelial cancers; and (6) determination of the relationship between macronutrients, including dietary fat and subsequent hormonal changes, to subsequent risk of breast, endometrial, and perhaps colon cancer.

## Carcinogen-DNA Adducts and Human Antibodies to Adducts

Our investigations of carcinogen metabolism in cultured human cells revealed that the major carcinogen-DNA adducts formed are identical to those found in experimental animals in which the chemical is carcinogenic. This important finding has encouraged us and others to search for adducts and antibodies to adducts in people exposed to environmental carcinogens. For example, roofers, coke oven workers, and aluminum plant workers as well as cigarette smokers are exposed to high levels of polynuclear aromatic hydrocarbons, including benzo-[a]pyrene (BP). We are currently comparing two very sensitive methods to detect BP-DNA. Both of the methods, USERIA (ultrasensitive enzymatic radioimmunoassay) using rabbit-produced polyclonal antibody and a biophysical method, synchronous fluorescence spectrophotometry, appear to detect about 1 adduct per  $1 \times 10^8$  nucleotides. Quantitation with both of the methods is possible. USERIA is most sensitive toward BP-DNA; for the synchronous fluorescence spectrophotometry, DNA has to be acid-hydrolyzed to release BP moieties as tetrals. Both qualitative and quantitative results from preliminary experiments comparing the methods have been promising. Another type of evidence of former or current existence of BP-DNA adducts comes from the presence of antibodies towards BP-DNA in serum from people exposed to BP. These antibodies have been detected by the ELISA technique from coke oven workers.

Aflatoxin B<sub>1</sub> (AFB)-contaminated food is the main source of AFB exposure. Both USERIA and synchronous fluorescence spectrophotometry are currently being applied in our laboratory to detect AFB-DNA.

With none of these methods have all of the persons among a certain exposed group been positive. The significance of this interindividual difference is as yet unknown. In addition, the formation of carcinogen-DNA adducts may represent only the initial stages of carcinogenesis and measures of later stages, i.e., tumor promotion and progression, are also needed to predict an individual's cancer risk.

## Xenobiotic Polymorphisms

Phenotyping of animal models and man for their xenobiotic metabolizing capabilities has, in recent years, been undertaken in the interests of predicting carcinogenic susceptibilities. The metabolism of exogenous agents is known to be genetically dependent and selective in vivo xenobiotic metabolic routes appear to be accessible to evaluation by the use of nontoxic doses of certain drugs. The determination of the rate of selective enzymatic modifications of test agents may thus provide a suggestion as to how susceptible an individual may be to the oncogenic potential of carcinogens activated by similar metabolic routes. Several agents including debrisoquine (DBQ), S-mephenytoin (SMPH), S-carboxymethyl-L-cysteine (SCMC), and sulfamethazine have been shown to be metabolized by enzymatic routes governed by separate genetic loci. We have initiated both an animal model study and clinical investigations. Since the primate colony from which these monkeys will be sampled has been and is presently involved in ongoing chemical carcinogenesis experiments, the results of the metabolic phenotyping can be compared to the susceptibilities of the monkeys to carcinogenesis.

Preliminary results of phenotyping three monkeys from each of three species, including rhesus, cynomolgus, and African green monkeys, are available. All of

the monkeys tested rapidly metabolized DBQ, SMPH, and SCMC. The metabolic ratio (concentration of parent drug to metabolite) was observed to range from 0.02 to 0.6 for DBQ, which is well below that reported for man. No differences between species are observed with the minimal sample size of three monkeys per group. MPH metabolism is extensive in these monkeys as well. From 6 to 52% of the administered dose of MPH is excreted as 40 H-MPH in 24 hours, which is greater than that reported for man. There are no significant differences in the rate of MPH hydroxylation between species.

The rate of sulfoxidation of SCMC is also high in these monkeys. The metabolic ratio ranged between 0.8 and 3.8, but the rhesus and cynomolgus monkeys metabolize SCMC significantly ( $P < 0.10$ ) faster than the African green monkeys. The average SCMC sulfoxidation index per species is  $1.4 \pm 0.3$ ,  $1.2 \pm 0.3$ , and  $2.7 \pm 0.9$  for rhesus, cynomolgus, and African greens, respectively. These rates are more rapid than that reported for man. Rhesus monkeys are significantly ( $P < 0.01$ ) slower N-acetylators of sulfamethazine than African green monkeys, while that of the cynomolgus monkeys fell between the former two species. The average rate of N-acetylsulfamethazine formation is  $51 \pm 4\%$ ,  $65 \pm 12\%$ , and  $76 \pm 9\%$  for rhesus, cynomolgus, and African green monkeys, respectively. This rate of sulfamethazine acetylation in rhesus monkeys compares well with previously reported values. These rates also compare well with the human data, classifying the rhesus as poor N-acetylators, the African greens as extensive N-acetylators, and individual cynomolgus monkeys in both categories.

The rapid rate of enzymatic hydroxylation observed in these monkeys for DBQ and MPH would suggest that these monkeys would be susceptible to carcinogenesis upon exposure to aromatic hydrocarbon carcinogens. AFB and methylazoxymethanol-acetate have been shown by Drs. Adamson and Sieber to produce tumors in these species. However, several other carcinogens, including benzo[a]pyrene, 3-methylcholanthrene, and cigarette smoke condensate did not induce tumors. Thus, there may be some correlation between the metabolic phenotype and chemical carcinogenesis susceptibility, but the data presently available are insufficient to draw firm conclusions.

Clinical studies are in collaboration with Dr. Jeff Idle, St. Mary's Hospital, London. We are using DBQ to phenotype lung cancer patients, their relatives, and individuals in various control groups. The extensive and poor metabolism phenotypes were further studied by measuring oncogene expression and presence of carcinogen-DNA adducts in tissues and cells, antibodies to these adducts, urinary levels of polycyclic aromatic hydrocarbons and carcinogen-guanine adducts, and chromosomal abnormalities.

### DNA Polymorphism

Classic genetic linkage studies have been used to predict the risk of developing diseases. Until recently, disease-associated polymorphisms could be assessed only by analyzing gene products, e.g., cell surface histocompatibility antigens or blood group substances commonly referred to as A,B,H. Advances in molecular biology have not made it possible to measure genetic polymorphisms at the DNA level. This approach utilizes restriction enzyme catalyzed endonucleolytic cleavage and DNA hybridization with gene-specific probes to detect base-pair substitution and fragment length polymorphisms. The potential of this technology is exemplified by the recent identification of individual DNA polymorphisms associated with diabetes mellitus and hemoglobinopathies. A similar molecular

approach using specific DNA probes, e.g., oncogenes or ectopic hormones, is being used to potentially detect and characterize DNA polymorphisms in individuals who are oncogenetically predisposed.

### Human T-cell Leukemia Virus

A human C-type retrovirus has been found to be associated with certain adult T-cell malignancies. Lymphocytes from these patients were established in long-term tissue culture using the human T-cell growth factor. Studies were carried out to examine the cell surface markers of cells infected with and producing the human T-cell lymphoma virus (HTLV). HLA typing was performed on these cells. The virus was transferred to human umbilical cord blood lymphocytes by coculture.

Cell lines established from patients with the adult T-cell leukemias and lymphomas express more than the expected two alloantigens controlled by the HLA-A or HLA-B locus. Alloantisera detecting these altered determinants are confined to the HLA-AW19 cross-reactive group and the HLA-B5 cross-reactive group. A monoclonal antibody detecting a polymorphic epitope on HLA alloantigens of these two cross-reactive groups was developed by Dr. Bart Haynes (Duke University School of Medicine). This monoclonal antibody is found to react with all cells infected with HTLV and producing products of this virus. Human umbilical cord lymphocytes infected with the virus by coculture also express altered HLA alloantigenic determinants. These alterations mirror those seen with tumor cell lines established from patients. Thus, the appearance of neoantigens suggests an association of HTLV provirus replication and HLA alloantigenic expression. Cell surface markers are examined on cell lines established from patients and cell lines established by coculturing with the human T-cell lymphoma virus. These cells are predominantly OKT4+ (helper phenotype). These cells also express increased HLA-DR and an antigen detected by the Tac antibody to the receptor for T-cell growth factor. A cytotoxic T-cell line, established from a patient with the HTLV-associated disease, kills autologous cells but is not cytotoxic for other cell lines established from patients with HTLV.

The cytotoxicity appears to be genetically restricted by HLA determinants A1 and DR3. These investigations are providing new insights into the host factors that influence the pathogenesis of this disease.

In culturing cells from patients with classic adult T-cell leukemia, two lines were established that had B-cell surface markers, produced immunoglobulins and were infected with HTLV. Supernatants from these cell lines are found to contain interferon alpha, both acid stable and acid labile. Further examination for soluble lymphokines produced by these cultured cells demonstrate production of B-cell growth factor. These observations suggest that B-cells may be infected in vivo with the T-cell tropic retrovirus. Furthermore, infection of B-cells with viruses can induce production of interferon alpha, a substance that is found in high levels in patients with certain autoimmune diseases and acquired immune deficiency syndrome (AIDS). Lymphokine production induced by virus infection may play a significant role in expansion of a transformed cell population to an apparent malignant state.

T-cell surface markers were examined in cells from homosexual individuals at high risk for AIDS and correlations were established with certain epidemiologic parameters. In studies of individuals who had contact with a Washington



population as well as a Denmark population, the most significant correlation was the altered T4/T8 ratios in contacts with New York homosexuals. Because of the high frequency of antibodies to products of the hepatitis B virus, lymphocytes from several patient populations and controls were probed for the presence of this virus. Patients with AIDS and chronic active hepatitis were positive for the presence of hepatitis B virus while individuals who had had an episode of the acute disease and at the time of the investigation were healthy as well as normal controls were negative for the presence of the virus. The results point out a significant biologic variation in infectivity of this virus.

Descriptions of these and other findings are given in more detail in the individual project reports that are on file in the Office of the Director, Division of Cancer Etiology.

#### OTHER ACTIVITIES

The Laboratory has been responsible for training intramural and extramural investigators in the techniques for (1) culturing human epithelial tissues and cells and (2) enzyme immunoassays to measure carcinogen-DNA adducts and onco-fetal antigens. Members of the staff have also organized and/or served on the program committees of both national and international scientific meetings. These meetings include Human Carcinogenesis Conference, Biochemical and Molecular Epidemiology of Cancer, IV World Congress on Lung Cancer, III International Congress on Cell Biology, Interspecies Comparisons in Tumor Promotion, and Tumor Promotion in Humans. During this fiscal year, members of the staff have also served as reviewers and on editorial boards of several journals and on intramural and extramural committees, e.g., NIH Handicapped Employees Advisory Committee, DCE Promotion Committee, Environmental Pathology Committee of the International Academy of Pathology, Advisory Board of the Danish Cancer Society, Cellular Physiology Grant Review Study Section, and Honor B. Fell Division, Tissue Culture Committee.

CONTRACTS IN SUPPORT OF LHC PROJECTS

UNIVERSITY OF MARYLAND (N01-CP-15738)

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Current Annual Level: \$450,000

Man Years: 9.17

Objectives: To provide a resource to the NCI 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 102 cases, including specimens from surgery patients (31) with and without cancer and at time of autopsy (71); autopsy specimens are collected from patients undergoing either immediate autopsy (8) (i.e., within 30 minutes after death) or routine autopsy (63) (i.e., between 2 and 12 hours after death). All tissues received at the NIH are usually residuals of materials taken for regular diagnostic and corrective purposes and not for research per se.

A. Surgical Specimens

A total of 31 surgeries resulted in tissue donations.

Bronchus: Tumor tissues from 20 cases of lung carcinoma were collected. Bronchial specimens uninvolved with tumor were provided from all of these cases and transported to the NIH. Twelve specimens of lung tumor were received. The tumors were defined and classified as described below.

Colon: Tumor tissues from 11 cases of colon carcinoma were collected. Colonic tissue uninvolved with tumor from 10 of these cases was transported to the NIH. In addition, 6 specimens from patients without cancer were collected and tissue from 5 of these cases was delivered to the NIH. There was 1 polyp, 1 villous adenoma, 1 adynamic colon, 1 diverticulitis, and 1 radiation adhesion. All of the tumors were defined and classified as described below.

B. Autopsy Specimens

Immediate Autopsy: There were 8 immediate autopsies. The specimens collected are shown below.

<u>Organ</u>	<u>Number of Specimens</u>
Colon	8
Bronchus with lung attached	8
Pancreatic duct	7
Liver	7

Tissues required for pathological examination and assessment of viability were retained by the contractor, but the major portions of the specimens were received at the NIH. Liver samples were quick-frozen in liquid nitrogen and stored at -70°C in the contractor's facility; other samples were shipped in ice-chilled L-15 medium.

Routine Autopsy: The numbers of specimens collected from 63 routine autopsies were as listed below:

<u>Organ</u>	<u>Number of Specimens</u>
Bronchus	38
Liver	25

## 2. Viability Evaluation

Bronchus: Small pieces of nontumorous bronchial epithelium from lung cancer patients (surgery) and from noncancer patients (immediate autopsy) were grown in explant cultures. Eighty percent of the cases were viable.

Pancreas: Pancreatic ducts from 7 immediate autopsy cases were collected. Two cases were cultured to test tissue viability and proliferative potential. The ducts survived 2 weeks in culture. Four cases were cultured using the Parsa Protocol (Cancer 47: 1543, 1981) and N-methyl-N'-nitrosourea (MNU) as the carcinogen. The culture portions for these cases were completed and post-culture explants were treated with MNU and implanted in nude mice along with one case using CMRL 1066 as control. Carcinogen-treated explants have not as yet developed tumors in the mice, but 1 CMRL 1066 control implant developed a "cyst-like" lesion. This protocol is being continued.

Parsa media has been less effective in explant organ culture than CMRL 1066. Controls to assess viability in CMRL 1066 with and without serum and growth-promoting factors (insulin, transferrin, EGF, etc.) were included. CMRL 1066 with 5% heat-inactivated fetal bovine serum was the most effective.

Pancreatic tissue from 10 routine autopsies were nonviable. Ischemic changes, areas of fat necrosis, and nonviable explant cells were observed in all cases.

Liver: Six liver specimens from immediate autopsies and 25 from routine autopsies were frozen and stored. Liver slices and cells were stored by the contractor and are available upon request for shipment to the NIH.

In the 12-month period, 7 livers from immediate autopsy and 2 from the organ donor program were used for hepatocyte isolation. Post-mortem changes were observed in several, but viable hepatocytes were isolated from these tissues. Approximately 10-20 gm of tissue was digested with a collagenase-dispase solution. The yields ranged from 0.1 to  $2 \times 10^6$  cells/gm of tissue with 70-90% viability. The low yields for some livers are not clearly understood. The effects of pathological changes (e.g., cirrhosis, fatty cells, cell necrosis, and post-mortem changes) on isolated hepatocytes are being studied. Four hours after seeding the freshly harvested cells, hepatocytes attached to the culture dishes. Hepatocytes from 2 organ donors and 3 of 7 immediate autopsy cases were received at the NIH for use in DNA transfection studies. Currently, the contractor is examining detoxification enzymes and ultrastructure of the cultured hepatocytes.

### 3. Epidemiological Profile Construction and Storage

Abstracting medical records, compiling donor histories, and computerizing these data continue as essential requirements of this project. In this period, 111 medical records were abstracted for surgery patients (75 for thoracic and 36 for colonic); donor histories were compiled for 65 patients (36 bronchus and 29 colon) via interviews using the standard questionnaire (developed by LHC and the contractor); and in data processing, a total of 176 colonic and thoracic records (medical and epidemiological) have been coded for computer storage and analysis.

The total number of cases with these data collected under the current contract from the 6 participating hospitals are listed below:

	<u>Univ. Hosp.</u>	<u>LRVA</u>	<u>Un. Mem.</u>	<u>St. Agnes</u>	<u>W. Va.</u>	<u>Med. Exam.</u>	<u>Total</u>
Bronchus	251	106	23	45	0	70	495
Colon	251	71	0	0	36	0	358
Total							853

To date, the activities completed in the effort to provide epidemiological profiles for donors of tissues delivered in this period are as follows:

	<u>Med.Rec.</u>	<u>Interviewed</u>	<u>Coded</u>	<u>Interview Refused By:</u>			
				<u>Patient</u>	<u>Doctor</u>	<u>Hospital</u>	<u>Med.Exam.</u>
Bronchus	475	326	460	35	4	23	44
Colon	315	259	306	20	5	0	0
Total	790	585	766	55	9	23	44

### 4. Definition and Classification of Nonneoplastic and Neoplastic Tissue

Epidemiologic data are provided to allow determination of the relationships between tumor type, selected risk factors, and e.g., in lung cancer and hepatoma, the amount of benzo[a]pyrene (BP) or aflatoxin B<sub>1</sub> (AFB), respectively, bound to DNA by the same patient's noncancerous epithelium.

Bronchus: The contractor provided the following characterizations:

Morphological and histochemical characterization of human primary lung carcinomas are routine. Characterization of tissues by immunocytochemistry has continued using the peroxidase-antiperoxidase method to demonstrate the presence or absence of various antigens. Tumor and normal tissues, abnormal and preneoplastic tissues are examined for beta human chorionic gonadotropin (HCG), calcitonin, adrenocorticotrophic hormone (ACTH), serotonin, alpha-fetoprotein (AFP), keratin, somatostatin, neuron specific enolase (NSE), calmodulin, and tubulin.

Normal and abnormal (but nonneoplastic) adult bronchi contain only mucosubstances, keratin, calmodulin, and tubulin. Keratin is seen only in aldehyde-fixed bronchi if the epithelium is neoplastic; in the basal layer of ethanol-fixed bronchi, keratin is seen in normal bronchial epithelium (including bronchial glands) and occasionally in columnar cells that reach the lumen. Calmodulin apparently increases levels at the cell borders.

Lung tumors have greater heterogeneity than tumors in the bronchial epithelium. Each marker is found at least occasionally in tumors; HCG is found in 80% of non-small cell tumors, and keratin is found in 75% of such tumors. Somatostatin

is seen in keratinizing areas and more diffusely in a smaller proportion of adenocarcinomas. NSE and serotonin are seen in endocrine tumors only. Appearance of other markers shows less predilection for types of lung tumors, and HCG seems to follow glycogen distribution.

Indirect immunofluorescent detection of tubulins was performed on cellular outgrowths of bronchial explants involved and uninvolved with tumor. Generally uniform, the non-malignant cells had mostly straight microtubules originating from assembly sites near the nucleus, while the variable tumor cells had irregular microtubular patterns in a mesh-like arrangement.

Colon: All tissues were characterized as described below:

1. Morphological (light microscopy [LM], transmission electron microscopy [TEM], scanning electron microscopy [SEM]) and histochemical examinations of normal, premalignant, and malignant human epithelium continue. The comprehensive description of the morphology of normal human colon is still incomplete, and some of the previously reported morphological markers of premalignancy may ultimately be declared normal aspects of different tissue segments. This possibility is suggested in electron microscopy (EM) data showing 3 colonic cell types (undifferentiated, endocrine, and mucous). In ascending segments, apical vesicles are EM dense on electron microscopic examination, but in the rectum, they are EM lucent. By LM, using histochemical stains, there are differences seen in ascending, transverse, and descending cells with mixed magenta and blue-purple staining; in the rectum, almost all cells stained blue, indicating highly acid mucus. HID-AB staining showed a large proportion of cells in all 4 regions staining brown-black, indicating high amounts of sulphomucin.

2. Human ascending, transverse, and rectal colonic epithelium from immediate autopsies are being maintained routinely in explant culture and provided to the NCI for xenotransplantation.

Pancreas: Pancreatic ducts from immediate autopsies were collected and perfused for cell isolation and culture. Although currently reinstated, collection of this tissue was temporarily discontinued as a necessary exclusion due to the rising cost of the resource and our inability to fund the full activity. All tissues received were characterized as described below.

Pancreatic tissues from immediate autopsy were examined by morphological techniques, histochemistry, immunohistochemistry, and freeze fracture. Pancreatic ducts were maintained using the contractor's organ explant and cell culture techniques, histocompatibility, immunohistochemistry, and freeze fracture. Routine autopsy and surgically derived tissues were examined to elucidate cellular alterations in pancreatic cancer. Previously, 40 cases (18 autopsy cases and 22 surgical cases) were under study, including both primary and metastatic tumors. Over 90% of these nonendocrine tumors looked like duct cell adenocarcinomas in routine LM.

Liver: Samples were collected and portions stored at  $-70^{\circ}\text{C}$ . The methods for culturing liver tissue and cells are still under study as indicated below.

Comparison of methods for the primary culture of human hepatocytes and rat hepatocytes are being continued using different media and substrates, including

human liver biomatrix. EM of zero-time samples is used to assess the viability of liver tissue at the time of perfusion. Comparisons are being made for optimal cell isolation between perfusion of intact lobes and wedge-shaped sections of lobes. Pieces of liver are also quick-frozen in liquid nitrogen for subsequent use in metabolic studies at the NCI. Preliminary results indicate that primary cultures of human liver cells can provide a mechanism for studying chemical metabolism and mutagenesis.

Proposed Course: During the coming year, emphasis will continue on providing and improving this unique resource to the NCI.

UNIVERSITY OF MARYLAND (N01-CP-31008)

Title: Resource for Human Esophageal Tissues and Cells from Donors with Epidemiological Profiles

Current Annual Level: \$80,399

Man Years: 1.27

Objectives: To provide tissue specimens and cells of human esophagus from epidemiologically defined donors to the Laboratory of Human Carcinogenesis for carcinogenesis studies; to provide fresh, well-characterized, and viable esophageal tissue for primary organ culture at the the NIH to create, characterize, and store monolayer cultures from esophageal tissues for delivery on request to the the NIH.

Major Contributions: Forty-four specimens were collected and characterized by the contractor. An epidemiological profile of the donors has been provided whenever possible. Tissues from the Medical Examiner (ME) sources possess chance viability in organ cultures, which decreases rapidly with time after death. Esophagi collected more than 8 hours post-mortem are unlikely to survive in vitro.

Monolayer cultures are developed according to the methods of Dr. Susan Banks-Schlegel (Exp. Cell Res. 146: 271-280, 1983). Explants from 110 "normal" uninvolved and 14 malignant human esophageal mucosa were cultured in this period. The cell banking facility is an integral part of this contract. Primary suspension cultures of cells from normal and malignant esophageal mucosa are frozen and thawed as cell stocks in viable condition. Cells were obtained from 8 normal human esophagi from medical examiner cases (immediate autopsies) and 4 esophageal cancers. Currently, there are approximately 2,000 vials of frozen stock, including 1900 epithelial cell suspensions (340 normal, 1500 malignant), 106 3T3 feeder cells, and 162 sarcoma-180 mouse tumor cells for the production of tumor-conditioned medium, both required for esophageal cell culture.

Morphological, cytochemical, and immunocytochemical characteristics were determined for each tissue collected and will be delivered to the NCI or elsewhere on request. Assays are also being conducted to determine specific biochemical markers occurring in normal, premalignant, and malignant human esophageal epithelium.

Proposed Course: The current tasks, including the in vitro testing of the quality of the cells now being generated and stored for in vitro carcinogenesis experimentation, will be continued and improved.

GEORGETOWN UNIVERSITY (N01-CP-31007)

Title: Collection and Evaluation of Human Tissues and Cells from Donors with an Epidemiological Profile

Current Annual Level: \$63,354

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 bronchoalveolar cells from bronchial lavage of normal smokers and nonsmokers, and (4) completed epidemiological questionnaires for medical and environmental histories.

Major Contributions: In this period, the contractor provided 15 specimens (1 benign, 14 malignant) from resected bronchus and peripheral lung, 34 specimens of pleural fluid for mesothelial cells, 27 sets of lavage samples (bronchoalveolar cells) and corresponding blood (mononuclear cells) preparations from 16 smoking and 11 nonsmoking normal volunteers. 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 the NCI.

Materials obtained from this contractor were used in ongoing studies in the In Vitro Carcinogenesis and Biochemical Epidemiology Sections of LHC. The human bronchial and peripheral lung tissues are used in ongoing studies of chemical carcinogenesis and human lung cancer. The mesothelial cell cultures from pleural fluids are used to examine in vitro effects of asbestos and other environmental agents that may be involved in the pathogenesis of malignant mesothelioma. The bronchoalveolar and peripheral blood mononuclear cells and the bronchoalveolar cells from smokers and nonsmokers were used in biochemical epidemiology studies to develop methods for monitoring human exposure to carcinogens and mutagens.

Proposed Course: To continue and improve provision of the specified tissues and cells to the NCI during the next contract period.

LITTON BIONETICS (N01-CP-15769)

Title: Resource for Xenotransplantation Studies of Carcinogenesis in Human Tissues in Athymic Nude Mice

Current Annual Level: \$252,000

Man Years: 2.43

Objectives: To provide an immunodeficient animal model, athymic nude mouse, (1) for long-term survival of human tissue xenografts; (2) for a continuing resource of athymic nude mice for long-term xenotransplantation, proliferation, and

tumorigenicity studies of normal, premalignant, and malignant human tissues; (3) to use human tissues to study the in vivo development of preneoplastic and neoplastic transformation induced in vitro and in vivo by carcinogens; and (4) to study the ability of selected agents and cellular manipulations to modify the effects of carcinogens on human tissues.

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 and the incorporation of labeled precursors into epithelial cells of the grafts.

Epithelium-denuded rat tracheas successfully serve as anchorage for human bronchus cells, which attach to and layer the luminal surface of the tracheas. Esophageal xenografts are characterized by epithelial growth and cyst formation.

In the breeding stock, 532 Swiss litters contained 3660 pups (6.9/litter), including 1810 nu/nu pups. Eighty-nine percent of these newborns survived, giving a total of 1610 or 2.3 surviving nudes/litter. During the year, the contractor maintains a monthly average colony population of 626 mice: 100 breeders, 174 newborns, 110 weanlings for new experiments, and 242 mice in experimental protocols.

In this period, 40 new experiments were initiated containing a total of approximately 467 mice. These included eighteen new (20 ongoing) experiments to study the growth rate and morphology of xenotransplanted HUT 294 cells, a bronchial epithelial cell line, implanted with and without a variety of X-irradiated feeder cells, irradiation of the host, and treatment of the host with anti-interferon, antiserum, or the implant with dimethylnitrosamine, cigarette smoke condensate, nickel (Ni), chromium, or arsenic; 14 new (13 ongoing) experiments to study (a) the morphology of xenotransplanted normal human esophageal (HE) tissue after treatment with N-methyl-N-nitro-N-nitrosoguanidine (MNNG), dimethylbenzanthracene, or dimethylsulfoxide; (b) growth of HE cells in antilymphocyte serum (ALS)-treated or X-irradiated mice; and (c) growth of HE carcinoma cells in normal nude mice; seven experiments to investigate xenographic characteristics of human mesothelial, pancreatic, and hepatic cells in nude mice; and one for effects of MNNG pretreatment of explant tissue and effects of MNNG in animals given azoxymethane after vascularization of the grafts. Other ongoing studies include experiments to study xenographic growth and morphology of human pancreas and bladder tissues with and without MNNG treatment or exposure to SV40 or SV40 + Ni<sub>3</sub>S<sub>2</sub> before xenotransplantation into ALS-treated nude mice. Five ongoing experiments to study xenographic morphology and growth of human colon were terminated.

Malignant transformation from tissue treated chemically in vitro continues to elude observation in xenografts. Tissue explants treated in vitro with carcinogens respond with epithelial abnormalities which, when xenografted, infrequently maintain transformation in the nude mouse for sufficiently extended periods. Squamous metaplasia occurs in grafts given carcinogens in vivo but has not become malignant. Emphasis is now being placed on increased immunosuppression as a means of effecting the change.

However, in this period, the successful xenotransplantation of oncogene transfected human bronchial epithelial (HBE) cell lines did produce tumors. One hundred percent of the nu/nu mice given subcutaneous injections of HBE cells



transformed in vitro by transfection with the Harvey ras gene developed progressively growing nodules.

Proposed Course: Continuation of the resource activities in the directions described and implied in the above discussion: (1) long-term testing of the effects of carcinogens and treatment regimens in vivo on human tissue xenografts in the athymic nude mouse; (2) examining for tumorigenic potential of human cells exposed to chemical and physical carcinogens in vitro; (3) xenotransplantation of transformed and nontransformed cells combined with other cells (species and type) with and without treatment with carcinogens, enzymes, or ionizing irradiation (cesium source); and (4) increased emphasis on further immunosuppression of the nude mouse to enhance its xenotransplantation capabilities. In this area, anti-mouse interferon antibodies, coinjection of human fibroblasts, antilymphocyte serum, and various monoclonal antibodies are being explored for this purpose. These efforts are ongoing and the results are at this writing insufficient for this report.

NATIONAL NAVAL MEDICAL CENTER (Y01-CP-30257)

Title: Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: \$16,144

Man Years: 1.0

Objectives: (1) To provide specimens of nontumorous bronchial and colonic epithelium (obtained at time of surgery for cancer or for benign lesions), with epidemiological profiles of medical and environmental histories for each donor, to the the NIH for the study of carcinogen activation and deactivation and (2) to determine the ability of human tissue to metabolize carcinogens to mutagens.

Major Contributions: Because of a staffing problem which occurred near the beginning of this new agreement, the National Naval Medical Center (NNMC) has delivered only 14 surgical specimens of human lung to the NCI. In December 1983, a technician was hired to fill the vacancy that was created by the resignation of the predecessor approximately 1 year ago. Unfortunately, there was unresolved difficulty early on and the position is once again vacant (March 19, 1984). Navy personnel is attempting to fill the slot position. We are currently continuing in the agreement with the intention to use a no-cost extension to recover the inactive period.

Proposed Course: A current no-cost, supplemental agreement to use the Navy technician for additional collections of tissues from the Walter Reed Army Hospital (WRAH) (Y01-CP-30504) cardiothoracic surgery department is now formulated. It is intended that, when the technician is hired, WRAH will increase the tissue yields and perhaps stabilize the technical aspects with more experienced management. In addition, specimens obtained from Walter Reed will increase the total volume without added cost.

VETERANS ADMINISTRATION HOSPITAL (Y01-CP-30255) (PREVIOUSLY LISTED AS Y01-CP-30205)

Title: Resource for Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: \$56,700

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; and (3) an epidemiological profile (including preoperative medical and environmental histories) for each donor.

Major Contributions: From lung and colon surgeries, the contractor delivered a total of 84 specimens: 38 lung (18 normal, 20 malignant); 24 colon (14 normal, 10 malignant); 10 bronchus; and 12 pleura. All tissues received were characterized by light and electron microscopy. Of the 42 patients who variously underwent pneumonectomy, lobectomy, bilobectomy, and local excision, 10 (24%) had squamous cell carcinoma, 6 (14%) had adenocarcinoma of the lung, and 6 (14%) had adenocarcinoma of the colon. Tissues delivered to the NCI came from cases with a sufficient excess after clinical diagnosis and characterization purposes were satisfied. Thirty-six of the 42 donors have epidemiological profiles completed and filed in the contractor's facility.

Thus, the contractor continues to obtain acceptable numbers and kinds of tissues from the surgical procedures involved, which in this period were as follows:

Right pneumonectomy	4
Left pneumonectomy	2
Right upper lobectomy	4
Right middle lobectomy	2
Right lower lobectomy	2
Left upper lobectomy	6
Mediastinal resection	2
Local excision	2
Colectomy	14
Total	38

By light microscopy, it was ascertained that 14 of the 38 patients did not harbor a malignancy, but rather had a number of other conditions ranging from normal epithelium to granuloma. The pulmonary malignancies were identified as follows:

Squamous cell carcinoma	10
Adenocarcinoma (lung)	6
Adenocarcinoma (colon)	6
Thymoma	2
Granuloma (pulmonary)	2
Emphysema	2
Lipoma	2
Colitis	2
Diverticulitis	2
Normal epithelium	4
Total	38

Proposed Course: Based upon mutual experiences to date, the contractor's activity should include the following: (1) continue obtainment and morphological examination of viable normal and abnormal human tissues from patients with an epidemiological profile and (2) increase obtainments to include more samples of esophageal, gastric, and colonic mucosa.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES (Y01-CP-00502)

Title: Hybridoma Resource

Current Annual Level: \$90,000

Man Years: 0.75

Objectives: To prepare and screen monoclonal antibodies to cell surface antigens and alkylated DNA for use in studying carcinogen and cocarcinogen actions in bronchial epithelium, including (1) identification of normal preneoplastic, and neoplastic bronchial epithelial cells; (2) characterization of transforming growth factors secreted by human cancer cells; and (3) measurement of DNA damage in normal and neoplastic bronchial epithelial cells caused by carcinogens and antitumor drugs.

Major Contributions: Mice have been injected with tobacco smoke condensate (TSC) adducted to DNA mixed with protamine, the latter serving as the adjunct. Spleen cells from mice producing antibody were fused with the HAT-sensitive NS-1 cells, cloned, and expanded, and the supernatants were screened for antibody. Further characteristics of specificity are being examined. In other studies, O<sup>6</sup>-ethylguanosine and N<sup>7</sup>-ethylguanosine were complexed specifically with protamine and used to immunize mice. Monoclonal antibodies resulting from this immunization were produced by the techniques described above. These antibodies react with ethylated-DNA. Specificity determinations are under way. Sera from individuals exposed to high levels of benzo[a]pyrene were assayed for antibodies to this compound adducted to DNA. Some of these individuals as well as others with lower levels of exposure were found to react with this compound.

Proposed Course: To develop monoclonal antibodies to a number of DNA adducts formed by environmental carcinogens, including those found in tobacco smoke; to produce monoclonal antibodies to the alkylated DNA adducts and surface antigens from chemically treated cells; and to analyze cell surface antigens for possible correlations with chemically induced DNA modifications.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

701CE04513-09 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Metabolism of Chemical Carcinogens by Cultured Human Tissues and Cells

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

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	Herman Autrup	Senior Staff Fellow	LHC	NCI
	Kirsi Vahakangas	Visiting Fellow	LHC	NCI
	John F. Lechner	Senior Staff Fellow	LHC	NCI
	Susan P. Schlegel	Senior Staff Fellow	LHC	NCI

COOPERATING UNITS (if any)

Univ. of Md. School of Medicine, Baltimore, MD (B. F. Trump);  
 Institute for Cancer Research, New York, NY (A. Jeffrey); Cancer Institute,  
 Peoples Republic of China (Sun Tsung-tang and Hsia Chu-chieh); Department of  
 Surgery, University of Nairobi, Nairobi, Kenya (J. Wakhisi)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

0.8

OTHER:

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.)

Human bronchus, colon, duodenum, esophagus, and pancreatic duct cultured either as explants or epithelial cells in chemically defined media provide an excellent in vitro system to study the metabolism of chemical carcinogens, including those found in tobacco smoke and the environment. Several classes of chemical carcinogens, polynuclear aromatic hydrocarbons, N-nitrosamines, hydrazines, aromatic amines, and mycotoxins, can be metabolically activated by human tissues. Fetal human liver, stomach, and esophagus cultured as explants metabolized the same group of compounds. The metabolic pathways leading to the formation of DNA adducts in explants and epithelial cell cultures have been defined for benzo[a]pyrene (BP), 7,12-dimethylbenz[a]anthracene, aflatoxin B<sub>1</sub> (AFB), and N,N-dimethylnitrosamine (DMN). The adducts between these carcinogens and DNA in human tissues are essentially the same as those found in experimental animals in which the chemicals are carcinogenic. Interindividual differences in carcinogen-DNA binding values vary 50- to 150-fold. The role of AFB in liver carcinogenesis has been further studied. We found that when urine samples collected in Kenya were analyzed for the presence of 2,3-dihydro-2-(7'-guanyl)-3-hydroaflatoxin B<sub>1</sub> (AFB-Gua I) by high pressure liquid chromatography, 11 of 128 samples had a detectable level of AFB-Gua I; its identity was confirmed by photon-counting fluorescence spectrophotometry. The positive samples were primarily from people living in low-lying areas and collected in the rainy season when AFB contamination of the food is highest.

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

Curtis C. Harris	Chief	LHC	NCI
Herman Autrup	Senior Staff Fellow	LHC	NCI
Kirsi Vahakangas	Visiting Fellow	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
Susan P. Schlegel	Senior Staff Fellow	LHC	NCI

Objectives:

To determine the metabolic pathways of chemical carcinogens in target tissues of experimental animals and humans. To measure interindividual and intertissue variations in the metabolism of carcinogens.

Methods Employed:

Explant cultures and epithelial cell cultures of human and animal tissues; isolation of cellular macromolecules; high pressure liquid chromatography; enzyme systems; and synchronous scanning fluorescence spectrophotometry.

Major Findings:

Cultured human bronchial mucosa can enzymatically activate procarcinogens (polynuclear aromatic hydrocarbons: 7,12-dimethylbenz[a]anthracene [DMBA], 3-methylcholanthrene [MCA], benzo[a]pyrene [BP], 6-nitrobenzo[a]pyrene [6-NO<sub>2</sub>-BP], and dibenz[a,h]anthracene [DBA]; N-nitrosamines: N-nitrosodimethylamine [DMN], N-nitrosodiethylamine, N-nitrosopiperidine, N-nitrosopyrrolidine [NPY], and N,N'-dinitrosopiperazine; a substituted hydrazine: 1,2-dimethylhydrazine [1,2-DMH]; a mycotoxin: aflatoxin B<sub>1</sub> [AFB<sub>1</sub>]; and an aromatic amine: 2-aminoacetylfluorene) into metabolites that bind to cellular macromolecules, including DNA.

The extrapolation of carcinogenesis data among animal species depends in part on qualitative and quantitative differences between metabolic activation and deactivation of procarcinogens. Therefore, the metabolism of benzo[a]pyrene (BP) has been extensively studied in explants of tracheobronchial tissues from experimental animals--hamsters, rats, mice, cows--and humans. The total metabolites as measured by both organic solvent-extractable and water-soluble metabolites of BP was substantial in the respiratory tract from humans and animal species susceptible to the carcinogenic action of BP. Furthermore, the results suggested that determination of both activation and deactivation pathways is important in assessing carcinogenic risk of a chemical. No qualitative difference in the profile of organosoluble metabolites (tetrols and diols being the major metabolites) was observed among the different species. The metabolism of BP-7,8-diol to BP tetrols was mediated not only by the mixed function oxidase system but also by the prostaglandin synthetase pathway. Addition of arachidonic acid to the culture medium enhanced the production of the BP-tetrols. The prostaglandin synthetase pathway did not activate BP

itself. The binding values of BP to cellular DNA were quite similar in all tissues, although slightly higher binding was observed in hamster trachea. Wide interindividual variation in the binding of BP to DNA was seen in tissues from outbred species. The major BP-DNA adducts in all animal species were formed by interaction of BP diol-epoxide with the 2-amino group of deoxyguanosine. Both stereoisomeric forms of ( $\pm$ )-(7 $\beta$ ,8 $\alpha$ )-dihydroxy-(9 $\alpha$ ,10 $\alpha$ )-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE I) reacted with deoxyguanosine, the (7R)-form being the most reactive. No difference in the relative distribution of the various adducts was seen among the species, except in the rat (DC, Wistar, and Buffalo), where BPDE-deoxyadenosine adducts accounted for 20% of the total modification. In cultured hamster trachea, the persistence of the different adducts was similar. In conclusion, the metabolism of BP is qualitatively similar in tracheobronchial tissues from both humans and animal species in which BP has been experimentally shown to be carcinogenic. The major DMBA-DNA adduct was also formed between the "bay-region" diolepoxide and the 2-amino group of deoxyguanine.

The metabolism of BP was studied in both epithelial and fibroblast cells initiated from the same bronchus specimens. The total metabolism was three-fold higher in the epithelial than in the fibroblast cells. No qualitative differences in the metabolic profile of BP between the explant culture and the epithelial cell cultures were observed.

Nontumorous esophagus cultured in a chemically defined medium metabolized BP, DMBA, AFB, DMN, and N-nitrosodiethylamine (DEN) to species that reacted with DNA. No detectable amount of radioactivity was associated with DNA after incubation with NPy. The major carcinogen-DNA adducts were (1) trans addition of (+) BP diol epoxide I at the 10 position to the 2-amino group of guanine; (2) with DMBA, addition of DMBA-3, 4-dihydroxy-1,2-epoxide to the 2-amino group of guanine; (3) with DMN, 7-methylguanine and 06-methylguanine (06-MeG/7-MeG = 0.3); and (4) with AFB, 2,3-dihydro-2-(N-guanyl)-3-hydroxy-aflatoxin B<sub>1</sub>.

The mean level of binding of BP in human esophageal DNA was lower than that in bronchus from the same individual and showed a 100-fold interindividual variation. As N-nitrosamines are potential esophageal carcinogens in rats, a comparative study in humans and rats on the metabolism of this group of compounds was performed. Both acyclic and cyclic N-nitrosamines were metabolized by rat esophagus. The highest level of metabolite binding was seen with N-nitroso-benzylmethylamine (BMN), an organotrophic carcinogen for the rat esophagus. The binding level was about 100-fold higher than in adult human esophagus. The results indicate significant quantitative and perhaps qualitative differences between cultured rat and human esophagus in their ability to activate N-nitrosamines.

Metabolism of various carcinogens in cultured human colon and duodenum has been investigated. Nontumorous tissue was collected at the time of either "immediate autopsy" or surgery from patients with or without colonic cancer. After 24 hours in culture, explants were exposed to radioactive-labeled carcinogen for another 24 hours, and the binding to cellular DNA was measured by radiometric methods. The following carcinogens were converted by human colon to species that bound to DNA: BP, 6-NO-BP, 1-nitropyrene, DMBA, AFB, DMN, DMH, and 3-amino-1,4-dimethyl-5H-pyrido(4,3b)indole. The latter is a potent fecal

mutagen formed by pyrolysis of tryptophan. The major carcinogen-DNA adducts were identified for BP, DMBA, and AFB and were found to be identical to the adducts formed in human bronchus. The mean level of binding of BP was higher in duodenum than in colon. A wide interindividual variation was observed. A positive correlation in the binding level of BP between bronchus and colon and duodenum from the same individual was seen.

Explant cultures of human fetal liver, stomach, and esophagus extensively metabolized chemical carcinogens into DNA binding species. The metabolism of N-nitrosamines--DEN, NPy, and BMN--was significantly higher in stomach than in the other tissue from the same fetus. Fetal esophagus did metabolize NPy into a DNA-binding metabolite in contrast to adult esophagus. The major BP-DNA and AFB-DNA adducts in fetal livers were similar to the adducts observed in other adult human tissues. The high pressure liquid chromatographic profile of organosoluble BP-metabolite was more complex than with adult tissues.

In order to access the role of AFB in human liver carcinogenesis, we collected urine samples in Kenya for analysis of AFB-Gua I, a "DNA-repair product." It has previously been shown that food samples collected are known to be contaminated with AFB, and a positive correlation exists between the dietary intake of AFB and the incidence of liver cancer. The urine samples collected at the outpatient clinic of Murang'a District Hospital was concentrated on C<sub>18</sub>Sep-Pak columns, and AFB-Gua I was isolated by high pressure liquid chromatography in two different systems. Eleven of 128 samples had a detectable level of a compound whose synchronous fluorescence spectrum was identical to chemically synthesized AFB-Gua I. The spectrum did not show any bathochromic shift when pH was made alkaline. These results are an indication of interactions between the ultimate carcinogenic form of AFB and cellular nucleic acids in vivo and further support the hypothesis that AFB may play an important role in the etiology of human liver cancer.

In order to understand the interaction of complex chemical mixtures, such as tobacco smoke and diesel exhaust, with biological systems, the postlabeling method of Randerath has been modified. Carcinogen-DNA adducts were formed when tobacco smoke condensate was incubated with DNA in the presence of liver microsomes. The identification of these adducts is presently being attempted.

#### Significance to Biomedical Research and the Program of the Institute:

Because most environmental carcinogens require metabolic activation to exert their carcinogenic effect, the study of their metabolic pathways and the reaction of the ultimate carcinogen with cellular macromolecules in potential human target tissues is important. Extension of these studies to more complex, potentially carcinogenic mixtures, such as tobacco smoke condensate, is important. The development of controlled culture conditions for human tissues provides a model system for these studies in intact human tissues. The use of explant culture also provides a link between studies in experimental animals and the human situation as the metabolism of the carcinogens can be studied at the same level of biological organization in both species; this information is essential for extrapolation of carcinogenesis data between species. Furthermore, human tissues obtained by immediate autopsy also will allow a comparative study in various organs from the same individual. From this study,

we hope to be able to identify an easily accessible cell type that can be used for the identification of individuals at high risk of developing chemically induced cancers.

Proposed Course:

To continue a combined laboratory-epidemiology study of carcinogen metabolism. To use ultramicroassays of carcinogen metabolism and carcinogen-DNA adducts so that metabolism and repair can be studied in biopsy specimens. Our attention will continue to focus on chemicals found in tobacco smoke and environmental agents, such as T<sub>2</sub> toxin. To compare the metabolism of chemical carcinogens in target tissues and possible "detector" cells, i.e., monocytes and macrophages, a study on the biological effect in relationship to level and type of carcinogen-DNA interaction will be continued.

Publications:

Atrup, H., Bradley, K., Shamsuddin, A. K. M., Wakhisi, J. and Wasunna, A.: Detection of putative adduct with fluorescence characteristics identical to 2,3-dihydro-2-(7'-guanyl)-3-hydroxyafatoxin B<sub>1</sub> in human urine collected in Murang's District, Kenya. Carcinogenesis 4: 1193-1195, 1983.

Atrup, H. and Harris, C. C.: Metabolism of chemical carcinogens by cultured human tissues. In Harris, C. C. and Atrup, H. (Eds.): Human Carcinogenesis. New York, Academic Press, pp. 169-194, 1983.

Atrup, H., Harris, C. C., Wu, S.-M., Bao, L.-Y., Pei, H.-T., Lu, S., Sun, T.-T. and Hsia, C.-C.: Activation of chemical carcinogens by cultured human fetal liver, esophagus, and stomach. Chem.-Biol. Interact. (In Press)

Harris, C. C., Grafstrom, R. C., Shamsuddin, A., Sinopoli, N., Trump, B. F. and Atrup, H.: Activation and deactivation of chemical carcinogens in cultured human tissues and cells. In Greim, H., Jung, R., Kramer, M., Marquardt, H. and Oesch, F. (Eds.): Biochemical Basis of Chemical Carcinogenesis. New York, Raven Press, pp. 123-237, 1983.

Harris, C. C., Vahakangas, K., Trump, B. F. and Atrup, H. Interindividual variation in carcinogen activation and DNA repair. In Omenn, G. (Ed.): Banbury Report: Genetic Predisposition in Responses to Chemical Exposures. New York, Cold Spring Harbor Laboratory, Vol. 16 (In press)

Hsia, C.-C., Tzian, B.-L. and Harris, C. C.: Proliferative and cytotoxic effects of fusarium T<sub>2</sub> toxin on cultured human fetal esophagus. Carcinogenesis 4: 1101-1107, 1983.

Nebelin, E., Atrup, H., Christensen, B. and Blomkvist, G.: Detection of metabolites of N-nitrosopyrrolidine and N-nitrosomethylethylamine in cultures of human bladder epithelial cells of normal origin. In Bartsch, H., O'Neill, I. K., Castegnaro, M. and Okada, M. (Eds.): N-nitrosocompounds - Occurrence and Biological Effects. Lyon, IARC Scientific Publication No. 41 (In Press)



Vahakangas, K., Autrup, H. and Harris, C. C.: Interindividual variation in carcinogen metabolism, DNA damage, and DNA repair. In Hemmenki, K. (Ed.): Methods of Monitoring Human Exposure to Carcinogenic and Mutagenic Agents. New York, Plenum Press (In Press)

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01CE04846-12 LHC
PERIOD COVERED October 1, 1983 to September 30, 1984		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Biochemical Analysis of Viral Infection and Its Control at the Cellular Level</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI:            Brenda I. Gerwin                      Research Chemist                      LHC            NCI		
COOPERATING UNITS (if any) Laboratory of Molecular Genetics, NICHHD, NIH (J. G. Levin); Personal Resources, Incorporated, Frederick, MD (A. Rein)		
LAB/BRANCH Laboratory of Human Carcinogenesis		
SECTION Carcinogen Macromolecular Interaction		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>In the course of studying a nonconditional polymerase mutant of B-tropic MuLV, we have found that enzyme pausing correlates with the presence of certain consensus sequences within multibranch loop structures. The pol mutant which synthesizes a truncated reverse transcriptase molecule is able to initiate reverse transcription and transfer from the 5' to the 3' end of genomic RNA but cannot read past one of the pause sites. Thus, the mutant terminates synthesis prematurely. The mutation in the pol gene has been sequenced and consists of the addition of one C residue to a 5 bp stretch of Cs. This addition results in immediate termination of translation since a TGA codon is brought immediately into frame. Analysis of this result in light of phenotypic characterization of the mutant allows us to conclude that the MuLV pol gene contains coding capacity for a protein of about 13 K daltons upstream of reverse transcriptase and a protein of about 40 K daltons downstream of the polymerase coding region and that the RNase H and DNA polymerase active sites are located in the N-terminal two-thirds of the reverse transcriptase molecule. In addition, we have determined the DNA sequence of 1.8 Kb from the 5' end of two molecularly cloned sarcoma viruses which either do (M1 MSV) or do not (HT1 MSV) produce virion structural proteins encoded in the 5' portion of the genome. We have shown that the primary differences occur in the 5' untranslated portion of the genome.</p>		

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

Brenda I. Gerwin	Research Chemist	LHC	NCI
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Objectives:

The goal of this project is to understand at the molecular level the life cycle of RNA tumor viruses, i.e., the expression of these viruses in cells infected both nonproductively and productively, as well as to understand the cellular control mechanisms applicable to these viruses. The topics of present interest are:

1. Analysis of the products of in vitro DNA synthesis by a mutant polymerase molecule. Further characterization of the mutant viral genome which codes for this protein.
2. Determination of the DNA sequences controlling the expression of the structural genes in Moloney MSV.

Methods Employed:

1. Products of DNA synthesis by mutant and wild-type enzymes are compared by gel electrophoresis, DNA blotting techniques, and filter hybridization. Computer analyses of pause sites are studied to detect structural and/or sequence features which result in enzyme pausing. Mutations are characterized with respect to base sequence by Maxam and Gilbert sequencing techniques after DNA cloning.
2. Maxam and Gilbert sequencing techniques are utilized to compare the relevant regions of the two viral clones. Constructs involving interchange of relevant portions of the virions are created to prove which changes are critical in the determination of function.

Major Findings:

1. We have shown that several intermediate DNA species arise from enzyme pausing during endogenous reverse transcription in MuLVs. Using the expected sequences of the intermediate and the known sequences of the AKR and Moloney MuLV genomes, we have located consensus sequences and multibranch loop structures which are significantly correlated with pause sites. In addition, through molecular cloning and DNA sequencing we have determined the nature of the molecular defect in the mutant. The mutant genome has six residues of cytosine where the wild-type has five. This change results in a frame shift which produces a premature termination of translation by bringing a stop codon into frame. Phenotypic characterization of this mutant has determined the molecular weight of the mutant enzyme. Since knowledge of the stop signal accurately positions the C terminus within the pol gene, the N terminus can be localized mathematically and the location of the wild-type C terminus deduced. These

calculations result in the finding that the MuLV pol gene contains enough information to encode a 13 K dalton protein 5' of reverse transcriptase and an approximately 40 K dalton protein 3' of the polymerase. By analogy with the known map of the avian leukemia viruses, we suggest that these proteins are a protease and an endonuclease, respectively. Furthermore, the location of the C terminus of the mutant reverse transcriptase indicates that the active sites for the RNase H and DNA polymerase activities reside within the amino terminal two-thirds of the molecule.

2. Sequencing of the 5' portion of the cloned murine sarcoma virus isolates (M1) and HT1 MSV has shown that M1 MSV which expresses the virion structural proteins has a 62 bp deletion in the untranslated region before p15 initiation, which results in a frame shift. HT1 MSV, which does not express structural proteins, has an added C residue in the same region, which also results in a frame shift. These two changes, however, result in shifts to different frames. At this point, a few hundred bases remain to be determined in order to be certain of which changes are significant.

Significance to Biomedical Research and the Program of the Institute:

1. Characterization of this polymerase mutant has increased our understanding of the mechanisms of RNA tumor virus replication and the genetic structure of the mammalian virions.
2. Information on genetic signals controlling gene expression are of major importance to understanding and manipulation of regulated growth of mammalian cells.

Proposed Course:

1. The characterization of the pol mutant has been completed in as much detail as is presently feasible and has been terminated.
2. The sequencing will be completed and constructs to prove which changes result in the phenotypic alterations of the two MSV genomes will be synthesized.

Publications:

Levin, J. G., Hu, S., Rein, A., Messer, L. and Gerwin, B. I.: Murine leukemia virus mutant with a frameshift in the reverse transcriptase coding region: Implications for pol gene structure. J. Virol. 51: 470-478, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05130-04 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Carcinogenesis Studies Using Cultures of Human Epithelial Cells

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

PI:	John F. Lechner	Senior Staff Fellow	LHC	NCI
Others:	Edward Gabrielson	Medical Staff Fellow	LHC	NCI
	George H. Yoakum	Senior Staff Fellow	LHC	NCI
	M. Edward Kaighn	Expert	LEP	NCI
	Curtis C. Harris	Chief	LHC	NCI

## COOPERATING UNITS (if any)

Univ. of Maryland School of Medicine, Baltimore, MD (B. F. Trump); Litton Bionetics, Rockville, MD (M. Valerio); Georgetown University 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 20205

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

1.0

## OTHER:

2.0

## CHECK APPROPRIATE BOXES)

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

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

Normal human bronchial epithelial (NHBE) cells inoculated at clonal density will multiply with an average generation time of 28 hours; the majority of the cells are small and migratory and have few tonofilaments. Adding human blood-derived serum (BDS) depresses the clonal growth rate of NHBE cells in a dose-dependent fashion. In contrast, 10 representative lines of human lung carcinomas either replicate poorly or fail to grow at all when inoculated at clonal density in serum-free medium; their rates of multiplication increase in direct proportion to the amount of BDS added to the optimized medium. BDS reduces the clonal growth rate of NHBE cells by specifically inducing the normal cells, but not lung carcinoma cells, to undergo squamous differentiation. In vitro carcinogenesis experiments with normal bronchial epithelial tissue and cell cultures have yielded populations of cells with abnormal characteristics. These phenotypically altered cells (PAC), which have keratin epithelial cell markers have extended population doubling potentials, abnormal human karyologies, and reduced response to differentiation control by serum. However, they are not tumorigenic. Human prostatic epithelial cells were transfected by protoplast fusion with the vHa-ras oncogene 34 population doublings post SV-40 virus infection. The transfected cells express the vHa-ras transcripts and phosphorylated P21 protein but, although they express two viral oncogenes, they are not tumorigenic.

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

John F. Lechner	Senior Staff Fellow	LHC	NCI
Edward Gabrielson	Medical Staff Fellow	LHC	NCI
George H. Yoakum	Senior Staff Fellow	LHC	NCI
M. Edward Kaighn	Expert	LEP	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To develop systems to study malignant transformation of human epithelial cells, including the following: (1) studying the in vitro carcinogenesis of bronchial epithelial cells using a defined system; (2) evaluating both the long-term and rapidly dividing explant cultures as model systems to study in vitro malignant transformation; and (3) studying the effects of chemical and physical cocarcinogens and promoters on the progression of carcinogen-induced, phenotypically altered cells (PAC) to malignancy.

Methods Employed:

Human bronchial tissues are obtained from the 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 human bronchial epithelial cells are developed from explant culture outgrowths. As many as 20 successive outgrowth cultures can be obtained from a single tissue by repeated transferring of the explant. Upon transfer of the explants to new dishes, the outgrowth cultures remaining in the original dishes are incubated in defined, serum-free medium to expand the population and are then subcultured. Markers to identify the normal epithelial cells in vitro (an important objective for this laboratory) include karyology; polygonal morphology; ciliary activity; scanning electron microscopic morphology; ultrastructural identification of tight junctions; desmosomes and tonofilaments; production of acidic and neutral mucopolysaccharides; and immunostaining of keratin, blood group antigens, and type IV collagen.

Long-term explant cultures are being used to scrutinize cellular changes induced by exposure to chemical carcinogens. Subsegmental bronchi are dissected free of peripheral lung tissue. The bronchi are then cut with a scalpel into 0.2 cubic cm<sup>3</sup> fragments. Four degassed Gelfoam sponges (2 square cm<sup>2</sup>) are placed in a 60 mm culture dish containing 3.0 ml of medium supplemented with 1% serum, and four or five bronchial fragments are placed on the surface of each sponge. This culture system is similar to organ culture in that the sponge supports keep the tissue fragments from becoming submerged. The cultures are incubated in an atmosphere box containing 50% O<sub>2</sub>:5% CO<sub>2</sub>:45% N<sub>2</sub> at 36.5°C. The box is rocked at 10 cycles per minute. The media (without and with incorporated carcinogens) are replaced at 4-day intervals. The tissues are incubated for 12 weeks before being dissociated and expanded as pure epithelial cell cultures.

Pure populations of bronchial epithelial cells are also exposed to chemical carcinogens for extended periods. Abnormal, mitotic cells are selected on the basis of aberrant terminal differentiation control and extended population doubling potential.

A clonally isolated culture of SV-40 virus infected human prostate epithelial cells was transfected by protoplast fusion with an E. coli strain carrying the plasmid, H1, that contains the vHa-ras oncogene on a 5.4 Kbp Eco RI fragment in the plasmid, pBR322. The SV-40 infected culture was infected with Kirsten virus. Cells that survived bath treatments were assessed for altered growth control properties and the presence of the oncogene RNA transcripts and translation products.

Both chemical and oncogene-altered epithelial cells are ultimately assessed for tumorigenicity in nude athymic mice.

#### Major Findings:

(1) Subsegmental bronchiolar tissues explanted onto Gelfoam supports were exposed to chemical carcinogens. After 12 weeks of incubation and continuous exposure, control cultures exhibited some squamous metaplasia, but the appearance of the glands was generally unremarkable, and cellular atypia was not noted. In contrast, epithelium continuously exposed to Ni<sup>2+</sup> (10 µg/ml, as NiSO<sub>4</sub>.6H<sub>2</sub>O) exhibited extensive cell growth, amorphous glands were common, and areas of cellular atypia with mitotic cells were frequent. Replicative cultures of epithelial cells were initiated from enzymatically dissociated, exposed, and control tissues. Cultures became quiescent after four (1:3) subculturing. After four additional weeks of incubation, colonies of mitotic epithelial cells appeared only in the cultures originating from the Ni<sup>2+</sup>-exposed tissues. These latter cultures expressed aberrant differentiation and growth control characteristics and are being assessed for tumorigenic properties. (2) A method for routinely initiating replicative epithelial cell cultures of human bronchus was developed. Large pieces of bronchus tissue were initially set up as explant cultures. After 5 to 10 days of incubation, very few fibroblastic cells were present among the epithelial outgrowth. At this time, the tissue explant was transferred to a new dish for reseeding a second wave of epithelial cell outgrowth. Sequential tissue transfer was repeated up to 20 times over a period of 1 year, and the epithelial cells were not discernibly different from the first outgrowth culture. (3) A defined (serum-free) medium (LHC-9) for normal human bronchial epithelial (NHBE) cells was developed. Clonal growth dose-response experimentation was used to tailor the concentrations of nutrients and growth factors to satisfy the requirements of the normal human bronchial epithelial cells. (4) Supplementation with as little as 0.25% fetal bovine blood-derived serum (BDS) resulted in a decrease in clonal growth rate; 8% supplementation completely inhibited growth by inducing terminal squamous cell differentiation. Human lung carcinoma lines were also incubated in LHC-9 medium without and with 8% BDS. The results showed that serum toxicity per se was not responsible for the observed inhibition of NHBE cell growth; all 10 carcinoma lines divided significantly more rapidly ( $p < 0.05$ ) in BDS-supplemented medium except for line A 427, which failed to grow at clonal density in LHC nutrient media. Thus, the carcinoma cells have both increased requirements for BDS mitogens and a greatly reduced response

to factors in BDS that induce the normal cells to undergo squamous differentiation. (5) The cytotoxicity of  $\text{NiSO}_4$  for NHBE cells was assessed by dose-response experiments. Both colony-forming efficiency (CFE) and population doublings per day (PD/D) were measured as a function of  $\text{NiSO}_4$  concentration. Concentrations less than  $5 \mu\text{g/ml}$  had no significant effect on growth parameters. However, an apparent differential cytotoxicity was noted with  $\text{NiSO}_4$  concentrations above  $10 \mu\text{g/ml}$ , i.e.,  $16 \mu\text{g/ml}$  of  $\text{NiSO}_4$  was required to reduce the CFE 50%, whereas only  $8 \mu\text{g/ml}$  of  $\text{NiSO}_4$  was required to halve the mean PD/D. The relatively greater cytotoxic effect on colony size was due to a progressive increase in the intercellular concentration of  $\text{Ni}^{2+}$  for more than 120 hrs. (6) Dose-response experiments were conducted to assess the carcinogenic potency of  $\text{NiSO}_4$ . These experiments were initiated by inoculating  $2 \times 10^5$  NHBE cells (2nd subculturing) into 100 mm culture dishes with media containing none and from  $0.5$  to  $20 \mu\text{g/ml}$  of  $\text{NiSO}_4$ . After 20 days of incubation, the cultures became confluent and mitotically quiescent, and the cells began to undergo squamous differentiation. After 40 days, foci of mitotic cells were observed in cultures exposed to either 10 or  $20 \mu\text{g/ml}$  of  $\text{NiSO}_4$ . After 75 days, approximately one PAC colony per 100,000 original cells at risk was noted. (7) Control cultures incubated for 75 days failed to grow when subcultured. In contrast, all of the PAC isolates that arose continued to multiply for three or more subculturings. However, seven of these clonally derived isolates ceased multiplication before the culture consisted of more than  $1 \times 10^5$  cells (a minimum of 17 population doublings assuming clonal origin of the culture). The remaining isolates could be subcultured an additional 6 to 12 times before multiplication ceased. These PAC cultures had characteristics noted for both the target (NHBE) cells and the carcinoma cells. The PAC cultures have reduced growth factor requirements and a greatly extended culture population doubling potential, reduced responsiveness to BDS and 12-O-tetradecanoylphorbol-13-acetate (TPA) differentiation-inducing signals and chromosomal abnormalities, all properties noted for the carcinoma cells. However, the altered cells have retained normal cell characteristics, i.e., they do not recognize BDS factors as mitogens nor are they tumorigenic. The phenotypically altered bronchial epithelial cells induced by prolonged exposure to  $\text{Ni}^{2+}$  may be premalignant bronchial epithelial cells, and a subsequent exposure to cocarcinogens might be required for these cells to progress to tumorigenicity. However, initial experiments to test this possibility by exposing PAC cultures to 7,12-dimethylbenz[a]anthracene have not yet been successful. (8) Transfection of the vHa-ras oncogene into SV-40 virus infected human prostatic epithelial cells causes a 50% reduction in the clonal growth rate followed by crisis and the emergence of more rapidly growing cells that may be immortal and express v-Ha ras transcripts and phosphorylated P21 protein. However, these cells are not tumorigenic.

#### Significance to Biomedical Research and the Program of the Institute:

The extrapolation of experimental animal data to man is a major problem in the study of carcinogenesis. One approach to provide a link is to develop model systems in cultured human tissues for carcinogenesis investigations. Such systems could be used for the identification of individuals who are highly susceptible to chemical carcinogens.



Proposed Course:

Experiments studying the effect of tumor-promoting agents on the PAC cells are underway. Experiments are also in progress to determine whether carcinogen-treated bronchial epithelial cells lose antigens commonly associated with normal cells and to demonstrate the growth characteristics ascribed to transformed cells. Changes in these properties may be indicative of premalignant transformation. Studies of the effects of multiple carcinogen exposures as well as cocarcinogenesis with physical and viral agents and transfected oncogenes on the development of neoplastic lesions in explant and cell cultures and xenotransplanted tissues will be continued and expanded.

Publications:

Lechner, J. F., Haugen, A., Tokiwa, T., Trump, B. F. and Harris, C. C.: Effects of asbestos and carcinogenic metals on cultured human bronchial epithelium. In Harris, C. C. and Autrup, H. (Eds.): Human Carcinogenesis. New York, Academic Press, 1983, pp. 561-585.

Lechner, J. F., McClendon, I. A., LaVeck, M. A., Shamsuddin, A. K. M. and Harris, C. C.: Differential growth control by platelet factors of squamous differentiation in normal and malignant human bronchial epithelial cells. Cancer Res. 43: 5915-5929, 1983.

Lechner, J. F., Stoner, G. D., Haugen, A., Autrup, H., Willey, J. C., Trump, B. F. and Harris, C. C.: In vitro human bronchial model systems for carcinogenesis studies. In Webber, M. M. and Sekely, L. (Eds.): In Vitro Models for Cancer Research. New York, CRC Press (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05133-05 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Epidemiological Studies Using Monoclonal Antibodies to Aflatoxin B1-DNA Adducts

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

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	Herman Autrup	Senior Staff Fellow	LHC	NCI
	Glennwood E. Trivers	Research Scientist	LHC	NCI
	Kirsi Vahakangas	Visiting Fellow	LHC	NCI
	Dean L. Mann	Medical Officer	LHC	NCI

COOPERATING UNITS (if any)

Boston University School of Public Health, Boston, MA (J. Groopman); MIT, Cambridge, MA (G. Wogan); Cancer Institute, Chinese Academy of Medical Sciences, Beijing, Peoples Republic of China (Sun Tsung-tang and Hsia Chu-chieh)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

2

PROFESSIONAL:

1

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

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

Immunological approaches to measure DNA damage caused by carcinogens may be useful in biochemical and molecular epidemiological studies to identify individuals at high cancer risk. Monoclonal antibodies to aflatoxin B1-adducted DNA and to aflatoxin B1 and its metabolites have been characterized and, in conjunction with competitive ultrasensitive enzyme immunoassay, used to quantitate aflatoxin B1-modified DNA in liver obtained from rats that received doses ranging from 0.01 to 1.0 mg of aflatoxin B1/Kg. In addition, a complementary biophysical approach, i.e., synchronous scanning fluorimetry with 3-dimensional image computer processing, has been developed to measure these adducts. At this time, the limit of detection is 1 aflatoxin B1 residue per 1,355,000 nucleotides. Enzyme radioimmunoassays using these monoclonal antibodies and synchronous scanning fluorimetry are being used to measure aflatoxin B1-DNA adducts in liver samples from individuals at high risk of developing liver cancer.

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

Curtis C. Harris	Chief	LHC	NCI
Herman Autrup	Senior Staff Fellow	LHC	NCI
Glennwood E. Trivers	Research Scientist	LHC	NCI
Kirsi Vahakangas	Visiting Fellow	LHC	NCI
Dean L. Mann	Medical Officer	LHC	NCI

Objectives:

Monoclonal antibodies and fluorimetric techniques will be used to detect carcinogen-DNA adducts in human tissue specimens and cells and will be used in experiments such as (1) determination of exposure of individuals to chemical agents, (2) adduct distribution in different organs, (3) DNA repair studies, and (4) experimental in vitro carcinogenesis.

Methods Employed:

Spleens from immunized mice are removed 2 to 3 days after the last immunization and minced in Dulbecco's phosphate-buffered saline. Spleen cells ( $10^8$ ) are mixed with myeloma cells ( $10^7$ ), fused with polyethylene glycol, and grown in selected medium. Myeloma cells will not grow in the selective hypoxanthine/aminopterin/thymidine (HAT) medium. Since spleen cells will not grow in culture, the only cells that survive are cell hybrids. Hybrid cells are dispersed in 96-well plates and incubated at 37°C. Cell growth after 14 days is recognized as a successful hybrid. Cells can then be cloned with thymus cells with a modified enzyme-linked immunosorbent assay. Cells producing specific antibody are recloned, expanded, and injected i.p. into mice for development of ascites tumor. Monoclonal antibodies from ascites and cell culture medium are then isolated and characterized. The characterization procedures recognize only aflatoxin bound to DNA and not free aflatoxin B<sub>1</sub> (AFB<sub>1</sub>)-guanine adducts or six other AFB<sub>1</sub> metabolites. These assays are performed using both enzyme-linked immunosorbent assay (ELISA), ultrasensitive enzyme radioimmunoassay (USERIA), and photon counting synchronous scanning fluorimetry techniques.

Major Findings:

Immune response to AFB<sub>1</sub>-DNA adducts was obtained by injecting methylated bovine serum albumin-AFB<sub>1</sub>-DNA conjugate or AFB<sub>1</sub> conjugate emulsified in Freund's complete adjuvant into mice. Hybridoma clones producing monoclonal antibodies against AFB<sub>1</sub>-DNA adducts or AFB<sub>1</sub> have been obtained and characterized. Competitive ELISA using these monoclonal antibodies reliably quantitated AFB<sub>1</sub> residue per 1,355,000 nucleotides. The competitive USERIA was determined to be at least 10- to 12-fold more sensitive than the competitive ELISA in analysis of AFB<sub>1</sub>-adducted DNA. Using biotinylated monoclonal antibody and an avidin enzyme conjugate, a competitive enzyme immunoassay has also been developed. In addition, monoclonal antibodies to aflatoxin B<sub>2</sub> have been found to react with AFB<sub>1</sub>-modified DNA. These antibodies will be useful in confirming results

obtained with our initial antibodies. Synchronous scanning fluorimetry with 3-dimensional image analysis is proving to be a highly sensitive and specific method for measuring DNA adducts and metabolites of AFB<sub>1</sub>. This methodology complements the immunological techniques and has been successfully used in measuring DNA adducts in the rat model of AFB<sub>1</sub>-induced liver carcinogenesis.

Significance to Biomedical Research and the Program of the Institute:

Methods are being developed to quantitatively measure carcinogen-DNA adducts in femtomole and less amounts. These methods will be useful in studies of the molecular interactions of carcinogens and the cell genome and in measurement of carcinogen-DNA adducts in biopsy specimens from people in high- and low-risk environments for cancer.

Proposed Course:

Studies are underway to make monoclonal antibodies that specifically recognize other carcinogen-DNA products as well as isolated base adducts. The monoclonal antibodies and USERIA are being utilized to search for carcinogen-DNA adducts in human biopsy specimens and to determine (1) their rate of formation and removal and (2) their value in predicting an individual's cancer risk.

Publications:

Harris, C. C.: Role of carcinogens, cocarcinogens and host factors in human cancer risk. In Harris, C. C. and Autrup, H. (Eds.): Human Carcinogenesis. New York, Academic Press, 1983, pp. 941-970.

Harris, C. C. and Sun, T.-T.: Multifactorial etiology of human liver cancer. Carcinogenesis 5: 697-701, 1984.

Shamsuddin, A. K. M. and Harris, C. C.: Improved enzyme immunoassays using biotin-avidin-enzyme complex. Arch. Path. Lab. Med. 107: 514-517, 1983.

Trivers, G. E., Harris, C. C., Rougeot, C. and Dray, F.: Development and use of ultrasensitive enzyme immunoassays. In Conn, P. M. (Ed.): Methods in Enzymology. New York, Academic Press, 1983, Vol. 103, pp. 409-434

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05192-04 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Repair of Carcinogen-Induced Damage in Human Cells

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

PI:	Curtis C. Harris	Chief	LHC	NCI
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Others:	Herman Autrup	Senior Staff Fellow	LHC	NCI
	John F. Lechner	Senior Staff Fellow	LHC	NCI
	Hans Krokan	Guest Researcher	LHC	NCI
	Albert J. Fornace, Jr.	Expert	ROB	NCI

## COOPERATING UNITS (if any)

Department of Physiology, Hershey Medical Center, Hershey, PA (A. E. Pegg);  
 Department of Pathology, University of Maryland School of Medicine, Baltimore, MD  
 (B. F. Trump); Karolinska Institute, Stockholm, Sweden (R. C. Grafstrom)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Carcinogen Macromolecular Interaction Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

Normal adult human tissues and cultured bronchial epithelial cells and fibroblasts exhibit O6-alkylguanine-DNA alkyltransferase activity in vitro by catalyzing the repair of the promutagenic alkylation lesion O6-methylguanine from DNA. The amount repaired by extracts of liver, peripheral lung, and colon extracts was proportional to the amount of extract protein. Repair of O6-methylguanine led to stoichiometric regeneration of guanine in the DNA and stoichiometric formation of S-methylcysteine in protein. Alkyltransferase activity varies in the different human tissues tested in the decreasing order of liver > colon > esophagus > peripheral lung > brain. Extracts of lung tissues, cultured human bronchial epithelial cells and fibroblasts had similar alkyltransferase activities. Various human tissues exhibit 2- to 10-fold higher alkyltransferase activity than corresponding rat tissues. Whereas the interindividual variation of the activity was 4- or 5-fold in 10 or more human lung and colon specimens, the interindividual variation in the inbred rat was less than 20%. The present results show that different human tissues and cells have a several-fold higher capacity to repair O6-methylguanine in DNA than rat tissues and that the repair process occurs via a mechanism similar to that previously shown in other mammalian cells and *E. coli*. Formaldehyde inhibits repair of O6-methylguanine and potentiates the mutagenicity of an alkylating agent, N-methyl-N-nitrosourea, in normal human fibroblasts. Because formaldehyde alone also causes mutations in human cells, formaldehyde may cause genotoxicity by a dual mechanism of directly damaging DNA and also inhibiting repair of mutagenic and carcinogenic DNA lesions caused by other chemical and physical carcinogens.

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

Curtis C. Harris	Chief	LHC	NCI
Herman Autrup	Senior Staff Fellow	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
Hans Krokan	Guest Researcher	LHC	NCI
Albert J. Fornace, Jr.	Expert	ROB	NCI

Objectives:

To understand the mechanism of repair of DNA damage by environmental agents in human epithelial tissues and cells and to investigate the genotoxicity of formaldehyde and other tobacco smoke-related aldehydes.

Methods Employed:

Culture of human epithelial and fibroblastic cells; alkaline elution techniques for detection of DNA single strand breaks (SSB) and DNA protein crosslinks (DPC); BND cellulose chromatography for measurement of repair replication; <sup>3</sup>H-thymidine incorporation in the presence of hydroxyurea for measurement of unscheduled DNA synthesis; isolation of cellular macromolecules; high pressure liquid chromatography. O6-Alkylguanine-DNA alkyltransferase activity found in extracts from a variety of human tissues was characterized and quantitated in three ways: (1) measuring the specific loss of labeled O6-methylguanine (O6-MeGua) from a <sup>3</sup>H-methylated DNA substrate, (2) measuring the production of protein containing S-[<sup>3</sup>H]-methylcysteine during the reaction with this DNA substrate, and (3) measuring the formation of [8-<sup>3</sup>H]-guanine in DNA when the extracts were incubated with a synthetic DNA substrate containing O6-MeGua labeled in the 8-position.

Major Findings:

We have investigated alkyltransferase activity in various human tissues and compared it with the corresponding rat tissues. The alkyltransferase activities of cultured normal human bronchial epithelial cells and fibroblasts were also compared. Extracts of human colon, esophagus, and lung had lower activities than those previously found in human liver samples that showed somewhat higher activities than human brain. When compared with the corresponding rat tissue, human tissue samples contained 2- to 10-fold higher levels of alkyltransferase activity.

We also compared O6-alkylguanine-DNA alkyltransferase activity in two types of cultured human bronchial cells. The removal of O6-MeGua was proportional to the protein content of the extracts in both cell types up to 2 mg of protein added. When assayed under linear conditions, human fibroblasts and epithelial cells removed  $193 \pm 45$  and  $137 \pm 28$  fmol O6-MeGua per mg protein (mean  $\pm$  S.D.), respectively. No significant difference was observed between either exponentially growing or highly confluent bronchial fibroblasts. When the

activity of Chinese hamster V79 cells was assayed under similar conditions as the human cells, the activity of V79 cells was so low that it could only just be detected in extracts obtained from one billion cells and corresponded to  $1.6 \pm 1.2$  fmol O6-MeGua removed per mg protein.

Formaldehyde (HCHO) is a common environmental pollutant found in tobacco smoke and a metabolite of demethylation reactions of drugs and carcinogenic N-nitrosamines. It is also a respiratory carcinogen in rats and a potential carcinogenic hazard in humans. Therefore, we have initiated a systematic study of the genotoxicity of HCHO in cultured human cells. The alkaline elution technique was used to study repair of DNA damage caused by HCHO in human bronchial epithelial cells and fibroblasts, skin fibroblasts, and DNA excision repair-deficient skin fibroblasts from donors with xeroderma pigmentosum (XP). Exposure of cells to HCHO resulted in DNA-protein crosslinks (DPC) and DNA single strand breaks (SSB) in all cell types. DPC were induced at similar levels and were also removed by all cell types, including the XP cells. By excision repair of HCHO-induced DNA damage, normal cells generated SSB that were also readily repaired. HCHO was only moderately cytotoxic to normal bronchial epithelial cells and fibroblasts at concentrations that induced substantial DNA damage. HCHO enhanced the cytotoxicity of both ionizing radiation and N-methyl-N-nitrosourea in both cell types. The results indicate that most DPC caused by HCHO can be removed without the involvement of DNA excision repair. Furthermore, HCHO also directly causes DNA SSB as well as SSB generated indirectly during UV-type excision repair. 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.

Since HCHO is formed in equimolar quantities with methylcarbonium ions during the metabolic activation of N-nitrosodimethylamine, we have recently examined the effects of HCHO on the repair of the promutagenic lesion O6-MeGua formed following N-nitrosodimethylamine metabolism. HCHO decreases O6-alkyltransferase activity, inhibits the removal of O6-MeGua, and in low concentrations, synergistically potentiates the cytotoxicity and mutagenicity of N-methyl-N-nitrosourea. In high doses (100 or 130  $\mu$ M), HCHO is detectably mutagenic itself. Therefore, exposure to HCHO may lead to the dual genotoxic mechanism of both directly damaging DNA and inhibiting repair of mutagenic and carcinogenic lesions caused by alkylating agents and physical carcinogens.

#### Significance to Biomedical Research and the Program of the Institute:

Methodologies developed for and utilized in studies of DNA damage and repair in animal (normal and tumor) cells can be successfully extended to similar investigations in cells cultured from human tissues susceptible to carcinogenesis. These investigations should aid in identifying mechanisms by which chemical and physical agents will damage the genetic material and exert carcinogenic and/or cocarcinogenic properties.

#### Proposed Course:

Identify endogenous and exogenous agents, especially those found in tobacco smoke or produced by tumor promoters that will damage DNA and/or affect its repair. To compare the levels of DNA damage (i.e., DNA SSB, DPC, or repair

replication) with levels and persistence of DNA adducts caused by chemical carcinogens. To continue correlation of the extent of DNA damage from various agents with biological effects, including toxicity, mutagenesis, and transformation assays.

Publications:

Grafstrom, R. C., Fornace, A. J., Jr. and Harris, C. C.: Repair of DNA damage caused by formaldehyde in human cells. Cancer Res. (In Press)

Grafstrom, R. C. and Harris, C. C.: Metabolism of N-nitrosamines and effects of formaldehyde on DNA repair in cultured human tissues and cells. In Rydstrom, J., Montelius, J. and Gengtsson, M. (Eds): Extrahepatic Drug Metabolism and Chemical Carcinogenesis. Amsterdam, Elsevier Biomedical Press, 1983, pp. 527-540.

Grafstrom, R. C., Pegg, A. E., Trump, B. F. and Harris, C. C.: O6-Alkylguanine-DNA alkyltransferase activity in normal human tissues and cells. Cancer Res. (In Press)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05193-04 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

**Alterations in Growth and Differentiated Properties in Human Carcinoma Cells**

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

PI:	Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Adi Gazdar	Medical Officer	NMOB	NCI

COOPERATING UNITS (if any)

University of Maryland School of Medicine, Baltimore, MD (E. McDowell);  
 VA Hospital, Washington, D.C. (P. Schafer); Litton Bionetics, Rockville, MD  
 (M. Valerio); University of Alabama, Birmingham, AL (K. Sexton)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Cancer of the human esophagus and lung represents a major cause of death in certain populations of people throughout the world. In focusing our efforts on these two organ systems, we have initially evaluated the usefulness of analysis of keratin protein patterns and cross-linked envelopes in the characterization of epithelial neoplasms. Distinctive qualitative and quantitative differences in the spectrum of keratin proteins were found in the carcinomas compared with their nontransformed counterparts. Analysis of keratin protein patterns appeared to be a useful adjunct in defining the type of tumor present. Moreover, assessment of cross-linked envelope-forming capabilities served as a specific marker for squamous differentiation, and the extent of envelope formation correlated well with the degree of squamous differentiation. The tumor with more well-differentiated squamous carcinomas formed more cross-linked envelopes. We have established human esophageal and lung carcinoma cell lines in cell culture to evaluate if their properties in vitro faithfully manifest those of the original tumor, thereby representing useful models of carcinogenesis in vitro. Moreover, we have compared the growth and differentiated properties of these carcinoma cells with their nontransformed counterparts. Numerous morphological and biochemical differences were observed between normal and malignant epithelial cells in culture. Significant changes in the array of keratins and in the proportions of cells making cross-linked envelopes were noted. The results we obtained paralleled findings with tumor masses indicating that the tumor cells in cell culture continue to maintain a program of gene expression reflective of that of the original tumor.

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

Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Adi Gazdar	Medical Officer	NMOB	NCI

Objectives:

The growth and differentiation of normal human epithelial cells will be analyzed and compared with those of their neoplastic counterparts. We will attempt to establish neoplastic epithelial cells in culture and examine the growth and differentiated properties of these cells compared with their normal counterparts. These studies are designed to provide insight into the specific changes that occur during malignant transformation. In particular, initial studies will evaluate the usefulness of analysis of keratin protein patterns and cross-linked envelopes in the characterization of epithelial neoplasms. Other defects in the pathway of terminal differentiation will also be assessed.

Methods Employed:

Human tissue is obtained from "immediate" autopsy (i.e., performed within 1 hour of death), from autopsy material that is less than 12 hours postmortem, or from surgical specimens. The epithelium and some adherent connective tissue are carefully dissected from the remainder of the tissue. In some instances, the epithelium is separated from the adherent stroma either by heat separation or by surgical excision, and the epithelium is analyzed for keratin proteins. In other cases, the epithelium is first cut into explants and used for radiolabeling of proteins. Tumors (minced) were radiolabeled in an analogous manner. When being used for cell culture, the epithelium or tumor is minced and trypsinized to obtain a single-cell suspension. The cells are grown on tissue culture dishes containing a layer of lethally irradiated 3T3 cells. The cells are fed every 3 to 4 days in medium containing 10% fetal calf serum plus various hormonal supplements and growth factors. The morphological and biochemical characterization of the cells is being assessed by a variety of techniques: light and electron microscopy, histochemical staining, immunofluorescent staining, radiolabeling of macromolecules, immunoprecipitation, polyacrylamide gel electrophoresis, autoradiography, and peptide mapping. The terminal differentiation of the cells is triggered by means of calcium ionophore, and the extent of terminal differentiation is assessed by cross-linked envelope formation. When using human tumor material, the epithelium is not separated from adherent stroma. Otherwise, the tumor and normal tissues are handled identically.

Major Findings:

Cancer of the human esophagus and lung occurs worldwide and frequently represents a major cause of death in the population. Therefore, we have chosen to focus our efforts on these two organ systems.

## 1. Human Esophageal Epithelium and Esophageal Carcinoma: Tumor and Cell Culture Studies

In contrast to the simplified keratin content of bovine, rabbit, and rat esophageal epithelium (composed mainly of a 57 and 46 or 51 kD keratin, depending on the animal species), human esophageal epithelium contains a quantitatively different array of keratin proteins, ranging in molecular weight from 37 to 61 kD. The pattern of keratin proteins from human esophageal epithelium differs qualitatively and quantitatively from that of human epidermis. Human esophageal epithelium lacks the 63, 65, and 67 kD keratins characteristic of human epidermis, consistent with the absence of a granular layer and an anucleate stratum corneum. Human esophageal epithelium contains a distinctive 61 kD keratin protein, which was either not present or present in only small amounts in human epidermis, and variable amounts of a 37 kD keratin. Whereas the 56, 59, and 67 kD keratins were the most abundant keratins in human epidermis, the 52, 57, and 61 kD keratins predominated in human esophageal epithelium. During in vitro cultivation, both human epidermal and esophageal keratinocytes produced colonies that were stratified; however, the morphologic appearance of these cultured epithelia differed. Only cultured human epidermal keratinocytes contained keratohyalin granules in the outermost layers and a prominent 67 kD keratin on immunoprecipitation, reflective of their in vivo program of differentiation. Otherwise, the keratin contents appeared similar. In conclusion, human esophageal epithelium exhibited intertissue and interspecies differences in the pattern of keratin proteins. During in vitro cultivation, human esophageal keratinocytes retained some aspects of their distinctive program of differentiation.

Analysis of keratin proteins extracted from human esophageal tumors revealed dramatic changes in the pattern of keratins. In addition to an overall reduction in the amount of keratin, most tumors were characterized by a complete loss of the major 52 and 61 kD esophageal keratins. The lower molecular weight keratins (48 to 50.5 kD) and the 57 kD keratin were conserved in the transformed phenotype. Injection of these tumors into nude mice and analysis of the tumors for keratin proteins revealed an even more dramatic shift in patterns of keratins. To analyze for alterations in the pathway of terminal differentiation in human esophageal carcinomas, we examined for the expression of another differentiated function, cross-linked envelopes. The formation of cross-linked envelopes, structures resistant to SDS and a reducing agent, was induced using the calcium ionophore, X-537A, and then the extent of terminal differentiation was estimated by calculating the percentage of cells that formed cross-linked envelopes. Compared to normal esophageal epithelial cells, human esophageal carcinoma cells exhibited a variable capacity to form cross-linked envelopes, ranging from unimpaired to a severely restricted capacity to form cross-linked envelopes. In general, there was some correlation between envelope-forming capabilities and the degree of tumor differentiation, with more differentiated tumors forming more envelopes. While the majority of the tumor examined (6 out of 8 cases) displayed a markedly reduced capacity to form envelopes, indicating a defect in the pathway of terminal differentiation, carcinoma cells from two tumors formed cross-linked envelopes at levels comparable to that of normal esophageal epithelium.

After repeated attempts at establishing esophageal epithelial tumors in culture, we have succeeded in establishing seven tumor cell lines. We have undertaken a

number of studies to examine various growth and differentiated properties exhibited by these human esophageal carcinoma cells. While the cells from most of the tumor cell lines looked typically epithelial, the cells were morphologically distinguishable from normal human esophageal epithelial cells when examined by phase contrast microscopy. In contrast to normal esophageal epithelial cells, which were uniform in appearance and polygonally shaped and underwent a very organized and orderly stratification process, human esophageal carcinoma cells were very pleomorphic, varied greatly in size and shape, and tended to pile up on one another in an unorganized manner. When grown under optimal growth conditions (Medium 199 containing 10% fetal calf serum and various growth supplements), human esophageal carcinoma cells reached a much higher saturation density ( $5$  to  $8 \times 10^6$  cells/60 mm dish) than their nontransformed counterparts ( $2.5$  to  $3 \times 10^6$  cells/60 mm dish). Their doubling times ranged from approximately 33 hours (similar to normal human esophageal epithelial cells) to 82 hours. Because transformed cells have been reported to exhibit reduced serum and growth factor requirements, we also examined the ability of the cells to grow under more stringent growth conditions (Medium 199 plus 2% fetal calf serum and hydrocortisone). Interestingly, although the carcinoma cells grew better than the normal cells under stringent growth conditions, they grew much more slowly than they had under optimal growth conditions (in most cases) and reached lower saturation densities (only 1 to  $1.5 \times 10^6$  cells/60 mm dish), suggesting that these esophageal carcinoma cells are not significantly altered in terms of their serum and growth factor requirements compared to their nontransformed counterparts. Similar to normal esophageal epithelial cells, the esophageal carcinoma cell still required cocultivation with a layer of irradiated 3T3 fibroblasts for growth at clonal densities. When assessed for anchorage-independent growth, all carcinoma cells displayed some capacity to grow in agarose, although the colony-forming efficiency and size of the colonies varied, depending on the cell line. Normal esophageal epithelial cells did not form colonies in agarose. When evaluating tumorigenicity in nude mice, the different esophageal carcinoma cell lines exhibited varying capacities to form tumors in nude mice.

Next, we investigated human esophageal carcinoma cell lines for the expression of certain differentiated functions associated with normal human esophageal epithelial maturation, namely keratin proteins, a major cytoskeletal component, and cross-linked envelopes as a measure of the extent of terminal differentiation. Radiolabeled keratin proteins were extracted using high salt and detergent, selectively immunoprecipitated with keratin antiserum, and analyzed on gels. Surprisingly, in contrast to the results with tumor masses (summarized above) in which specific changes in keratin proteins characterized the malignant phenotype, the esophageal carcinoma cell lines exhibited a greater variability in the spectrum of keratin proteins associated with malignant transformation. While some cell lines exhibited the loss of the major 52 kD keratin, others did not. Moreover, some cell lines showed a loss of the 57 kD keratin, which was never found to be missing in studies with esophageal tumor masses, and two lines expressed a high molecular weight 67 kD keratin. Human esophageal tumors in nude mice always revealed loss of the 61 and 52 kD keratins, similar to tumor masses. The patterns of keratin proteins in tumors formed by injection of the carcinoma cell lines into nude mice are being compared to the original cell line and to cell lines reestablished from the nude mice tumors. In contrast to the typical perinuclear arrangement of keratin filaments found within normal esophageal epithelial cells, keratin filaments in esophageal carcinoma cells were usually distributed uniformly

throughout the cytoplasm or located peripherally, frequently in association with desmosomes. To assess the extent of terminal differentiation in cultured normal human esophageal epithelial cells and esophageal carcinoma cells, cells were induced to terminally differentiate with calcium ionophore, and then the percentage of cells with cross-linked envelopes, structures resistant to detergent and a reducing agent, was determined. Analogous to findings with tumor masses, some, but not all, esophageal carcinoma cell lines were found to exhibit a reduced capacity to form cross-linked envelopes. Therefore, these results suggest that expression of the transformed phenotype sometimes, but not always, leads to a defect in the pathway of terminal differentiation as assayed by this marker. These cell lines are currently being examined for alterations in the expression of a number of other properties commonly ascribed to transformation.

## 2. Human Bronchial Epithelium and Bronchiogenic Carcinomas: Tumor and Cell Culture Studies

Most human lung tumors arise from the area of the bronchus. They have been classified by the World Health Organization on the basis of their histological appearance and synthetic product(s) into four major classes specifically: squamous cell carcinoma, adenocarcinoma, large-cell carcinoma, and small-cell lung carcinoma. Keratins have been found to be useful not only for delineating the epithelial nature of the tumor but also as an adjunct in defining the type of tumor present. For instance, well-differentiated squamous cell carcinomas, urotheliomas, and mesotheliomas tend to be strongly keratin positive. Adenocarcinomas tend to be weakly positive to negative. Both adenocarcinomas and squamous cell carcinomas of the lung contained keratin proteins, as demonstrated by immunocytochemical and electron microscopic data. However, the amount of keratin varied depending on the tumor type (decreased in adenocarcinomas) and the degree of squamous differentiation (decreased in poorly differentiated tumors) (studies performed in collaboration with Drs. Elizabeth McDowell, Benjamin Trump, and Tom Wilson at the University of Maryland). Since the lung tumors were not easily classified on the basis of immunoperoxidase staining or ultrastructural localization of keratin, we analyzed keratin-enriched protein fractions of these tumors by one-dimensional gel electrophoresis to investigate their usefulness in distinguishing these lung neoplasms. Keratin extraction data and keratin immunoprecipitation data revealed that there were distinct qualitative and quantitative differences useful in distinguishing adenocarcinomas from squamous cell carcinomas of the lung.

Human lung tumor cell lines established from the major histological types of lung cancer were examined by immunofluorescent staining techniques for their pattern of intermediate filament (IF) (keratin, vimentin, and neurofilament triplet protein) expression. The cell lines had been established by Dr. Adi Gazdar and collaborators at the NCI-Medical Oncology Branch, Bethesda, MD. All cell lines examined, both small-cell lung carcinoma (SCLC) and non-SCLC (squamous cell carcinoma [SQC], adenocarcinoma [AC], large-cell carcinoma [LCC], and mesothelioma) contained keratin, consistent with their epithelial derivation. These lung carcinoma cell lines also expressed vimentin, the characteristic intermediate filament of mesenchymal cells in vivo, similar to previous reports demonstrating a coexpression of vimentin and keratin in carcinoma cells in vitro. In light of the proposed "neuroectodermal" origin of SCLC, cell lines were also studied for neurofilament expression. Two of

four SCLC tumor cell lines, as well as non-SCLC cell lines, showed no reactivity with antibodies to neurofilament triplet protein. Two of the SCLC cell lines stained weakly with anti-neurofilament antibody. Examination of specific keratin patterns in human lung tumor cell lines by selective immunoprecipitation with keratin antiserum and SDS-polyacrylamide gel electrophoresis indicated that low molecular weight forms of keratin protein (44- to 52-kD) were present in cell lines derived from SCLC and non-SCLC types of lung cancer. Tumor cell lines exhibiting squamous differentiation (by light microscopic criteria) also displayed a preponderance of intermediate molecular weight forms of keratin (57- and 59-kD) and exhibited another feature of terminal keratinocyte differentiation (cross-linked envelope formation). Mesothelioma cell lines had varying keratin profiles, perhaps related to their pleomorphic nature and the simultaneous expression of two types of IF *in vivo*, the relative amounts of which correlate with their morphologic appearance. The keratin profiles of the lung cancer cells provide further evidence of a biochemical link between SCLC and non-SCLC types of bronchogenic carcinoma.

The biochemical characterizations of the various epithelia and tumors have been compatible with immunological approaches using specific antibodies, as demonstrated in studies performed in collaboration with Dr. J. Said at Cedars-Sinai Medical Center and Drs. G. Pinkus and J. Corson at Harvard Medical School.

#### Significance to Biomedical Research and the Program of the Institute:

Most human cancers are epithelial in origin. A better understanding of the complex process of neoplasia will require both a full understanding of the normal program of differentiation in human epithelial cells and how it is altered during malignant transformation. Advances in the ability to grow human epithelial cells in culture will undoubtedly facilitate attempts to unravel the mechanism(s) involved in malignant transformation.

#### Proposed Course:

Studies aimed at understanding the control of differentiation and the sequence of events involved in malignant transformation of epithelial cells will continue.

#### Publications:

Banks-Schlegel, S. P. and Harris, C. C.: Aberrant expression of keratin proteins and cross-linked envelopes in human esophageal carcinomas. Cancer Res. 44: 1153-1157, 1984.

Banks-Schlegel, S. P., McDowell, E. M., Trump, B. F., Wilson, T. S. and Harris, C. C.: Keratin proteins in human lung carcinomas: Combined use of morphology, keratin immunocytochemistry, and keratin immunoprecipitation. Am. J. Pathol. 114: 273-286, 1984.

Corson, J. M., Weiss, L. M., Banks-Schlegel, S. P. and Pinkus, G. S.: Keratin proteins in synovial sarcomas: An immunohistochemical study of 24 cases. Hum. Pathol. (In Press)

Said, J. W., Banks-Schlegel, S. P., Sassoon, A. F. and Shintaku, I. P.: Involucrin in squamous and basal cell carcinomas of the skin--an immunohistochemical study. J. Invest. Dermatol. (In Press)

Said, J. W., Nash, G., Sasson, A. F., Shintaku, I. P. and Banks-Schlegel, S.: Involucrin in lung tumors--a specific marker for squamous differentiation. Lab. Invest. 49: 563-568, 1983.

Schlegel, R., Banks-Schlegel, S., Vimadalal, S. D. and Said, J. W.: Keratins in carcinoid tumors. Am. J. Pathol. 49: 511-512, 1983.

Thomas, P., Said, J. W., Nash, G. and Banks-Schlegel, S. P.: Profiles of keratin proteins in basal and squamous cell carcinomas of the skin: An immunohistochemical study. Lab. Invest. 50: 36-41, 1984.

Walts, A. E., Said, J. W., Shintaku, I. P., Sassoon, A. F. and Banks-Schlegel, S.: Keratins of different molecular weight in exfoliated mesothelial and adenocarcinoma cells--an aid to cell identification. Lab. Invest. (In Press)

Willingham, M. C., Banks-Schlegel, S. P., and Pastan, I. H.: Immunocytochemical localization in normal and transformed human cells in tissue culture using a monoclonal antibody to the src protein of the Harvey strain of murine sarcoma virus. Exp. Cell Res. 149: 141-149, 1983

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05291-03 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

DNA Adducts in People Exposed to Benzo[a]pyrene

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

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	Nuntia Sinopoli	Visiting Fellow	LHC	NCI
	Kirsi Vahakangas	Visiting Fellow	LHC	NCI
	Dean L. Mann	Medical Officer	LHC	NCI
	Glennwood E. Trivers	Research Scientist	LHC	NCI
	John Minna	Chief	NMOB	NCI

COOPERATING UNITS (if any) Mt. Sinai School of Medicine, New York, NY (R. R. Boesch); Univ. of Md. School of Medicine Baltimore, MD (B. F. Trump); Georgetown Univ. School of Medicine Washington, DC (H. Yeager); Univ. of Ca. School of Medicine, Los Angeles, CA (W. Wright)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Biochemical Epidemiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

Benzo[a]pyrene (BP) is a ubiquitous carcinogen found in tobacco smoke, burning of fossil fuels, and our diet. Formation of BP diol epoxide (BPDE)-DNA adducts due to human exposure is most likely to be at very low levels that are beyond the sensitivity of routine radioimmunoassay and chromatographic analyses. Thus, ultrasensitive enzymatic radioimmunoassay (USERIA), enzyme-linked immunosorbent assay (ELISA), and photon counting synchronous scanning fluorimetry have been employed to detect and quantitate BPDE-DNA adducts in humans at high cancer risk due in part to BP exposure. DNA isolated from white blood cells of asphalt workers (roofers) and foundry workers and DNA from lung tissue, bronchial washings, and alveolar macrophages of lung cancer patients and smokers are being investigated. BP-DNA adducts have been detected in several of the high-risk individuals. Antibodies to BPDE-DNA adducts were also found in sera from those workers. These data suggest that the activation of BP to its ultimate carcinogen as well as formation of adducts with DNA occurs in humans.



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

Curtis C. Harris	Chief	LHC	NCI
Nuntia Sinopoli	Visiting Fellow	LHC	NCI
Kirsi Vahakangas	Visiting Fellow	LHC	NCI
Dean L. Mann	Medical Officer	LHC	NCI
Glennwood E. Trivers	Research Scientist	LHC	NCI
John Minna	Chief	NMOB	NCI

Objectives:

Using rabbit anti-benzo[a]pyrene diol epoxide (BPDE) antibodies and the most sensitive immunoassays available, BPDE-DNA adducts and human antibodies to these adducts will be determined in high-risk individuals. Results should help us in further understanding activation and mechanism of carcinogenesis in humans.

Methods Employed:

Twenty-five to 40 ml of peripheral blood was obtained from 28 male volunteers who were active in their occupation as roofers for over 20 years. The blood samples were centrifuged at 100 x g for 15 minutes, and the "buffy coat" was homogenized in 5 volumes of HKM:0.25 M sucrose buffer (0.05 M HEPES, pH 7.3; 0.024 M (KCl; 0.05 M MgCl<sub>2</sub>) using a glass homogenizer. The homogenate was centrifuged for 10 minutes at 300 x g at 4°C. The pellet was suspended in HKM-sucrose buffer containing 0.5% Triton 100 and centrifuged for 10 min at 4°C. The pellet was suspended in HKM-sucrose buffer and recentrifuged. The final pellet was resuspended in 5 ml HKM-sucrose buffer containing 1% SDS and 1 M NaCl. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added, and the mixture was vigorously agitated for at least 20 minutes followed by centrifugation at 10,000 x g for 10 minutes. The aqueous epiphase was removed by winding onto a glass rod. Residual ethanol was removed by nitrogen and DNA dissolved in water. Purity and quantitation of DNA were determined by absorbance at 260 nm and 280 nm using a Beckman DU8 spectrophotometer and a fluorimeter. The final volume of DNA solution was adjusted to 1 mg DNA/ml water, and the solution was rendered single-stranded by boiling. Single-stranded DNA was then stored at 4°C until tested. DNA was similarly isolated from lung tissue, bronchial washings, and alveolar macrophages. DNA from foundry workers was received in purified form, ready to be tested.

Competitive enzyme immunoassays, USERIA and ELISA, were performed on the test DNA samples by using rabbit anti-BPDE-DNA antibody. Polyvinyl U-bottom 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with unmodified DNA (control) and BPDE-modified DNA (1 ng/well for USERIA and 5 ng/well for ELISA). Standard competitive inhibition curves were obtained by mixing serial dilutions of known standard BP-DNA with rabbit antisera. Percentage inhibition of the test samples was determined from the standard curves. All tests and assays were done in duplicate, and the standard deviation was less than 10%.

Major Findings:

Metabolic activation of benzo[a]pyrene (BP) to its ultimate carcinogenic form and the binding of BPDE to DNA are important steps in BP carcinogenicity in experimental animals. Since people of certain occupations are exposed to high concentrations of BP, we have used immunoassays, ELISA and USERIA; and synchronous scanning fluorimetry to measure BPDE-DNA adducts in white blood cells from 2 of these occupational groups. Seven of 28 samples from roofers and 7 of 20 samples from foundry workers were positive for BPDE-DNA adducts (range: 2 to 120 femtomoles BPDE/50  $\mu$ g DNA). Positive BPDE-DNA samples of bronchial lavage cells were obtained from 3 out of 5 donors. The donors who had positive samples were smokers of either tobacco or marijuana. Control DNA obtained from 2 human lymphocyte cell lines was negative. Photon counting synchronous scanning fluorimetry is also being used to confirm the results of the enzyme immunoassays.

Because carcinogen-DNA adducts are antigenic when injected into experimental animals, we formulated a hypothesis that people may make specific antibodies to carcinogen-DNA adducts formed after exposure and activation of carcinogens. In sera from 11 of 40 coke oven workers, high titers of antibodies to BP-DNA adducts have been detected.

Significance to Biomedical Research and the Program of the Institute:

Demonstration of carcinogen-DNA interaction in human tissue will enable us to better understand the mechanism of carcinogenesis in humans. Although white blood cells may not be the prime target for certain carcinogens, the presence of carcinogen-DNA antigenicity in these cells not only suggests a widespread distribution of the carcinogen but also provides an opportunity to screen high-risk individuals with relatively simple procedures. Antibodies to carcinogen-DNA adducts may be useful as an indicator of past exposure and metabolic activation of carcinogens.

Proposed Course:

Since BP-DNA antigenicity suggests the presence of BPDE-DNA adducts in humans, we are in the process of further documenting this result using biophysical approaches to measure carcinogen-DNA adducts. Anti-BP-DNA antibodies found in donors who are exposed to BP will also be characterized as to immunoglobulin type. Biochemical and molecular epidemiological studies of high-risk individuals are planned.

Publications:

Harris, C. C.: Carcinogenesis studies using cultured human tissues and cells. Cancer Res. 43: 1880-1883, 1983.

Harris, C. C. and Autrup, H. (Eds.): Human Carcinogenesis. New York, Academic Press, 1983, 986 pp.

Harris, C. C. and Suemasu, K.: Multiple primary neoplasms. Cancer Res. 43: 5629-5630, 1983.

Harris, C. C. and Trump, B. F.: Human tissues and cells in biomedical research. Surv. Synth. Path. Res. 1: 165-171, 1983.

Harris, C. C., Vahakangas, K., Autrup, H., Trivers, G. E., Shamsuddin, A. K. M., Trump, B. F., Boman, B. M. and Mann, D. L.: Biochemical and molecular epidemiology of human cancer risk. In Scarpelli, D. and Craighead, J. (Eds.): The Pathologist and the Environment. New York, Alan R. Liss, 1984 (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05293-03 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Oncogene Transfection of Human Cells

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

PI:	George H. Yoakum	Senior Staff Fellow	LHC	NCI
Others:	Louise Malan-Shibley	Microbiologist	LHC	NCI
	Brent E. Korba	Staff Fellow	LHC	NCI
	John F. Lechner	Senior Staff Fellow	LHC	NCI
	Paul Amstad	Visiting Fellow	LHC	NCI
	Hans Krokan	Guest Researcher	LHC	NCI
	Dimitrios Boumpas	Visiting Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Carcinogen Macromolecular Interaction Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.5

## OTHER:

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.)

Genetic studies of human cell DNA repair and carcinogenesis have been initiated by protoplast fusion transfection of a variety of types of human cells with specific cellular and viral genes known to play a role in human carcinogenesis. The role of the ras gene in normal human bronchial epithelial cell carcinogenesis is being studied by analyzing the progression of Harvey murine sarcoma virus (vHa-ras)-transfected HBE cells through the states of resistance to squamous cell differentiation, immortalization, anchorage independent growth and tumorigenicity in athymic nude mice. The advantages of genetic transfection vis-a-vis virus/helper-virus experiments to study the biological activities of viral genes includes the ability to produce virus-free human cell lines that stably carry and express virus gene products. This circumvents the biohazards associated with virus shedding cell cultures and the technical problems of human cells releasing transforming viruses during xenotransplantation experiments testing the tumorigenicity of transformed human cells. Complete characterization of vHa-ras-transfected human bronchial epithelial cells, including restriction mapping of transfection loci, selection of tumorigenic and nontumorigenic clones, and determination of conditions required for expression of tumorigenic phenotypes will provide information to elucidate the mechanism of ras-mediated carcinogenesis in an important progenitor cell of human lung cancer.

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

George H. Yoakum	Senior Staff Fellow	LHC	NCI
Louise Malan-Shibley	Microbiologist	LHC	NCI
Brent E. Korba	Staff Fellow	LHC	NCI
Paul Amstad	Visiting Fellow	LHC	NCI
Hans Krokan	Guest Researcher	LHC	NCI
Dimitrios Boumpas	Visiting Fellow	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
George Mark	Cancer Expert	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The primary goals of this research project are development and application of a genetic approach to problems of human carcinogenesis at the molecular level. This research program focuses on the genetic role of Ha-ras in normal human bronchial epithelial cell (NHBE) transformation by transfection of MSV-Ha ras into human bronchial epithelial (HBE) cells, and the mechanism of biological responses involved in these carcinogenic processes following oncogene transfection.

Methods Employed:

We have developed a method to transfect a variety of human cell types, normal fibroblastic and epithelial cells, i.e., carcinoma cells and transformed fibroblasts. Introduction of exogenous genes (human or viral) to human cells is essential to development of effective research programs in human carcinogenesis at the genetic and molecular levels. This permits the construction of human cell lines from normal human cells carrying oncogenes for characterization of in vitro carcinogenic potential. The human Ha-ras<sup>T</sup> tester cells have been developed for carcinogenesis studies (TBE-1). The protoplast fusion method of transfection for transfer of plasmids stably transfers genes into human cells at frequencies greater than  $10^{-3}$  units. We are transfection-testing the following potential oncogene constructs on pSV2 neo-plasmids: (1) vHa-ras, (2) v-myc, (3) adenovirus Ela, (4) v-raf, (5) v-raf/v-myc, and (6) v-raf/pEx HTLV/v-myc, by transfection into normal human bronchial epithelial cells, cord blood lymphocytes and TBE-1 (HBE with vHa-ras<sup>T</sup>).

Standard nucleic acid hybridization analysis methods will be utilized to characterize the genetic organization of human recombinant cell lines constructed for these studies. This includes slot-blot DNA or RNA hybridization to detect the presence and expression of transcripts in human cell transfectants and Southern hybridization of restriction digested nuclear DNA after gel electrophoresis and transfer to nitrocellulose to map genomic DNA inserts and characterize transfected gene structures.

Biological analysis of human recombinants constructed for carcinogenesis studies will employ (1) standard tissue culture methods to determine the culture longevity, growth rate, production of autogenous growth factor(s), and anchorage independent growth (soft agar growth); (2) tumorigenicity assays by xenotransplantation in athymic nude mice; (3) determination of karyotypic status, isozyme phenotype, immunocytochemical staining for keratin, human chorionic growth hormone, and analysis of surface antigens; and (3) determination of the effects of oncogene expression on DNA repair processes.

#### Major Findings:

The primary areas of progress are (1) development of a method to transfect normal human cells at high frequency and (2) application of this method to oncogene testing in normal human bronchial epithelial cells. The transfection of vHa-ras into NHBE cells and the clonal isolation of vHa-ras-transfected human bronchial epithelial cells that are tumorigenic when injected into nude mice provides insight into the mechanism of Ha-ras carcinogenesis.

Oncogene transfection of primary cultures of human cells. Transfection of primary human bronchial cultures with a plasmid carrying the vHa-ras oncogenic complementary DNA results in alteration of cell growth properties, resistance to inducers of squamous cell differentiation, immortality, and progression to anchorage independent growth and tumorigenicity. The characterization of the recombinant cell line (TBE-1) has established cells from clonal isolation at various stages of development for carcinogenic complementation studies with (1) other oncogenes and (2) chemical and physical carcinogens.

#### Significance to Biomedical Research and the Program of the Institute:

The development of a method to efficiently transfect a variety of normal human cell types is of general significance to biomedical research programs that employ human somatic cell genetics and molecular biology. The use of this procedure to isolate vHa-ras oncogene-transfected human bronchial epithelial recombinant cell lines with known tumorigenic potential provides a unique opportunity to study Ha-ras oncogenesis in vitro in a human progenitor cell.

#### Proposed Course:

1. Oncogene transfection of normal primary human cultures will be used to study the role of oncogenes in normal human cells in vitro.
2. The oncogenic role of vHa-ras will be determined by characterization of the transfected recombinant cell line with assayable tumorigenic potential, and the interaction of vHa-ras with other oncogenes.

#### Publications:

Yoakum, G. H.: Protoplast fusion: A method to transfect human cells for gene isolation, oncogene testing and construction of specialized cell lines. BioTechniques 2: 24-30, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05321-02 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Carcinogen-DNA Adducts by Synchronous Fluorescence Spectrophotometry

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

PI: Kirsi Vahakangas Visiting Fellow LHC NCI

Others: Glennwood E. Trivers Research Scientist LHC NCI  
Curtis C. Harris Chief LHC NCICOOPERATING UNITS (if any) Univ. of Southern California, Los Angeles, CA (W. Wright);  
Inst. of Public Health, Oslo, Norway (A. Haugen); Georgetown Univ. School of  
Medicine, Washington, DC (H. Yeager); Cancer Inst., Chinese Academy of Medical  
Sciences, Beijing, Peoples Republic of China (Sun Tsung-tang & Hsia Chu-chieh)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Biochemical Epidemiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

Sensitive methods to detect carcinogen-DNA adducts are needed to study the mechanism of carcinogenesis as well as a measure of true exposure to carcinogens. Synchronous fluorescence spectroscopy is a more sensitive method compared to conventional luminescence methods. Compared to USERIA (ultrasensitive enzymatic radioimmunoassay), its sensitivity appears to be at the same range, if a large volume of samples can be analyzed. By this method, benzo(a)pyrene- and aflatoxin-DNA adducts were detected not only in various tissues of persons with known exposure to these carcinogens but also in some persons without known exposure. According to the preliminary data, the relationship between the fluorescence intensity and the amount of carcinogen-DNA adducts seems to be in linear correlation.

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

Kirsi Vahakangas	Visiting Fellow	LHC	NCI
Glennwood E. Trivers	Research Scientist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The specificity and sensitivity as well as the quantitation by the synchronous fluorescence spectrophotometry are being worked out.

Benzo(a)pyrene (BP)- and aflatoxin B<sub>1</sub> (AFB)-DNA adducts are assayed in various tissues from people exposed to BP (e.g., smokers, foundry workers) and AFB (e.g., people eating food contaminated by aflatoxin).

Methods Employed:

DNA is modified in vitro with tritiated benzo(a)pyrene-diolepoxide (BPDE) and AFB for reference material.

DNA is isolated from cells or tissues of animals and humans exposed in vivo by a method including extractions by organic solvents, RNase and proteinase treatments, and ethanol precipitations. Isolated DNA is dissolved in buffer solution and frozen for storage.

At the moment, we have two machines for fluorescence measurements. One consists of a Perkin-Elmer MPF-44B fluorescence spectrophotometer attached to a photon counter (Artec) to give enough sensitivity. The other machine is a Perkin-Elmer fluorescence spectrophotometer 650-40 with a Perkin-Elmer 3600 data station. The latter makes it possible to manipulate the data (e.g., even the curves, do derivative spectroscopy, subtract background).

In conventional luminescence spectroscopy a luminescent substance is excited at fixed wavelength while the intensity distribution pattern of emission (emission spectrum) is monitored by scanning of the emission wavelength. An excitation spectrum can be obtained by scanning the excitation wavelength while the emission is monitored at a fixed wavelength. In synchronous fluorescence spectroscopy (as first suggested by Lloyd; Nature, 231: 64, 1971) both excitation and emission wavelengths are changed simultaneously while a constant wavelength interval is kept between them.

For both BP-DNA and AFB-DNA-adducts, a delta lambda of 34 nm is used. For both, only one peak is seen in the synchronous spectrum, the maximum of the emission being at 378 nm for BP-DNA and 436 nm for AFB-DNA. We are currently developing a computer modeling system to assist us in further analysis of carcinogen-DNA interactions by this method.



Major Findings:

1. Benzo(a)pyrene-DNA: The emission peak at 378 nm seems to be specific for the products of BPDE (BP-DNA, isolated nucleotides modified with BPDE, and BP-tetrols and -triols formed from BPDE). We are able to detect about 200-400 fmol/ml by MPF-44B with the photon counter. More sensitivity is gained by 650-40 with 3600 data station, the limit being about 20-50 fmoles/ml. BPDE attached to DNA gives less fluorescence than isolated adducts or tetrols, so that for the most accurate quantitation, the hydrolysis of DNA is necessary. The simplest and the most reliable way to do this is being worked out. A rat study to confirm the quantitation after in vivo exposure has been carried out.

BP-DNA adducts have been found by this method in lymphocytes from peripheral blood of coke oven workers, aluminum plant workers, placental DNA from both smokers and nonsmokers and alveolar macrophages.

2. Aflatoxin-DNA: The emission peak at 436 nm seems to be specific for AFB and AFB-DNA or AFB-guanine adducts. The correlation between the fluorescence intensity and the amount of adducts is linear. We expect to be able to detect one adduct in  $5 \times 10^6$  nucleotides by this method.

In in vivo rat studies in which three doses of AFB were administered, DNA adducts were easily detected in the liver of the treated rats, and a dose-response effect was observed.

AFB-DNA adducts have been found in urine from people living in geographical areas where AFB is known to contaminate food products to significant extent.

Significance to Biomedical Research and the Program of the Institute:

Sensitive methods to detect carcinogen-DNA adducts are needed to settle the relationship between the carcinogen exposure, actual binding to DNA, persistence of the adducts, and cancer formed in these same individuals.

Because the amount of adducts reflects not only the amount of carcinogen which entered into the body but also the amount of activated metabolites formed within the body, the level of adducts is a probably far more accurate measure of the true exposure to the carcinogen than, e.g., the detection of carcinogen in the air.

Proposed Course:

The method described here is one of the several methods used in our laboratory to detect carcinogen-DNA-adducts. The results gained by this method will be compared with those obtained by other methods.

According to the preliminary results, the quantitation by this method seems to be possible, and it is going to be worked out.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05322-02 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Role of Hepatitis B Virus in Hepatocellular Carcinoma

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

PI:	Brent E. Korba	Staff Fellow	LHC	NCI
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Others:	George H. Yoakum	Senior Staff Fellow	LHC	NCI
	John F. Lechner	Senior Staff Fellow	LHC	NCI
	Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

## COOPERATING UNITS (if any)

Department of Pathology, University of Maryland School of Medicine, Baltimore, MD (B. F. Trump); Cancer Institute, Chinese Academy of Medical Sciences, Beijing, Peoples Republic of China (Sun Tsung-tang and Hsia Chu-chieh)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Carcinogen Macromolecular Interaction Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

3.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

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

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

A plasmid carrying a subgenomic fragment of hepatitis B virus (HBV) encoding the core antigen (HBc) gene has been stably introduced into a human mycoepidermoid carcinoma cell line using a protoplast fusion transfection procedure developed in this laboratory. This cell line was chosen as a model recipient since it exhibits many of the properties associated with normal epithelial cells. Transfected cells producing a low basal level of HBV core antigen exhibit cytotoxic responses. Expression of the HBc gene is observed only when cells are cultured in a complex serum-free media developed in this laboratory for growth of normal human epithelial cells. However, the addition of fetal calf serum does not alter HBc gene expression. Treatment of cultures with 5'-azacytidine further stimulates HBc gene expression to a lethal level. A direct correlation exists between this high-level expression and loss of 5-methylcytosine at a specific DNA site in the promoter region, 280 base pairs upstream from the HBc structural gene. Alteration of DNA methylation levels within the HBc structural gene do not affect expression. Demethylation is necessary but not sufficient for expression since growth under specific conditions is also required. Primary control of the HBc gene is at the level of transcription since quantities of HBc-specific mRNA are well correlated with the observed levels of gene expression. Regulation of the HBc gene with human cells involves a complex interaction between host cell responses to nutritional factors and DNA cytosine methylation levels within the HBc gene promoter.

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

Brent E. Korba	Staff Fellow	LHC	NCI
George H. Yoakum	Senior Staff Fellow	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To determine the relationship between hepatitis B virus (HBV) and hepatocellular carcinoma. Studies are directed toward the development of a model system using cultured human epithelial cells into which HBV DNA and/or chemical carcinogens can be introduced. These experiments will eventually be extended to the study of these agents in primary cultures of normal human hepatocytes.

Methods Employed:

The protoplast fusion method of gene transfection (as modified in this laboratory) is used to transfer HBV-containing plasmids into human cells. This procedure permits transient expression of transferred genes in 70% to 90% of recipient cultures for 6 to 12 days and allows for subsequent selection of dominant markers to produce a population containing stably integrated plasmid DNA. For these studies a fragment of HBV containing the core antigen (HBcAg) gene (Hbc gene) was inserted into the Bam HI site of pSV2gpt using standard recombinant DNA techniques. NCI H292, a mucoepidermoid carcinoma cell line, was used as a model recipient since these cells exhibit many of the properties associated with normal epithelial cells.

Expression of the Hbc gene was followed using commercially available radioimmune assay kits for the detection of HBeV antigen (HBeAg). Since HBeAg is reported to be a proteolytic product of HBcAg, this kit quantitatively cross-reacts with HBeAg.

Presence of integrated viral DNA sequences was detected by Southern blot analysis of chromosomal DNA extracted from isolated nuclei. Whole cell RNA was extracted using guanidine thiocyanate and centrifugation through CsCl and analyzed by Northern blotting techniques. The levels of specific nucleic acid species were quantitated by densitometric analysis of autoradiographic films.

Major Findings:

Transfection of NCI 292 cultures with plasmids containing a subgenomic fragment of HBV encoding for HBcAg (pKYC200) was used to determine some of the factors that regulate the expression of the Hbc gene and to explore the effects of expression of the Hbc gene on human epithelial cells separate from the rest of the HBV genome. Within 48 hours following transfection, NCI 292 cultures containing pKYC200 (GTC2) displayed a marked cytopathic response (i.e.,

vacuolation, granulation). Cultures receiving the vector, pSV2gpt, appeared identical to the parental cultures. Selection of GCT2 cultures for the gpt<sup>+</sup> marker produced a cell population that expressed the HBC gene at a virtually undetectable level in RPMI 1640 medium with 10% fetal calf serum (FCS). Growth of these cultures in a modified MCDB 151 medium (LHC4) with 5% FCS significantly elevated the level of HBC gene expression. Treatment with 5'-azacytidine further stimulated expression, resulting in increased cytotoxicity. Analysis of integrated HBV DNA in GTC2 cells indicates that loss of DNA cytosine methylation at a specific Hpa II site, 280 base pairs upstream from the start of the HBCAg structural gene, is directly correlated with the 5'-azacytidine-induced expression. Demethylation at this site immediately precedes the peak of HBCAg production. However, methylation levels at this site were the same in both the expression permissive medium, LHC4, and the nonpermissive medium, RPMI 1640. Thus, cytosine demethylation in the promoter region is necessary but not sufficient for expression of the HBC gene in human cells, requiring in addition an interaction of host responses to nutritional factors. Methylation levels at another Hpa II site, within the HBCAg structural gene, are unrelated to changes in expression. Levels of HBC-specific mRNA in GTC2 cells have been found to be well correlated with changes in HBC gene expression. Quantitative analysis shows that the levels of mRNA rise and fall in approximate proportion to the relative amounts of HBCAg produced. This demonstrates that control of the HBC gene in human cells is at the level of transcription.

#### Significance to Biomedical Research and the Program of the Institute:

Hepatitis B virus, in addition to being the major cause of viral hepatitis, has been epidemiologically linked to hepatocellular carcinoma and acquired immune deficiency syndrome (AIDS). To understand the mechanism of HBV pathology during acute and chronic disease processes, it is essential to separate the various viral genetic elements and to study their biological effects and molecular biology in a model cell system in vitro. This project demonstrates the value of such an approach by revealing the importance of the core antigen in the cytotoxic response of cells to infection with HBV, a role previously unassigned to any specific HBV gene. The methylation state of chromosomal DNA has been implicated as a general controlling factor in carcinogenesis. The system described here provides a unique opportunity to study, at the molecular level, the role of DNA cytosine methylation in controlling the expression of a specific gene of established biological importance in human cells.

#### Proposed Course:

Most recently, our efforts have been directed toward a detailed study of the regulation of the HBC gene in human cells. Future plans include a further characterization of the factors that regulate HBC gene expression and its biological consequences. Experiments are currently underway to transfect primary cultures of normal human hepatocytes with cloned HBV DNA in collaboration with Dr. I.-C. Hsu of the University of Maryland School of Medicine. A similar collaboration with Dr. Sun Tsung-tang of the Cancer Institute, Chinese Academy of Medical Science is also planned. These experiments are the initial steps in a long-term study of HBV-related carcinogenesis in vitro that includes the treatment of HBV-transfected cultures with chemical carcinogens, particularly aflatoxin B<sub>1</sub>.

Publications:

Yoakum, G. H., Korba, B. E., Lechner, J. F., Tokiwa, T., Gazdar, A. F., Seeley, T., Siegel, M. E., Leeman, L., Autrup, H. and Harris, C. C.: High frequency transfection and cytopathology of the hepatitis B virus core antigen gene in human cells. Science 222: 385-389, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01CE05324-02 LHC
PERIOD COVERED October 1, 1983 to September 30, 1984		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Human Lung Carcinoma/Bronchial Epithelial Cell Hybrid Genetics		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)		
PI:	Edward W. Gabrielson	Medical Staff Fellow      LHC      NCI
Others:	John F. Lechner	Senior Staff Fellow      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 20205		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Genetic changes related to carcinogenesis are being studied using hybrids of human lung carcinoma cells with normal human bronchial epithelial cells. Initial studies suggest that a limited population doubling potential (mortality) is a 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.		

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

Edward Gabrielson	Medical Staff Fellow	LHC	NCI
John Lechner	Senior Staff Fellow	LHC	NCI
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.

Methods 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 HAT (hypoxanthine, aminopterin, and thymidine) and ouabain. This selection media is toxic to both the normal parent (ouabain) and the carcinoma parent (HAT).

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:

Initial fusions of HUT 292, a human lung carcinoma cell line, with normal human bronchial epithelial cells and selection of hybrids as described above has resulted in the isolation of several clones. With extended culturing, all of these clones to date have demonstrated a limited doubling potential.

Significance to Biomedical Research and the Program of the Institute:

This system will hopefully provide new information regarding the nature of the genetic changes of cancer.

Proposed Course:

Hybrids will be cloned and characterized for (a) population doubling potential, (b) chromosomal karyotype, and (c) tumorigenicity in athymic nude mice.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05325-02 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Chemical Carcinogenesis and 5-Methylcytosine Patterns in DNA

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

PI:	Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	John F. Lechner	Senior Staff Fellow	LHC	NCI
	Bruce M. Boman	Medical Staff Fellow	LHC	NCI

COOPERATING UNITS (If any)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

5.0

PROFESSIONAL:

3.0

OTHER:

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 pattern of 5-methylcytosine residues in mammalian DNA has recently been found to be crucial to the control of genetic expression. Decreases in DNA 5-methylcytosine content are known to alter the level of differentiation of cells in culture. Thus, changes in DNA 5-methylcytosine patterns may be critical to the process of carcinogenesis. In this regard, we have developed a new method that enables the determination of genomic 5-methylcytosine levels in a microgram of DNA. The effects of chemical carcinogens on 5-methylcytosine levels in micro-systems, tissue biopsies, and human epithelial cells can now be studied. These studies have also shown that the expression of hepatitis B core antigen and human gammaglobin are correlated with the loss of specific methylation sites in the respective promoter regions of these genes. Human tumor DNAs will, therefore, be probed for DNA methylation pattern alterations in selective DNA sequences and genes. Since chemical carcinogens have been shown to decrease genomic 5-methylcytosine levels in BALB/3T3 cells, DNA from carcinogen-treated human epithelial cells will also be probed for changes in 5-methylcytosine patterns.



PROJECT DESCRIPTIONNames, 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
John F. Lechner	Senior Staff Fellow	LHC	NCI
Bruce M. Boman	Medical Staff Fellow	LHC	NCI

Objectives:

To determine the relationship between the changes in 5-methylcytosine patterns in DNA and the carcinogenesis process. It is known that tumor cells contain altered methylation patterns in some genes and DNA sequences as compared to normal tissue. It is not known, however, if these changes in 5-methylcytosine patterns initiate carcinogenesis, occur during carcinogenesis, or are the result of this multistep process. Studies are directed to determine the correlation between the ability of chemical carcinogens to inhibit the formation of 5-methylcytosine and carcinogen-induced oncogenic transformation. Specific genes and DNA sequences that may be more susceptible to carcinogen-induced decreases in 5-methylcytosine are also sought. These susceptible DNA sequences may be the same areas observed to be undermethylated in tumor cells. The methylation patterns of human tumor DNAs will be probed in the search for demethylated genes and/or DNA sequences that may be specific for the tumor type or tissue of origin.

Methods Employed:

This laboratory has developed and utilized human bronchial tissue and epithelial cell culture as a model for carcinogenesis studies. This system also provides a model for the study of the effects of chemical carcinogens on the methylation patterns in the DNA of normal human epithelial cells. DNAs are isolated from carcinogen-treated epithelial cultures; restricted with Hpa II, Msp I, and other enzymes sensitive to cytosine modification; and probed with specific DNA sequences and genes. The genomic levels of 5-methylcytosine will also be monitored in treated cells by a sensitive <sup>32</sup>P post-labeling 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 hours post carcinogenic treatment. Epithelial cell DNA methylation patterns will be compared to those of various carcinoma cell lines and human tumors. High molecular weight DNAs will be isolated from human tumors; subjected to the same enzyme restriction, gel electrophoresis, as above; and probed for alterations in methylation patterns in specific genes and DNA sequences.

Major Findings:

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 modification 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-<sup>3</sup>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 <sup>32</sup>P. The labeled nucleotides are then separated by TLC and the ratio of 5-methylcytidine to the total cytidine and 5-methylcytidine determined. This highly sensitive <sup>32</sup>P post-labeling 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.

Two separate studies have suggested that only one or two Hpa II methylation sites are important to the expression of selective genes. The human gamma-globin gene in mouse erythroleukemia cells containing the human chromosome 11 was found to require the conversion of a few 5-methylcytosines to unmethylated cytosine residues at Hpa II sites in the 5' leading sequences in order to be in an "allowable" state for gene expression. Subsequent treatment of the hypomethylated cells with hexamethylene bisacetamide (HMBA) was required, however, to induce active gamma-globin expression in these cells. HMBA is a known inducer of globin synthesis and differentiation of erythroid cells and has been shown to alter the configuration of chromatin. Thus, the methylation pattern may be the first level of regulation of gene expression. The conversion of a quiescent gene to an active state may require demethylation followed by some endogenous or exogenous inducing agent.

This has been further supported by the finding that the expression of the transfected HBV core antigen gene in a carcinoma cell line required both the loss of methylation and subsequent cell divisions in a proper medium. The components in the medium necessary for core antigen expression are not yet known.

#### Significance to Biomedical Research and the Program of the Institute:

A basic understanding of the mode of initiation of carcinogenesis by chemical agents should provide clues to the genetic mechanisms taking place during this multistep process. The elucidation of the effects of carcinogens on DNA methylation patterns may advance the understanding of the basic processes involved in tumor formation. Since the majority of human tumors are of epithelial origin, an understanding of the 5-methylcytosine pattern changes in selective genes may provide further clues to the genetic alterations that take place during carcinogenesis.

Proposed Course:

Future studies will depend on the results of the above studies. There are two areas that may be profitable to pursue. The first would be to develop a model system that would allow for the determination of the ability of chemical carcinogens to alter the methylation of a single or limited number of sites in a known DNA sequence (HBV core antigen or gammaglobin gene) and correlate this to an observable biological effect. The second area would be to study the level of expression of genes or specific DNA sequences determined to be undermethylated in tumor and/or transformed epithelial cells. The pattern of methylation of these genes or DNA sequences may also be monitored for changes during the transformation process in normal epithelial cells as well.

Publications:

Wilson, V. L. and Jones, P. A.: Chemical carcinogen-mediated decreases in DNA 5-methylcytosine content of BALB/3T3 cells. Carcinogenesis (In Press)

Wilson, V. L., Jones, P. A. and Momparler, R. L.: Inhibition of DNA methylation in L1210 leukemic cells by 5-Aza-2'-deoxycytidine as a possible component of chemotherapeutic action. Cancer Res. 43: 3493-3496, 1983.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05326-02 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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)

PI:	Dean L. Mann	Medical Officer	LHC	NCI
Others:	William Blattner	Research Scientist	EEB	NCI
	Marvin Reitz	Research Scientist	LTCB	NCI

## COOPERATING UNITS (if any)

Laboratory of Microbiology and Immunology, NIDR (J. Oppenheim); Uniformed Services University of the Health Sciences, Bethesda, MD (M. Newman)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Biochemical Epidemiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

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

HLA typing was performed on lymphocytes from patients with a common disease or families in which more than one member shared the same disease. Subpopulations of patients with Sjogren's syndrome as well as families with this disease and with additional different autoimmune conditions demonstrated specific HLA-DR antigen combinations in each autoimmune subset. In families with Hodgkin's disease, homozygosity at the HLA-DR MT1 locus more frequently occurred in the diseased individuals than in the nondiseased family members. Families of patients with adult T-cell leukemia (ATL), including individuals with antiviral antibodies, demonstrated HLA linkage, with a lod score of 2.5. HLA typing of AIDS (predominantly Kaposi's sarcoma) demonstrated an increased frequency of the HLA-DR5 antigen as well as HLA-DR MT2. Expression of HLA-A,B,C and HLA-DR was studied and compared in established cell lines from patients with adult T-cell lymphocytic leukemia (T-ALL) and HTLV-I-associated adult T-cell leukemias. No differences between the two diseases in DNA methylation were observed for the HLA-A,B,C antigens. HLA-DR, however, was highly methylated in the T-ALLs and only partially methylated in the HLA-DR-expressing ATL cell lines. Using fresh cells from patients (HLA-DR negative) with ATL, this same pattern of methylation was observed. After short-term culture (48 hours), HLA-DR was expressed on the cell surface, and no differences in the methylation patterns were observed. The results suggest a post-transcriptional control (in certain instances) for the expression of HLA-DR antigens. Expression of HLA-DR was studied in cord blood lymphocytes. It was observed that these monocytes, in contrast to adult monocytes, express low levels of HLA-DR. These cells could be induced to express these determinants with various lymphokines and gamma-interferon. Cytotoxic T cells were generated to autologous HTLV-infected cells. Cytotoxicity of these cells was restricted to other HTLV-infected cell lines that shared the HLA-A1 determinant.

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

Dean L. Mann	Medical Officer	LHC	NCI
William Blattner	Research Scientist	EEB	NCI
Marvin Reitz	Research Scientist	LTCB	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. 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 the Laboratory of Immunology, Department of Surgery, Uniformed Services University for the Health Sciences, under an interagency agreement. The association of HLA types with disease was examined for significance by statistical methods. HLA-A,B and DR cDNA probes were provided as gifts from several U.S. and European laboratories. DNA and RNA were prepared from cell lines or peripheral blood lymphocytes. The DNA was digested with appropriate restriction in the nucleases, electrophoresed, and probed with B32 nick-translated HLA probes. Cytotoxic T-cells were generated by exposure to autologous HTLV-infected cell lines. The T-cells were cloned and tested for cytotoxicity to HTLV-infected cell lines.

Major Findings:

This project continues to provide significant information relevant to histocompatibility antigens that are expressed and genetic control in the relationship to diseases. Much of the HLA typing that has been done on this project over the last year has related to HTLV studies. HLA typing has been used to monitor infections with HTLV in a variety of recipient cell lines. This typing provides useful information on expression of unusual HLA antigens in the infected cell lines but in addition the capability of monitoring the particular cell that is growing in these cultures. The results of studies on subsets of Sjogren's patients with different autoimmune diseases demonstrates that certain HLA-DR antigens and combinations are found in association with a particular disease manifestation. The family studies in Hodgkin's disease demonstrated an

unusually high frequency of homozygosity of HLA-DR antigens, which we have previously demonstrated to be found on the alpha chain of the DR dimer. This finding suggests the possibility of a genetic alteration in expression of a molecule and susceptibility to the disease. We have previously demonstrated the appearance of an HLA-associated class I (HLA-A,B,C) antigen in cells infected with the human T-cell lymphoma virus (HTLV). This epitope is defined by a monoclonal antibody, 4D12, which is known to precipitate an HLA class I molecule. This epitope appears in all cell lines that are productively infected with the HTLV. This monoclonal antibody also reacts with an epitope that is shared by restricted HLA alloantigenic specificities in several HLA-A and -B locus cross-reactive groups. This observation suggested a possible role for the HLA antigens induced by virus infection and disease susceptibility. We postulated that this determinant, if shared by normal histocompatibility antigens in the diseased individuals, may appear as self and, therefore, escape immune surveillance allowing virus shedding, reinfection of cells, and the development of the adult T-cell leukemia. We tested this hypothesis in part by HLA typing families in which certain individuals had the adult T-cell leukemia and other family members were demonstrated to have been infected by the virus but do not at the present have the leukemia. The susceptibility to the disease and its association with an HLA haplotype were demonstrated by a lod score of 2.5. Furthermore, it was observed that those individuals who had the leukemia all had the HLA type for the cross-reactive group of antigens that shares the common epitope detected by the 4D12 monoclonal antibody. This hypothesis was also studied in the experiments in which cytotoxic T cells were generated against autologous HTLV-infected cell lines. The particular individual from whom these cells were generated has been a long-term survivor of this disease. These cytotoxic T cells were demonstrated to react in an HLA-A1 restricted fashion. This cell line also was cytotoxic to other HLA-A1-bearing HTLV-infected cell lines. This latter result demonstrates the HLA restriction for the generation of cytotoxic cells against this virus-infected cell line and recapitulates data generated in other labs showing HLA restriction of cytotoxic T cells to virus-infected autologous cells. The increased frequency of HLA-DR5 in AIDS patients has been found by other investigators. Our new observation is that the HLA-DR MT2 antigen is much higher in frequency than any other HLA genetically controlled determinant. Since the locus for MT2 is linkage disequilibrium with HLA-DR5, this finding suggests that there is a strong association for susceptibility of the disease with this gene or gene product. HTLV infection of T lymphocytes induces the expression of HLA-DR antigens. This expression appears to coincide with the expression of viral proteins. Examination of the potential mechanisms for this alteration of control of expression demonstrated that HLA-DR expression was under post-transcriptional control in virus-infected cells.

#### Significance to Biomedical Research and the Program of the Institute:

A variety of diseases have been demonstrated to be associated with the human major histocompatibility complex. These associations and their influence in the disease process are not well understood. Further defining HLA association with disease severity demonstrates that the associations are complex and that the disease entity in and of itself probably results from gene interaction within this major histocompatibility complex. Furthermore, the demonstration that the human immune system can recognize small alterations in amino acid composition of a potential immunogen demonstrates the exquisite sensitivity of the human

immune response. Alterations in immune response are well documented in patients with cancers. Whether these alterations occur as a result of or as part of the disease process and disease susceptibility remains to be determined. However, detailed analysis of the genes and gene products within the major histocompatibility complex will further our knowledge as to the role of these genes in the human immune response system and in responses that may be related to carcinogenesis.

#### Proposed Course:

This project represents the combined efforts of a number of investigators, both clinical and laboratory. The project is now being directed at families in which multiple cases of disease, particularly cancer, appear in a single family. Most diseases that appear to have an association with HLA have this association with the HLA-DR region of the major histocompatibility complex. Recent studies have demonstrated that this is a complex region involving at least three loci. Our work will begin to focus on the molecular heterogeneity in this region in cancer-prone families to determine particular gene structure as well as expression and to investigate the possibility of altered HLA-DR antigens in cancer patients. Attempts will be made to correlate the combination of genes within the major histocompatibility complex with the disease. We will continue to examine alterations in immune response in in vitro assays in an attempt to correlate susceptibility and disease risk with altered immune responsiveness.

#### Publications:

Clarke, M. F., Mann, D. L., Murray, C. and Reitz, M. S.: Differential methylation of Class I histocompatibility antigen genes in T-cell lines derived from two different types of T-cell malignancies. Leuk. Res. (In Press)

Mann, D. L., Mendell, N., Kahn, C. R., Johnson, A. H. and Rosenthal, A.: In vitro lymphocyte proliferation response to therapeutic insulin components: Evidence for genetic control by the human major histocompatibility complex. J. Clin. Invest. 72: 1130-1138, 1983.

Mann, D. L. and Moutsopoulos, H.-M.: HLA-DR alloantigens in different subsets of patients with Sjogren's syndrome and in family members. Ann. Rheu. Dis. 42: 433-436, 1983.

Mitsuya, H., Matis, L. A., Megson, M., Bunn, P. A., Murray, C., Mann, D. L., Gallo, R. S. and Broder, S.: Generation of an HLA-restricted cytotoxic T-cell line reactive against cultured tumor cells from a patient infected with human T-cell leukemia/lymphoma virus (HTLV). J. Exp. Med. 158: 994-999, 1983.

Muchmore, A., Megson, M., Decker, J., Knudsen, P., Broder, S. and Mann, D. L.: Inhibitory activity of antisera to human Ia-like determinants: Comparison of intact and pepsin digested antibodies. J. Immunol. 131: 725-730, 1983.

Reitz, M. S., Mann, D. L., Trainor, C. D., Eiden, M. and Clarke, M. F.: DNA methylation and expression of HLA-DR alpha. Mol. Cell. Biol. (In Press)

Rosenthal, A. S., Mann, D. L. and Kahn, C. R.: Genetic control of the immune response to insulin in man and animal. In Gupta, P. (Ed.): Immunology of Diabetes in Man and Animal. New York, Plenum Press, 1984, pp. 51-71.

Stiehm, E. R., Sztein, M. B., Steey, P. S., Mann, D., Neuland, C., Blaese, M. and Oppenheim, J. J.: Deficient DR antigen expression on human cord blood monocytes: Reversal with lymphokines. Clin. Immunol. Immunopathol. 30: 430-436, 1984.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05327-02 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Development and Use of Antibodies to Detect Carcinogen-DNA Adducts

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

PI: Dean L. Mann Medical Officer LHC NCI

Others: Curtis C. Harris Chief LHC NCI  
 Glennwood E. Trivers Research Scientist LHC NCI

## COOPERATING UNITS (if any)

Department of Pathology, Uniformed Services University for the Health Sciences, Bethesda, MD (M. Newman)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Biochemical Epidemiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1

## OTHER:

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 use of antibodies in sensitive immunoassays provides a means of detecting carcinogens adducted to DNA. In population studies, monoclonal antibody technology is a useful tool in developing reagents to detect these chemical structures. Using different monoclonal antibodies that detect different epitopes of aflatoxin adducted to DNA, patterns of reactions appear to detect different metabolic products of this carcinogen. Hetero-antibodies for benzo(a)pyrene (BP)-DNA adducts have been used to identify BP-DNA in individuals who by occupations have been exposed to high levels of these compounds. Monoclonal antibodies to alter carcinogens adducted to DNA are being developed. Serum from some individuals has clearly demonstrated antibodies to BP-DNA. It is therefore important to develop a system of production of human monoclonal antibodies. To this end, a number of established myeloma and B-cell lines have been screened for the capability to be fused with human B cells. A model system has been established whereby chronic lymphocytic leukemia cells have been fused to a human myeloma cell line. The product of this cell line was the predominant cell surface immunoglobulin found on the chronic lymphocytic leukemia cells. This immunoglobulin was then used to produce a monoclonal antibody selected on the basis of restricted reactions to the specific immunoglobulin. Thus an anti-idio-type monoclonal antibody has been produced. The results demonstrate the capability of using human materials, B cells, and myeloma cell lines to produce monoclonal antibodies.

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

Dean L. Mann	Medical Officer	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Glenwood E. Trivers	Research Scientist	LHC	NCI

Objectives:

The objectives of this project are to produce monoclonal antibodies which would react against chemical compounds known to be associated with or to be carcinogenic in animal model systems. The chemicals under study are those found in the environment and includes drugs that are known to produce cancer in man. We are attempting to perfect techniques to produce human monoclonal antibodies by fusing peripheral blood B lymphocytes from individuals sensitized to a particular compound with a human myeloma cell. Once the antibodies are developed, they will be used to screen populations exposed to environmental chemicals as well as DNA from individuals treated with chemotherapy.

Methods Employed:

Standard hybridoma technology is being used in this study with variations and innovations developed in this laboratory to produce human monoclonal antibodies. In the mouse system, the carcinogen-DNA adducts are combined with protamine or other adjuvants and injected into mice. After repeated immunizations, the sera from the mice are screened for antibodies to the immunogen. Once antibodies develop, the spleens are removed and fused to a HAT-sensitive mouse myeloma cell line. The established fusion product is cloned by limiting dilution and, after an appropriate period of growth, culture supernatants are tested for antibodies to the immunogen. Clones are selected for specific reactivity, expanded in tissue culture systems, and injected into the peritoneal cavity of mice in order to produce ascites fluid and high titered antibody. Analogous techniques are used in the human system. The variation that has been applied in this laboratory is to isolate peripheral blood B lymphocytes and to use these lymphocytes as fusion partners to produce monoclonal antibodies. The assay systems for antibody production are the ELISA and USERIA techniques. These techniques employ an enzyme conjugated anti-immunoglobulin directed against the monoclonal antibody which in turn detects the antigen under study. Appropriate dilutions of the monoclonal antibody are made, and the conjugated anti-immunoglobulin and substrate are added as a means of detection. The USERIA assay employs a radioactive substrate to increase the sensitivity of the methods of detection.

Human sera were tested for antibodies to carcinogen-DNA adducts using the above techniques. Sera are screened using DNA and DNA adducted with the specific carcinogen in the solid phase. After determining binding patterns, selected dilutions are tested in competitive assays to determine specificity of antibody binding. Antibodies detecting specific cell surface antigens are assayed using the fluorescence-activated cell sorter. The monoclonal antibody is exposed to

the cell, the excess removed by washing, and a fluorescein-conjugated anti-immunoglobulin is added. All cells showing a forward light scatter pattern are examined for fluorescence.

### Major Findings:

Preliminary results indicate that successful immunizations have been accomplished with DNA conjugated with O<sup>6</sup>ethyl and N<sup>7</sup>-methyl guanosine derivatives. Heterosera detecting benzo(a)pyrene (BP)-DNA adducts were used to assay for this adduct in DNA from lymphocytes from individuals with high occupational exposure, laboratory workers, and vegetarians. BP-adducted DNA was found in individuals from each group with a higher frequency in those with occupational exposure. Using a competitive assay, sera from these individuals were tested for antibodies to BP-DNA. Some individuals from each group were positive.

A prototype for developing monoclonal antibodies in a human system was established. Under study are several individuals from one family with chronic lymphocytic leukemia (CLL). Surface immunoglobulins of different heavy chain types have been demonstrated on the CLL cells from the different family members. The object is to isolate the immunoglobulin being produced by the CLL cell using hybridoma technology. Cells from a patient were fused with a human nonproducing myeloma cell line; the cells were cloned, and cultured supernatants were assayed for the immunoglobulin isotypes. Cells from the patient were used to immunize mice, and antibody derived from this immunization was produced by fusion of spleen cells from the mouse with a mouse myeloma cell line. Antibodies from clones of this fusion were selected to react specifically with the Ig isotype (IgM) being produced by the human-human fusion. An antibody has been developed which appears to react exclusively with the IgM protein produced by the CLL cell. Furthermore, this monoclonal antibody reacts with the CLL cells from other family members with this disease. This demonstrates the capability of producing human-human fusion products with the resultant production of human immunoglobulins. The potential for production of human monoclonal antibodies has been established.

### Significance to Biomedical Research and the Program of the Institute:

The use of monoclonal antibody production technology is a powerful tool in biochemical epidemiology. The production of antibodies specific for chemicals, drugs, or their metabolites that are associated with carcinogens can be applied to studies in which populations are screened for exposure to these environmental carcinogens. In addition, these monoclonal antibodies can be used to attempt to define specific compounds in complex materials known to be associated with carcinogenesis. Antibodies with individual specificities to a particular compound can be used to isolate the compounds and to identify specific chemicals which may be adducted to DNA. In addition, these antibodies can be used to isolate segments of DNA which have specific carcinogen complexes in order to determine the potential alteration in gene expression in cells exposed to chemical carcinogens.

Proposed Course:

The project will continue to attempt to develop a variety of monoclonal antibodies against carcinogen-DNA adducts. In turn, these antibodies will be used to study DNA from populations known to be exposed to these potential carcinogens in the environment either in the workplace or in the general population. With the appropriate inhibition techniques, sera from patients exposed to these potential chemical substances (carcinogens) will be assessed for presence of antibodies to these compounds produced by the host. Systematic screening of exposed populations as well as control populations will be performed. Attempts will be made to develop human monoclonal antibodies using B cells from individuals who have demonstrated antibody to carcinogen-DNA adducts. In vitro sensitization techniques will be developed and explored in these studies.

Publications:

Blattner, W. A., Greene, M.-H., Goedert, J. J. and Mann, D. L.: Interdisciplinary studies in the evaluation of persons at high risk of cancer. In Harris, C. C. and Autrup, H. (Eds.): Human Carcinogenesis. New York, Academic Press, 1983, pp. 913-939.

Harris, C. C., Vahakangas, K., Autrup, H., Trivers, G. E., Shamsuddin, A. K. M., Trump, B. F., Boman, B. M. and Mann, D. L.: Biochemical and molecular epidemiology of human cancer risk. In Scarpelli, D. and Craighead, J. (Eds.): The Pathologist and the Environment. New York, Williams and Wilkins (In Press)

Neuland, C. Y., Blattner, W. A., Mann, D. L., Fraser, M. C., Tsai, S. and Strong, D. M.: Familial chronic lymphocytic leukemia. J. Natl. Cancer Inst. 71: 1143-1150, 1983.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05328-02 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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)

PI:	Dean L. Mann	Medical Officer	LHC	NCI
Others:	Mika Popovic	Senior Investigator	LTCB	NCI
	Marvin Reitz	Research Scientist	LTCB	NCI
	Robert Gallo	Chief	LTCB	NCI
	William Blattner	Research Scientist	EEB	NCI
	Jeffrey Clark	Senior Investigator	EEB	NCI

## COOPERATING UNITS (if any)

Uniformed Services University for the Health Sciences, Bethesda, MD (M. Newman)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Biochemical Epidemiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

The human T-cell lymphoma virus, HTLV-I, has been found to be associated with certain adult T-cell malignancies. Cell surface antigen profiles of HTLV-infected lymphocytes established in long-term cultures and a variety of cells infected by coculture methods were studied. The majority of cell lines established by coculture demonstrated the specific phenotype of OKT4, HLA-DR, the T-cell growth factor receptor, TAC, and an HLA-associated antigenic determinant that is detected by the monoclonal antibody, 4D12. A second but related retrovirus, HTLV-II, has also been studied under similar experimental circumstances. A cell line established from a patient with acquired immune deficiency syndrome demonstrated the presence of the HTLV-I retrovirus. This retrovirus in coculture experiments infected OKT4 cells, as well as some cells with the suppressor phenotype, OKT8. The kinetics of expression of the cell surface antigens, HLA-DR, 4D12, and TAC were studied. Short-term cultures of peripheral blood lymphocytes (tumor cells) from patients were established and examined for the expression of cell surface antigenic determinants. Within 6 hours, HLA-DR, 4D12, and TAC were expressed. In the short-term cultures, viral demethylation was not observed as opposed to established cell lines. Hamster cells were fused with an HTLV-I cell line, cloned, and examined for the expression of the 4D12 determinant, HLA antigens, and human chromosomes. HLA expression was always associated with chromosome 6; however, the 4D12 expression was only associated with the presence of a virus infection. The results suggest that the 4D12 epitope expression is a product of the HTLV. Two B-cell lines were established from patients with adult T-cell leukemia and found to be HTLV positive. Alteration in immune function was demonstrated by HTLV infection of T-cell clones that had restricted immune response potential.

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

Dean L. Mann	Medical Officer	LHC	NCI
Mika Popovic	Senior Investigator	LTCB	NCI
Marvin Reitz	Research Scientist	LTCB	NCI
Robert Gallo	Chief	LTCB	NCI
William Blattner	Research Scientist	EEB	NCI
Jeffrey Clark	Senior Investigator	EEB	NCI

Objectives:

To study the cell surface phenotypes, both HLA and mature lymphocyte markers, in cell lines established from patients with the human T-cell lymphoma viruses (HTLV) I and II. The study is designed to determine whether certain subpopulations of lymphocytes are infected with the virus and to examine the effect of viral infection in the expression of cell surface antigens. Studies were performed to assess the possibility that alteration in immune function occurred in antigen-specific and restricted responsive T cells.

Methods Employed:

HLA typing was carried out using alloantisera detecting 16 alleles at the A locus, 26 alloantigens of the B locus, and 6 alloantigens of the HLA-C locus. HLA-DR and MT antigens were detected by alloantisera detecting 10 determinants in the series. Other cell surface markers were examined using the fluorescence-activated cell sorter and monoclonal antibodies directed against cell surface determinants that define subpopulations and/or specific functional subsets of lymphocytes. Indirect immunofluorescence was employed in these studies. Cell lines were established from patients with human T-cell lymphoma virus infection and/or those individuals with the associated adult T-cell leukemia. The cultures were established using PHA and T-cell growth factor and maintained in the presence of T-cell growth factor. The exception that was observed was the spontaneous growth of the B-cell lines, which were demonstrated to be infected with HTLV. Both HTLV-I and HTLV-II were transferred into recipient cells by coculture techniques. The donor cells were irradiated and placed in culture with the recipient cell line. The cells were assayed for the presence of the HTLV gag proteins, p19 and p24, periodically. RNA and DNA were prepared from cells by the standard methodologies. DNA and RNA were probed with the HTLV nick-translated probes.

Major Findings:

Alterations in the expression of HLA alloantigens and other cell surface determinants were found in cells expressing the proviral products of HTLV. In all cell lines infected with the virus, there is a consistent pattern of expression of surface antigens. These antigens include the epitope detected by the 4D12 monoclonal antibody, which in turn detects an epitope that is shared with some HLA allotypic antigens. HLA-DR was present in all T-cell lines

infected with the HTLV-I and -II. The receptor for T-cell growth factor, TAC, was also present in all cells that had been infected with these viruses. A study was performed to examine the kinetics of this reaction and to investigate the control of the expression of the proviral DNA products. In peripheral blood lymphocytes from patients with acute T-cell leukemia and/or lymphoma, it was observed that the provirus was highly methylated. In contrast, cells established in long-term culture demonstrated demethylated proviral DNA sequences. Peripheral blood lymphocytes were placed into culture and examined at intervals of 12, 24, and 48 hours. When viral messenger RNA appeared in the cell, no changes in methylation patterns of the proviral DNA were found. However, by this time, cell surface antigens that are mentioned above appeared on the cell surface. There was one significant alteration in the cell surface antigen expression in the HTLV-II-infected cell lines. This was the lack of expression of the antigen detected by the monoclonal antibody, 4D12. When the cord blood lymphocytes from the same individual were infected with HTLV-I or -II, only the HTLV-I-infected cells expressed this epitope. HTLV-I and -II differ in the env region of the provirus. The results suggest that viral proteins coded for in the env region bear the epitope detected by this monoclonal antibody. The question relating to HLA epitope expression was further examined. Hamster cells were fused with HTLV-I-bearing cell lines, the fusion product cloned, the cells expanded and examined for the presence of human chromosomes, and the expression of cell surface determinants coded for by the major histocompatibility complex (chromosome 6) and the antigen bearing the epitope, 4D12. The clones that were infected with HTLV expressed the 4D12 antigen. Those clones not infected with HTLV but possessing chromosome 6 expressed HLA antigens. Other clones having neither chromosome 6 nor the virus did not express either of the above antigens. Antibodies to the HTLV-I virus have been detected in certain types of leukemias in other areas endemic for this virus. The frequency of this antibody reaction is above that of expected rates of infection for the general population. This is true in a group of patients from Jamaica who had chronic lymphocytic leukemia. The chronic lymphocytic leukemia cells (B cells) were examined for cell surface antigens and probed for HTLV proviral DNA. Cell lines were established from this same patient. The cell lines proved to be T cells by surface markers expressing the 4D12 antigen, HLA-DR, and TAC. These cells were positive for proviral DNA. The chronic lymphocytic leukemia cells, however, were negative for proviral DNA and only expressed the HLA-DR antigens, as well as the antigens that represent the usual B-cell phenotypes. This observation suggests the possibility of an indirect mechanism for the influence of this virus infection on the other neoplastic conditions. Cloned cytotoxic and antigen (KLH)-responsive T cells were obtained from Drs. Flomenberg and Volkman and infected with HTLV types-I and -II. The cells were assayed for specific response. Antigen restricted cytotoxicity was lost with infection with both viruses. With KLH response, the T cells lost their ability to discriminate, responding to both HLA-DR antigen-restricted and -nonrestricted antigen-presenting cells. The loss of specific T-cell function suggests that T-cell tropic viruses produce an alteration of immune response that is seen in patients with this disease.

#### Significance to Biomedical Research and the Program of the Institute:

The isolation of a type C retrovirus and its demonstrated association with human T-cell malignancies by Dr. Gallo and his associates has been an important advance in our understanding of neoplastic disease processes. With the isolation

and capability of transferring this virus to other human cells, it is now possible to study mechanisms of regulation of malignant transformation. One important feature of malignant transformation is the particular type of cell that the virus can infect as well as alterations that may occur in the expression of cell surface antigenic determinants with viral infection. The observation that altered HLA antigen expression occurs with viral infection raises the interesting possibility that control of infection and tumorigenesis may be related to HLA antigen expression. The coincident appearance of altered HLA expression with viral replication strongly suggests viral regulation of HLA gene expression or that the virus encodes for proteins that bear HLA alloantigenic determinants. It has been documented in other studies that the human T-cell lymphoma virus can infect individuals without producing any neoplastic disease process. The alteration in expression of HLA alloantigens may be a mechanism for control of tumorigenesis in that the viral replication induces the expression of an antigen that may be recognized as self or as foreign depending on the HLA type of the individual, and thus result in immunological control of replication and the disease process. The demonstration that cytotoxic T cells can be generated from a patient with this malignancy and that there is a restriction in this cytotoxicity suggests that a mechanism like this may be in some way controlling a human malignancy.

#### Proposed Course:

Studies will continue examining the cell surface phenotypes of HTLV-infected cells. We now intend to examine the immunologic response of individuals who are infected with HTLV who have no evidence of the leukemia and compare this response with the response of individuals who have the disease that has been associated with infection by this retrovirus. These will be done both serologically and at the cellular level. Cells will be examined from patients and HTLV carriers for specific cytotoxic T cells. Since both the infected individuals and the patients have antibodies against the retrovirus, we will examine the differences in the antibody specificity, looking particularly at the envelope proteins of the provirus.

#### Publications:

Mann, D. L., Popovic, M., Murray, C., Neuland, C., Strong, D. M., Sarin, P., Gallo, R. C. and Blattner, W. A.: Cell surface antigen expression in newborn cord blood lymphocytes infected with HTLV. J. Immunol. 131: 2021-2024, 1983.

Mann, D. L., Popovic, M., Sarin, P., Murray, C., Reitz, M. S., Strong, D. M., Hayes, B. F., Gallo, R. C. and Blattner, W. A.: Cell lines producing human T-cell lymphoma virus altered HLA expression. Nature 305: 58-60, 1983.

Popovic, M., Lange-Wantzin, G., Sarin, P. S., Mann, D. and Gallo, R. C.: Transformation of human umbilical cord blood T-cells by human T-cell leukemia/lymphoma virus (HTLV). Proc. Natl. Acad. Sci. USA 80: 5402-5406, 1983.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05338-02 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Characterization, Mode of Action, and Evolution of the Oncogene raf

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

PI:	George E. Mark, III	Expert	LHC	NCI
Others:	Takis S. Papas	Research Scientist	LMO	NCI
	Ravi Dhar	Research Scientist	LMV	NCI
	Dean L. Mann	Medical Officer	LHC	NCI
	John F. Lechner	Senior Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Ulf R. Rapp	Research Scientist	LVC	NCI
	John Minna	Medical Officer	NMOB	NCI

COOPERATING UNITS (if any) Laboratory of Microbial Immunity, NIAID (M. Howard); Laboratory of Molecular Genetics, NICHD, NIH (M. E. Digan); Arthritis and Rheumatism Branch, NIADDDK, NIH (J. Mountz and Y. Rosenberg); Litton Bionetics, Frederick, MD (T. Benyajati); Cornell University, Ithaca, NY (R. MacIntyre)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.5

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

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

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

A new replication-defective, acute transforming retrovirus (3611-MSV) was recently isolated from mice and was molecularly cloned. Two gag-containing polyproteins (P75 and P90) are found in nonproducer transformed cells. The nucleotide sequence of 1.5 kilobases encompassing the transforming gene (v-raf) of 3611-MSV has been determined. v-raf sequences were found to have been inserted into the p30 region of an ecotropic murine leukemia virus (MuLV) with the concomitant deletion of the 2.4 kilobases extending to the middle of the polymerase gene. A 5-nucleotide direct repeat exists at each end of the v-raf sequences. From the deduced amino acid sequence, a hybrid gag-raf polyprotein would have a molecular weight of approximately 75 kilodaltons. Consistent with the gag-x structure, we found that only the P75 polyprotein is modified by the fatty acid myristate, whereas only the P90 polyprotein is glycosylated. Comparison of the deduced v-raf amino acid sequence with other oncogenes revealed domains homologous to the src family of oncogenes. The phylogenetic organization of this family determined from the predicted amino acid sequences suggests that the raf oncogene evolved prior to those members which exhibit tyrosine-specific phosphorylating activity. The Drosophila and yeast raf homologs have been recognized and the former has been cloned. In situ hybridization to Drosophila polytene chromosomes localizes the raf homolog (D-raf-1) to position 2F on the X chromosome. Analysis of developmentally staged Drosophila RNAs reveals the D-raf-1 sequence is expressed only in 12-20 hour embryos. Both humans and mice express raf RNA in specific tissues in the form of mRNAs of approximately 3.5 kb and 3.3 kb, respectively. A human fetal liver cDNA library has been the source of several raf-specific clones recently characterized. c-raf-related sequences have been localized to human chromosome 3, a chromosome whose alterations are associated with small cell lung carcinoma and other familiar carcinomas. Human cells derived from small cell carcinomas express v-raf-specific RNA, as do some other established human lung carcinoma cell lines.

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

George E. Mark, III	Expert	LHC	NCI
Takis S. Papas	Research Scientist	LMO	NCI
Ravi Dhar	Research Scientist	LMV	NCI
Dean L. Mann	Medical Officer	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Ulf R. Rapp	Research Scientist	LVC	NCI
John Minna	Medical Officer	NMOB	NCI

Objectives:

Activated cellular oncogenes are involved in tumorigenesis - perhaps proximally, perhaps distally. The recognition, isolation, and characterization of these potentially deleterious, cellular sequences will ultimately lead to an understanding of the biochemical functions which drive a cell to cancerous growth. Most oncogenes have been highly conserved through evolution since the appearance of metazoan organisms; hence, the belief that their role in normal cellular development and differentiation is vital. Recently, a new transforming, replication-defective retrovirus has been isolated. Initial characterization of 3611-MSV revealed it to contain a partially deleted structural gene (gag) fused to a nonviral sequence. The objectives of this project are two-fold: (1) to discern its mode of action from studies of its intracellular localization, its required protein domains, and its evolutionary ancestors and (2) to identify its roles in normal cellular functions and human malignancy.

Methods Employed:

Nucleic acid sequencing protocols described by Maxam and Gilbert for the chemical cleavage of terminally radiolabeled oligonucleotides were employed. RNAs were extracted from cultured cells utilizing phenol heated to 65°C and poly-A-containing transcripts were selected by dt-cellulose chromatography. Analysis of RNAs was either quantitative (dot blotting samples onto nitrocellulose membranes followed by hybridization with a nick-translated <sup>32</sup>P-labeled probe) or qualitative (electrophoretic separation of RNA species, their transfer to DBM-paper, and detection through hybridization with specific, radiolabeled probes). A Drosophila melanogaster genomic library made in Charon 4A was obtained from Dr. Tip Benyajati (Litton Bionetics, Inc., Frederick, MD) and screened for raf oncogene-containing phage by conventional methods after reducing the stringency of hybrid selection. Data were analyzed with the aid of the computer facilities at the Frederick Cancer Research Facility, Frederick, Maryland. Staged Drosophila embryos were obtained from Dr. Carl Wu, Laboratory of Biochemistry, Division of Cancer Biology and Diagnosis, NCI. A yeast genomic library made in λMG was obtained from Dr. David Kabat (New Jersey School of Medicine).

Major Findings:

1. Characterization of v-raf, a new oncogene. A novel, acutely transforming replication-defective retrovirus (3611-MSV) has been isolated and characterized. DNA sequence determination of the acquired oncogene (v-raf) and its flanking viral elements has provided essential information pertaining to its acquisition, composition, and mechanism of action. The parent of 3611-MSV was an ecotropic murine type C virus; that is, no prerequisite recombinational events need occur prior to the transduction of the cellular oncogene. Deletion mutants, constructed using BAL-31 exonuclease, indicated that the active portion of the transforming gene was contained within 890 nucleotides, commencing 35 nucleotides from its 5' point of acquisition. Two polyproteins were detected in transformed cells, both represented the translation product of the molecularly fused viral gag gene and the raf oncogene, terminating at an amber codon within the cellular sequences. To determine the intracellular localization of the v-raf protein (or c-raf protein from normal cells), three polypeptides were synthesized, coupled to KLH, and used to immunize rabbits. The reactive antisera which was obtained has been tested. v-raf-transformed 3T3 cells exhibit cytoplasmic and perinuclear localization when the antiserum is employed in a standard fluorescent antibody staining analysis. The amino acid sequence, predicted from the nucleic acid sequence of v-raf, revealed a close relationship between the members of the tyrosine kinase-oncogene superfamily and v-raf. The predicted protein sequence of v-raf and v-mht, the avian homolog, also demonstrates striking similarities between these sequences and those of v-fms and v-erb B. In contrast to the more recently evolved members of the src family of oncogenes, only the above sequences display features of transmembrane glycoproteins. Since the v-erb B product has been shown to share homologous regions with the EGF receptor, it is likely that the raf and fms oncogenes also represent growth factor receptors. Consistent with this proposal, raf expression can be found in specific tissues and is inducible upon stimulation with particular biomolecules. Preliminary observations suggest raf expression may be down-regulated by a specific growth factor.

The role of these sequences in human malignancy is not fully understood. Preliminary experiments indicate that cells derived from human oat cell carcinomas express significantly more raf-related RNA than normal bronchial epithelial cells. Oat cell carcinomas are uniquely metastatic. This may be in part the consequence of the cell's expression of a new growth factor receptor (raf) since metastases contain 10 to 40 times more raf RNA than any of the oat cell carcinoma cell lines investigated.

Raf-specific cDNA clones have been isolated from a human fetal liver cDNA library made in  $\lambda$ gt10. Restriction map characterization of four individual clones showed one of these clones to be closely related to the c-raf-2 pseudogene sequence. DNA sequencing suggests this locus is not c-raf-2, but represents a new proto-oncogene sequence that is related to the c-raf-1. In all likelihood, the c-raf-2 pseudogene arose by reverse transcription of this new proto-oncogene and not the c-raf-1 sequence. Also, differences between this clone and the c-raf-2 locus suggest that splice acceptor choices exist for the 3' terminal exon ultimately found in the mature mRNA.

2. Raf-related sequences have been recognized in *Drosophila*, yeast, and *Dictyostelium* genomes. The yeast sequence is being characterized in collaboration with Dr. Ravi Dhar. The *Drosophila* genome contains two raf-related sequences, one (D-raf-1) more homologous to the murine sequence than the other (D-raf-2). In situ hybridization of D-raf-1 to polytene chromosomes (performed by Dr. Ross MacIntyre, Cornell University) localized this gene to the X chromosome, 2F region, near the zeste and white loci. This region may be involved in neural/endodermal differentiation. Four complementation groups within the 2F region have been described; all mutants in these groups are lethal. Investigation of D-raf-1 transcription during development and morphogenesis (performed in collaboration with Dr. Mary E. Digan, Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, NIH) reveals expression limited to the late embryonic stages of differentiation. The specific embryonic tissues responsible for this expression are being determined by in situ hybridization techniques. This will be of interest since the mammalian tissues which express raf normally are not present in invertebrates.

#### Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of the novel retroviral transduced oncogene, v-raf, has resulted in the reevaluation of the mode of action of the tyrosine kinase-oncogene superfamily. This will result in a fresh assault on the mechanisms of carcinogenesis mediated through these highly conserved sequences. Ultimately, the nature of the biochemical functions which drive a cell to cancerous growth will be discerned.

#### Proposed Course:

The expression of raf-related RNA sequences in human malignancies and the relationship of this potential cellular oncogene to neoplastic transformation will be investigated. Clones derived from cDNA copies of "normal" c-raf RNA will be analyzed for transforming activity by transfection into suitable recipient cells. If an activated c-raf gene is transforming, the localization of its product will be compared with that of its "normal" cellular counterpart. The interplay of other oncogenes on the raf sequence will also be investigated.

Investigations of the role of the raf oncogene in oat cell carcinomas and other lung neoplasias are continuing. Primary human tissues are being obtained through collaborations with Dr. Bernard J. Poiesz, VA Medical Center, Syracuse, NY, and Dr. York E. Miller, VA Medical Center, Denver, CO.

The characterization of raf as a normal cellular transmembrane glycoprotein (possibly a growth factor receptor) which is expressed in specific cells has led to investigations of this polypeptide. A cell line has been found which expresses large amounts of raf RNA as do stimulated primary cells. These will be used to isolate the c-raf polypeptide so that biological and biochemical characterization can be accomplished.

Experimental approaches directed toward uncovering a mechanism of carcinogenesis will be facilitated by employing the genetic tools available in *Drosophila* and yeast biology. The latter system holds the most promise since genes may be shuttled in and out of yeast by homologous recombination. Thus, the environment

of a specific gene is not altered as a consequence of manipulation. v-raf-related sequences are presently being cloned from yeast.

Publications:

Jansen, H. W., Lurz, R., Bister, K., Bonner, T. I., Mark, G. E. and Rapp, U. R.: Homologous cell-derived oncogenes in avian carcinoma virus MH2 and murine sarcoma virus 3611-MSV. Nature 307: 281-284, 1984.

Kan, N. C., Flordellis, C. S., Mark, G. E., Duesberg, P. H. and Papas, T. S.: A unique onc gene sequence is transduced by avian carcinoma virus MH2 (v-mht) and murine sarcoma virus (v-raf). Science 223: 813-816, 1984.

Kan, N. C., Flordellis, C. S., Mark, G. E., Duesberg, P. and Papas, T. S.: Nucleotide sequence of avian carcinoma virus MH2: Relation of MH2-specific sequences to other oncogenic avian and murine sarcoma viruses. Proc. Natl. Acad. Sci. USA 81: 3000-3004, 1984.

Mark, G. E. and Rapp, U. R.: Primary structure of v-raf predicts relatedness to the src family of oncogenes. Science 224: 285-289, 1984.

Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr. and Stephenson, J. R.: Structure and biological activity of v-raf, a novel oncogene transduced by a retrovirus. Proc. Natl. Acad. Sci. USA 80: 4218-4222, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05341-02 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Model Systems for the Study of Physical Carcinogens at the Cellular Level

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

PI:	John F. Lechner	Senior Staff Fellow	LHC	NCI
Others:	Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Brenda I. Gerwin	Research Chemistry	LHC	NCI
	Edward Gabrielson	Medical Staff Fellow	LHC	NCI

COOPERATING UNITS (if any)

Georgetown University School of Medicine, Washington, DC (H. Yeager); Veterans Administration Hospital, Washington, DC (P. Schafer); University of Maryland School of Medicine, Baltimore, MD (B. F. Trump); Litton Bionetics, Kensington, MD (M. Valerio)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

0.5

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.)

Asbestos and glass fibers were found to be 100 times more cytotoxic for human mesothelial cells and 10 times more cytotoxic for normal human bronchial epithelial cells than for normal human bronchial fibroblasts. Chrysotile was the most cytotoxic fiber tested. Focal hyperplasia and epidermoid metaplasia were observed in explants of human bronchial tissue two weeks after a single exposure to amosite asbestos; both intracytoplasmic and intranuclear asbestos fibers were seen by X-ray microanalysis in these lesions. However, these atypical lesions proved to be transient and were not detected in tissue examined 12 weeks post exposure. Normal human bronchial cells were also exposed to amosite, but no abnormal cells were recovered from these cultures. On the other hand, colonies of phenotypically altered mesothelial cells arose in cultures two subculturings post amosite exposure. The control cultures ceased growth during the fourth subculture, whereas the amosite-exposed cultures continued to multiply for more than 18 subculturings. The exposed cells had a near-normal modal number of chromosomes through the sixth subculturing. At the ninth subculture, 80% of the metaphases had dicentric chromosomes, and the modal number had increased to 77.

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

John F. Lechner	Senior Staff Fellow	LHC	NCI
Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
Edward Gabrielson	Medical Staff Fellow	LHC	NCI

Objectives:

To study the carcinogenicity of asbestos fibers in human mesothelial and bronchial epithelial in vitro systems. These studies include the following: (1) develop defined media for replicative mesothelial cell cultures, (2) evaluate cytotoxicity of asbestos fibers in mesothelial and bronchial epithelial cells, (3) evaluate the effects of asbestos fibers on progression of chromosome rearrangements in mesothelial cells, (4) evaluate asbestos as a cocarcinogen for cultured bronchial epithelial cells, and (5) evaluate the role of the cytoskeleton in the mode of action of asbestos-caused carcinogenesis.

Methods Employed:

Human bronchial tissues are obtained from a medical examiner and "immediate" autopsy donors. Replicative cultures of normal bronchial epithelial cells are developed from explant culture outgrowths. Upon transfer of the explants to new dishes, the outgrowth cultures are incubated in defined, serum-free medium to expand the population, then subcultured. Mesothelial cells are obtained from pleural effusions from donors without cancer. The fluid is centrifuged, and the pelleted cells are resuspended and inoculated into 100 mm culture dishes at a ratio of one dish per 50 ml of pleural fluid. The cells are dissociated using trypsin when the cultures attain subconfluency. The cultures are further expanded and either cryopreserved or used according to experimental protocols.

Several criteria are used to establish the identity of the cells grown in culture. Markers for bronchial epithelial cells include karyology; polygonal morphology; ultrastructural identification of tight junctions, desmosomes, and tonofilaments; production of acidic and neutral mucopolysaccharides; immunostaining of keratin; blood group antigens and type IV collagen; population doubling potential; clonal growth rate; and mitogenic responsiveness to peptide growth factors and hormones. Mesothelial cells are identified by several criteria, including immunofluorescent staining with antikeratin antibodies; a variable cell morphology depending on the presence (fusiform) or absence (cobblestone) of EGF and hydrocortisone in the growth medium; histochemical staining for hyaluronic acid-mucin; the presence of long, branched microvilli; and the normal human karyotype of Giemsa-banded metaphases.

Fiber cytotoxicity is assessed using clonal growth dose-response assays. Sixty-millimeter dishes are inoculated at clonal density. Twenty-four hours later, the medium is replaced with medium containing increasing concentrations

of fibers. After 3 days of exposure, the fiber-treated and control cultures are rinsed twice with medium, then reincubated in fiber-free medium. Ten days post inoculation, the colonies are fixed in 10% formalin and stained with 0.25% crystal violet.

Bronchial tissue is exposed to amosite asbestos by pipetting fiber suspensions onto the epithelial surface. The explants are then submerged in medium in a stationary position for 2 hours before culturing in a rocked, controlled atmosphere chamber. The culture medium is replaced with fresh medium without fibers the next day and then at 2-day intervals. The tissues are periodically examined by light and electron microscopy.

Replicative cultures of mesothelial cells are exposed to amosite asbestos by including the fibers (2 µg/ml) in the growth medium. After 4 days of incubation, the medium is replaced with medium without fibers and at 4-day intervals thereafter. Two weeks later, the cells are trypsin dissociated and subcultured. The following day, the cultures are reexposed to amosite asbestos. Unexposed control cultures are carried in parallel. Giemsa banding of mesothelial metaphases is conducted to monitor chromosomal rearrangements post asbestos exposure.

#### Major Findings:

Investigations into the mechanism of asbestos carcinogenesis have shown the following: (1) Amosite asbestos (100 to 1000 µg/ml) caused focal epithelial hyperplasia and atypical squamous metaplasia in human tracheobronchial explants. (2) Amosite fibers were shown by both scanning and high-voltage transmission electron microscopy to penetrate cultured epithelial cells. Short fibers (< 12 µ) were found in the cytoplasm of the cells within 6 hours, whereas longer fibers incompletely entered the cells. The epithelial cells did not show marked cell surface activity, and only small membrane sleeves around noncoated fibers were observed at the points of asbestos penetration. (3) To measure toxicity, asbestos (UICC samples, 0.1 to 100 µg/ml) were added to human bronchial epithelial cells that had been subcultured 24 hours previously at clonal density. When compared to glass fibers, asbestos caused a statistically significant ( $p < 0.05$ ) decrease in cell population doubling rate. Chrysotile was approximately 10-fold more cytotoxic than either amosite or crocidolite. A similar order of toxicity was observed when human bronchial fibroblastic cells were used; however, these cells tolerated approximately 100-fold more fibers for the equal level of cytotoxicity. Both intracytoplasmic and intranuclear asbestos fibers were seen by X-ray microanalysis in the hyperplastic lesions. (4) Conditions for replicative cultures of mesothelial cells were developed. The clonal growth rate of mesothelial cells was first determined in nine different medium formulations. Medium M199 was the worst (0.32 PD/D), whereas LHC-Basal supported the most rapid growth rate (0.63 PD/D). Thus, LHC-Basal was selected as the standard nutrient mixture for mesothelial cells. Further, experimentation assessing numerous growth factor and hormonal supplements showed that insulin, retinoic acid, EGF, and hydrocortisone spared the serum requirement (to < 3%) and enhanced the clonal growth rate of the mesothelial cells to > 0.85 PD/D. In addition, interleukin 1 (IL-1) further stimulated the growth rate to > 1.0 PD/D. (5) Keratin, hyaluronic acid-mucin, and branching microvilli, all markers for mesothelial cells, were detected on



the cells grown in the optimized medium. As assessed by Giemsa banding, the cells remain chromosomally normal until senescence (35 PD in culture). (6) Asbestos fibers are 10-fold more cytotoxic for mesothelial cells than for bronchial epithelial cells. (7) Phagocytosis of asbestos fibers by human mesothelial cells proved to be rapid; fibers were observed penetrating the cells within 2 hours after exposure. The fibers were engulfed end-first, and a sleeve of membrane surrounded the stalk of longer fibers (> 20  $\mu$ ) and then migrated up the fibers until they were engulfed. (8) Asbestos-exposed mesothelial cells had a near-normal modal number of chromosomes through six successive subcultures; however, chromosome rearrangements were noted. Dicentrics were found in 10% of the fourth passage metaphases; at the sixth passage, more than half of the metaphases contained dicentrics. At the ninth subculture, 80% of the metaphases had dicentric chromosomes, and the modal number had increased to 77. (9) The mitotic spindle poison colcemid is also significantly more cytotoxic for mesothelial cells than for lung fibroblastic cells. In addition, cells grown without growth factors acquire an epithelial cytoskeleton (keratin) and switch to a fibroblastic cytoskeleton (vimentin) when growth factors are present. The epithelial form of the cell is more sensitive to colcemid than is the fibroblastic form.

#### Significance to Biomedical Research and the Program of the Institute:

Although asbestos fibers have been epidemiologically associated as a cocarcinogen for human malignancies other than mesothelioma, these fibers are considered to be complete carcinogens for mesothelial cells. In fact, no other consequential etiologic agent other than fibrous structures, i.e., zeolites, ceramics, and, occasionally, glass, has been identified as a causative agent for pleural and peritoneal mesothelioma. Mesothelioma is a rarely encountered malignancy. However, the latency period for this disease averages 40 years, and with the marked increase in the use of asbestos during and since World War II, an epidemic of mesothelioma has been predicted for the latter part of this century. Carcinogenesis studies with animals have shown that mesothelioma can be caused by intrapleural or intraperitoneal injections of asbestos. The extrapolation of experimental animal data to man is a major problem; one approach is to develop model systems in cultured human tissues for carcinogenesis investigations.

#### Proposed Course:

Growth conditions for human mesothelial cells will be continually improved. Currently, the best clue to the mechanism by which asbestos transforms mesothelial cells is the rapid appearance of chromosomal aberrations. Chromosome rearrangements resulting from exposure to asbestos fibers have occasionally been observed with other types of cells though rarely in human cell cultures. This characteristic suggests that the mesothelial cell has unusual properties that increase its responsiveness to fibrous agents. Mesothelial cells have the remarkable ability to regulate their cytoskeletal composition; the content of keratin or vimentin in the cytoskeleton reflects the growth conditions. This uniquely fluid cytoskeleton, which may be more easily perturbed by penetrating asbestos fibers than are the cytoskeletons of other cell types, may impart mesothelial cells with an increased risk for chromosomal instability, which, in turn, results in their transformation. This proposed model will be tested by assessing the carcinogenic potency of mitotic poisons as both carcinogens and

cocarcinogens with asbestos for mesothelial cells grown as fibroblastic and as epithelial cells. In addition, the possibility of unique chromosomal rearrangements resulting in the production of autocrine growth factors and/or oncogenes will be evaluated in asbestos-exposed mesothelial cells.

Publications:

Tokiwa, T., Sato, J., Lechner, J. F. and Harris, C. C.: Effect of amosite asbestos on cultured human mesothelial cells. Igaku no Ayumi (Japan) 127: 807-808, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05402-01 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Lymphokine Production From Human T-Cell Leukemia Virus Infected Cell Lines

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

PI:	Dimitrios Boumpas	Visiting Fellow	LHC	NCI
Others:	Dean L. Mann	Medical Officer	LHC	NCI
	Mika Popovic	Senior Investigator	LTCB	NCI

COOPERATING UNITS (if any)

Laboratory of Immunology, Department of Surgery, Uniformed University for Health Sciences, Bethesda, MD (C. Neuland)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Two B-cell lines were established as spontaneous outgrowth of cell cultures from patients with adult T-cell leukemia. Both of these cell lines spontaneously produce alpha interferon (alpha-IFN) and one of them produces a mixture of acid stable and acid labile alpha-IFN. In addition, both cell lines spontaneously produce a B-cell growth factor.

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

Dimitrios Boumpas	Visiting Fellow	LHC	NCI
Dean L. Mann	Medical Officer	LHC	NCI
Mika Popovic	Senior Investigator	LTCB	NCI

Objectives:

To study the production of lymphokines such as interferon (IFN) and B-cell growth factor (BCGF) from human T-cell leukemia virus (HTLV)-infected B-cell lines.

Methods Employed:

Cell lines under study are cultured in RPMI 1640 supplemented with 10% fetal calf serum. Supernatants are collected from the cell lines 48 hours after media change, centrifuged and concentrated 8-10 times. Concentrated supernatants are next dialyzed against phosphate-buffered saline overnight at 4°C and stored at -20°C until the day of the IFN or BCGF assay.

IFN Assay: Human interferon is assayed using inhibition of vesicular stomatitis virus (VSV) plaque formation in human amnion (WISH) cells grown in microtiter plates. Neutralization assays are performed by incubating at 37°C for 1 hour equal volumes of IFN dilutions with the appropriate dilution of anti-IFN antisera. Stability of IFN at pH 2 is tested by dialyzing the samples at pH 2 for 24 hours and then reconstituting at pH 7.2 for 24 hours. A > 4-fold loss in the IFN titer after treatment at pH 2 is considered as acid lability.

BCGF Assay: B cells are obtained from frozen spleen cells by using the Fab technique. The cells are stimulated with insolubilized anti-IgM and are added into 96-well microtiter plates,  $1 \times 10^5$  cells per well. Serial dilutions of the samples are added and the plates are incubated for 80 hours at 37°C in a 5% CO<sub>2</sub> incubator and labeled for an additional 16 hours with tritiated thymidine. Finally, the samples are harvested and the incorporated radioactivity is determined with a liquid scintillation counter.

Major Findings:

1. HTLV-infected T-cell lines do not produce IFN either spontaneously or after induction with appropriate inducers.
2. Epstein-Barr Virus (EBV) and/or HTLV-infected B-cell lines spontaneously produce a mixture of acid-stable and acid-labile  $\alpha$ -IFN.
3. HTLV-infected B-cell lines spontaneously produce BCGF.

Significance to Biomedical Research and the Program of the Institute:

1. The establishment of immortalized HTLV-infected B-cell lines should prove useful for studies of the acid-labile  $\alpha$ -IFN and the BCGF which otherwise are difficult to obtain in large quantities. These HTLV-infected B-cell lines should also provide sources of messenger RNA for the genetic cloning of the acid-labile  $\alpha$ -IFN and the BCGF.
2. The establishment of B-cell lines producing acid labile  $\alpha$ -IFN should prove useful for the clarification of its role in the pathogenesis of systematic lupus erythematosus, rheumatoid arthritis and acquired immunodeficiency syndrome.

Proposed Course:

B-cell growth factor (BCGF) produced from these two cell lines will be used to elucidate the relationship between the raf oncogene and the receptor for the BCGF. In addition, the effect of the BCGF on the expression of the raf oncogene will be studied in B cell lines with high and low expression of raf.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

201CE05403-01 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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)

PI:	Brenda I. Gerwin	Research Chemist	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	John F. Lechner	Senior Staff Fellow	LHC	NCI
	George H. Yoakum	Senior Staff Fellow	LHC	NCI
	Brent E. Korba	Staff Fellow	LHC	NCI
	Colin S. Cooper	Visiting Fellow	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.6

PROFESSIONAL:

1.0

OTHER:

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.)

Two new projects have been initiated to determine the (1) the genes and/or gene products altered in asbestos transformed human mesothelial cells and (2) the chromosomal localization of the hepatitis B core antigen gene introduced into human lung carcinoma cells. In addition, we have determined that a diol-epoxide of benz(a)anthracene introduces single-strand breaks in DNA at guanidine and adenosine residues. The reactivity of specific bases is different if the same sequence is reacted as double-stranded or single-stranded DNA.

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

Brenda I. Gerwin	Research Chemist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
George H. Yoakum	Senior Staff Fellow	LHC	NCI
Brent E. Korba	Staff Fellow	LHC	NCI
Colin S. Cooper	Visiting Fellow	LMO	NCI

Objectives:

The goal of this project is to understand, at the molecular level, regulatory interactions of nucleic acids and proteins which govern the growth potential and differentiation state of the cells under analysis. The topics of present interest are:

1. Determination of which cellular factors have been altered in the asbestos-mediated transformation of human mesothelial cells.
2. Chromosomal localization of the hepatitis B core gene(s) inserted into cells of a human lung mucoepidermoid carcinoma.
3. Determination of the site of single-strand breaks in DNA treated with a diol-epoxide of benz(a)anthracene.

Methods Employed:

1. Asbestos-transformed cells and normal cells are grown in tissue culture and whole cell RNA is prepared by organic extraction and CsCl density gradient centrifugation. RNA is bound to nitrocellulose membranes and tested with radioactive DNA from known "onc" genes in order to determine whether any of these genes that are silent before the asbestos treatment are expressed after the treatment.
2. Human cells containing the hepatitis B core antigen gene after protoplast fusion are isolated as single-cell clones. DNA is obtained from these cells, cut with appropriate restriction enzymes, size-separated by gel electrophoresis, transferred to nitrocellulose membranes, and analyzed for junction fragments to determine the approximate number of core gene insertions in each cell clone. Clones which contain one or few insertions will be studied by in situ hybridization to determine the chromosomal location of the inserted gene(s).
3. Maxam-Gilbert sequencing plus diol epoxide treatment of a known DNA were compared to identify the residues at which breaks occurred.

Major Findings:

1. These studies have been recently initiated and have yet to yield definitive results.
2. The original mass culture of core-antigen containing cells seemed to give rise to either bipolar or cuboidal cells on occasion. Cell clones were selected for variant morphologies with cloning cylinders. Thus far, these morphologies have remained constant in different growth media and through selection for a marker introduced into the cells with the hepatitis core antigen.
3. Breaks occur at guanines and adenines, but the reactivity of specific positions is not equivalent in double-stranded and single-stranded DNA.

Significance to Biomedical Research and the Program of the Institute:

1. Since asbestos is an agent which increases the risk of lung cancer, the asbestos-transformed mesothelial cells are an excellent in vitro model in which to study the molecular events induced by the asbestos treatment that result in the immortalization of these cells. This alteration(s) when described might be the same as that induced in human lung cells by continued irritation by asbestos fibers. Identification of a critical step in the process of oncogenesis might lead to specific therapeutic strategies to block or reverse the process.
2. It has been shown that the hepatitis B core antigen when expressed in these cells is cytopathic. The cells are generally able to suppress the transcription of the gene except after azacytidine treatment or in hormonally enriched growth medium. Knowledge of the genetic organization and control of this inserted gene should increase our understanding of and ability to manipulate the physiological growth regulation of mammalian cells.
3. The determination of the nature of chemical damage caused to DNA by known carcinogens is relevant to understanding the mechanism of oncogenic alteration of cells.

Proposed Course:

1. When newly expressed genes are identified, we will attempt to define their gene products and mode of action in the oncogenic process.
2. We propose to gain the requisite data as to the number of insertions and chromosomal location of the hepatitis oncogene in order to see whether these suggest useful experiments with respect to the regulated expression of this gene.

Publications:

Cooper, C. S., Gerwin, B. I. and Scheiner, L. A.: Sites of single-strand breaks in DNA treated with a diol-epoxide of benz(a)anthracene. Carcinogenesis 4: 1645-1649, 1983.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05405-01 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Transfection of TBE-1 with v-myc, Burkitt's myc, raf, and raf/myc Oncogenes

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

PI:	Paul Amstad	Visiting Fellow	LHC	NCI
Others:	George H. Yoakum	Senior Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Carcinogen Macromolecular Interaction Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

Different oncogenes were transfected into GYB33 cells. TBE-1 is a cell line established in our laboratory that represents Harvey ras-transfected human normal epithelial cells. TBE-1 cells have an extended lifespan and can be considered as immortal. These cells, however, do not induce tumors in nude mice. TBE-1 cells were stably transfected with the v-myc oncogene. The ability of these cells to form tumors in nude mice is now being investigated. GYB33 cells passed through soft agar were also transfected with raf, raf/myc, and Burkitt's myc oncogenes.

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

Paul Amstad	Visiting Fellow	LHC	NCI
George H. Yoakum	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Using the protoplast fusion method of transfection, TBE-1 cells and TBE-1 cells cloned from soft agar were transfected with different oncogenes: v-myc, Burkitt's myc, raf, and raf/myc. The goal of these experiments is to study whether secondary transfection of oncogenes into cells that have already been transfected with an oncogene leads to transformation.

Methods Employed:

TBE-1 cells were grown in RPMI medium supplemented with 10% serum to 70% confluence. Then cells were subjected to protoplast fusion. The protoplasts were prepared as follows. Plasmid-carrying derivatives of HB 101 were grown in 250 ml of L-broth to  $2 \times 10^8$  to  $5 \times 10^8$  cells per milliliter. Chloramphenicol was added to a final concentration of 200  $\mu\text{g/ml}$ , and incubation at  $37^\circ\text{C}$  was continued for 18 to 20 hours to amplify the plasmid copy number. After centrifugation, cell pellets were placed on ice and protoplasts were prepared as follows: (1) the pellets were resuspended in 2.5 ml of HBS-20 buffer, (2) 0.8 ml of freshly mixed lysozyme of 10 mg/ml in HBS-20 was added, and (3) incubation at room temperature for 15 to 45 minutes was followed by microscopic observation of the conversion of E. coli cells to spheroplasts to determine when the reaction was complete. After the lysozyme had converted 85% to 90% of the cells to spheroplasts, the mixture was placed on ice, 0.4 ml of 1.25 mCaCl<sub>2</sub> was added to stop the lysozyme, and 2.5 ml of 0.25 mEDTA was added to chelate excess Ca<sup>2+</sup>. This mixture was diluted by slow addition to 2.5 ml of HBS-9 buffer, resulting in a preparation containing approximately  $2 \times 10^9$  protoplasts per milliliter. The fusion was done according to the method described previously. Three days after transfection, the cells were subcultured and selected for resistance to G418, the structural analog of neomycin.

DNA from TBE-1 cells transfected with v-myc oncogene physically linked to a neomycin-resistance marker was isolated by a standard procedure, blotted on nitrocellulose paper, and probed to a nick translated neoprobe. Southern blot and Northern blot analyses are currently done. Further, the v-myc gene-transfected TBE-1 cells are analyzed for colony formation in soft agar and tumor formation in nude mice.

Major Findings:

TBE-1 cells, which are normal bronchial epithelial cells transfected with a Harvey ras oncogene, have been secondarily transfected with a v-myc oncogene physically linked to a neomycin-resistance marker. The integration of the

v-myc/neo construct into the TBE-1 genome was assessed by slot blotting analysis. TBE-1 cells passed through soft agar were secondarily transfected with Burkitt's myc, raf, and raf/myc oncogenes.

Significance to Biomedical Research and the Program of the Institute:

Combinations of activated oncogenes may be responsible for some of the pleiotropic changes seen during tumor progression. The ability to transfect a second oncogene allows the study of these combined actions.

Proposed Course:

To characterize the phenotypic properties of the transfected human cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05406-01 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Construction of a Genomic Library From the Lung Carcinoma, A1146 DNA

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

PI: Paul Amstad Visiting Fellow LHC NCI

Others: Brenda I. Gerwin Research Chemist LHC NCI  
George E. Mark, III Expert LHC NCI  
George H. Yoakum Senior Staff Fellow LHC NCI  
Hans Krokan Guest Researcher LHC NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Genomic human gene libraries are useful tools to isolate and clone human genes. In our laboratory we thought it would be useful to have a human gene library in order to characterize oncogenes on the genomic level.

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

Paul Amstad	Visiting Fellow	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
George E. Mark, III	Expert	LHC	NCI
George H. Yoakum	Senior Staff Fellow	LHC	NCI
Hans Krokan	Guest Researcher	LHC	NCI

Objectives:

To construct and use a human gene library for the characterization of oncogenes.

Methods Employed:

Standard techniques of recombinant DNA work were employed. The phage, EMBL<sub>4</sub>, which can be selected for inserted DNA, was used as a cloning vector.

Major Findings:

To date optimal conditions of DNA ligation and in vitro packaging reaction  $5 \times 10^5$  pfu/ $\mu$ g DNA have been obtained, representing an average packaging efficiency in the range of  $2 \times 10^5$  pfu/ $\mu$ g to  $10^6$  pfu/ $\mu$ g for an optimized system. In order to get a complete library, at least  $10^6$  plaques are needed, with a cloned insert between 10 and 15 Kb.

Significance to Biomedical Research and the Program of the Institute:

Gene libraries of normal and neoplastic human lung cells will be useful for determining structures of normal and transforming genes.

Proposed Course:

To construct libraries using DNA from normal human bronchial epithelium.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05407-01 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Effects of Tumor Promoters and Cocarcinogens on Human Esophageal Cells

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

PI:	Koji Sasajima	Expert	LHC	NCI
Others:	Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
	James C. Willey	Medical Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

## COOPERATING UNITS (if any)

Department of Pathology, University of Maryland School of Medicine, Baltimore, MD (B. F. Trump)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

In Vitro Carcinogenesis Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

Acute effects of putative cocarcinogens and tumor promoters were investigated on cultured human esophageal epithelial cells in serum-free LHC-8 and 199 media with 10% fetal calf serum. The effects were evaluated on clonal growth rate (CGR), cross-linked envelope (CLE) formation, and the enzymatic activities of ornithine decarboxylase (ODC) and plasminogen activator (PA). 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) and the other compounds were inhibitory to clonal growth. None of the compounds induced CLE formation under the conditions used. TPA significantly induced ODC in media 199 but inhibited it in LHC-8 media. Capsaicin induced ODC in both media. TPA and capsaicin induced PA in both media. The other compounds did not change or rather decreased ODC and PA activities.

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

Koji Sasajima	Expert	LHC	NCI
Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
James C. Willey	Medical Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

1. To clarify the effects of putative carcinogens and tumor promoters on human normal esophageal cells in culture.
2. To detect the essential biochemical and morphological changes in human carcinogenesis.

Methods Employed:

1. Clonal Growth Assay: Normal primary esophageal cells, cocultivated with an irradiated layer of 3T3 fibroblasts and grown in media 199 + 10% fetal calf serum and growth supplements (5 x 10<sup>-7</sup>M hydrocortisone, 15 ng/ml EGF, 10<sup>-10</sup> M cholera toxin, 5 µg/ml insulin and 5 µg/ml transferrin), were disaggregated and inoculated either into medium 199 or serum-free LHC-8 medium (medium MCDB 151 with growth supplements, T3, and 35 µg protein of pituitary extract [PEX]). The cells cultivated in serum-containing medium 199 were cocultivated with a layer of irradiated 3T3 fibroblasts, whereas cells grown in serum-free 199 medium or LHC-8 medium were inoculated onto surface coated dishes (10 µg/ml fibronectin, 10 µg/ml bovine serum albumin, and 30 µg/ml Vitrogen collagen). Cells (10<sup>3</sup>) were inoculated into 60 mm dishes. After 6 days of incubation in media with or without test compounds, the cells were fixed and stained with 0.25% crystal violet. The clonal growth rate, expressed as population doubling per day, was determined by taking the mean number of cells per clone from 18 randomly selected colonies on duplicate dishes.

2. Cross-Linked Envelope (CLE) Formation: Cells (2 x 10<sup>5</sup> cells per 35 mm well) were inoculated into 6-well plates. After 48 h of incubation, the number of cells was calculated with a grid and the media were replaced with 2 ml of media containing the test compounds in 0.8% agar. After 6 h of incubation with the test compounds, 2 ml of 4% SDS and 20 mM DTT were placed on top of the agar. Following 4 h of incubation at 37°C, the number of cross-linked envelopes was counted and the percentage of total cells containing CLEs was determined.

3. Ornithine Decarboxylase (ODC) and Plasminogen Activator (PA) Assays: Cells (10<sup>5</sup> per 16 mm well) were inoculated into 24-well culture plates containing either serum-free 199 or LHC-8 medium with or without test compounds and incubated for 6 h at 37°C. ODC was then measured as the release of <sup>14</sup>C<sub>2</sub> from labeled ornithine during a 1-hour incubation. PA was measured by using benzylcarbonyl-glycyl-L-prolyl-L-arginyl (<sup>14</sup>C-Anilide) as a substrate. After 5 hours of incubation, plasminogen was added (final concentration 0.3 U/ml), and incubation was

continued for 1 more hour.  $^{14}\text{C}$ -Anilide was then extracted with Econofluor and radioactivity was counted. Specific activity of ODC and PA was calculated as nmole/h/mg protein.

4. Morphology: Cells were cultivated in medium with or without test compounds and examined through phase contrast microscopy at various periods.

#### Major Findings:

Data on clonal growth rate were obtained for 12-0-tetradecanoyl-phorbol-13-acetate (TPA), T-2 toxin, cigarette smoke condensate (CSC), ethanol, CSC + 1% ethanol, and capsaicin. Clonal growth rates of normal esophageal cells were  $1.0 \pm 0.08$  (PD/D) in 199 + 10% FCS and  $0.9 \pm 0.07$  in LHC-8 medium containing 0.1% DMSO as carrier. All compounds tested were inhibitory to clonal growth. Doses of 50% growth inhibition were estimated as 10 nM of TPA, 6 nM of T-2 toxin, 8  $\mu\text{g}/\text{ml}$  of CSC, 0.8  $\mu\text{g}/\text{ml}$  of CSC and 1% ethanol, 540 nM of ethanol and 40  $\mu\text{M}$  of capsaicin. CSC at 0.01  $\mu\text{g}/\text{ml}$  slightly accelerated clonal growth to 103-108% of control. Three different concentrations of each compound were tested for CLE, ODC, and PA assays. Rates of CLE, a marker for terminal differentiation of esophageal cells, were less than 1% in both media. All of these compounds did not change CLE formation under these conditions.

ODC activities in esophageal cells were detected in both 199 and LHC-8 media. Activity was 8- to 10-fold higher in LHC-8 media than in 199 media. ODC was low in LHC-0 media (LHC-8 without EGF and PEX) and was induced 2- to 3-fold by adding EGF and PEX to LHC-0. TPA (10 and 100 nM) induced ODC to 250-350% in serum-free 199 media but decreased it to 40% of control in LHC-8 media. Capsaicin (100  $\mu\text{M}$ ) induced ODC in both 199 and LHC-8 medium to 200% and 240%, respectively.

TPA (1 to 100 nM) induced significant PA activity in both media ( $p < 0.05$ ). Capsaicin, at a concentration of 100  $\mu\text{M}$ , induced PA in both media. T-2 toxin (100 nM) dramatically decreased ODC and PA in LHC-8 medium.

The other compounds were not effective or rather inhibitory to ODC and PA. All compounds induced minor morphological changes on esophageal cells.

#### Significance to Biomedical Research and the Program of the Institute:

We have investigated the effects of TPA and capsaicin and found them to be relevant in the human system to the phenomena associated with malignant transformation in animal models. This system is useful for examination of tissues from one individual and from different individuals and in the detection of inhibitors of tumor promotion. These investigations may help to identify the mechanisms and preventive compounds associated with human carcinogenesis.

#### Proposed Course:

Selected compounds should provide the comparison of the effects on normal esophageal cells and cancer cell lines. Further investigations include observation of the effects through experimental human carcinogenesis in vitro.

#### Publications:

None



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05408-01 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Drug Metabolism Phenotyping of Nonhuman Primates

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

PI:	Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Susan M. Sieber	Deputy Director	DCE	NCI

## COOPERATING UNITS (if any)

Department of Pharmacology, St. Mary's Hospital, London, England (J. R. Idle)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Carcinogen Macromolecular Interaction Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

5

## PROFESSIONAL:

3

## OTHER:

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.)

Phenotyping of animal models and man for their xenobiotic metabolizing capabilities has, in recent years, been undertaken in the interests of predicting carcinogenic susceptibilities. The metabolism of exogenous agents is known to be genetically dependent, and selective in vivo xenobiotic metabolic routes appear to be accessible to evaluation by the use of nontoxic doses of certain drugs. The determination of the rate of selective enzymatic modifications of test agents may thus provide a suggestion as to how susceptible an individual may be to the oncogenic potential of carcinogens activated by similar metabolic routes. Several agents including debrisoquine, S-mephenytoin, S-carboxymethyl-L-cysteine, and sulfamethazine have been shown to be metabolized by enzymatic routes governed by separate genetic loci. Thus, these agents will be used to phenotype nonhuman primates on the basis of their abilities to metabolize these compounds. Since the primate colony from which these monkeys will be sampled has been and is presently involved in ongoing chemical carcinogenesis experiments, the results of the metabolic phenotyping can be compared to the susceptibilities of the monkeys to carcinogenesis.

PROJECT DESCRIPTIONNames, 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
Susan M. Sieber	Deputy Director	DCE	NCI

Objectives:

To determine the relationship between the genetically dependent rates of selective enzymatic reactions and the chemical carcinogenic susceptibilities in nonhuman primates, man's phylogenetically closest relative. Previous work has demonstrated that chemical carcinogens are activated by selective enzymatic routes in vitro and in rodent animal models. Some correlation has been found between the rates of activation of some carcinogens and the carcinogenic susceptibilities of rats as well. Several studies have clearly demonstrated human polymorphisms in the rates of some of these metabolic routes. However, the correlation of these human metabolic phenotypes with cancer susceptibility has to be based solely on epidemiological data. Thus, more phenotyping data are needed from animal models phylogenetically closer to man than rodents and where carcinogenesis data are available.

Methods Employed:

Subefficacious doses of debrisoquine (DBQ), S-mephenytoin (MPH), S-carboxymethyl-L-cysteine (SCMC), or sulfamethazine will be administered p.o. to monkeys, and 24-hour urine samples collected and analyzed for parent drug and metabolite(s). The methods for quantitation of these compounds in urine have been previously reported in human studies. These four agents were chosen because they each represent a metabolic phenotype governed by separate genetic loci. The enzymatic reactions monitored by these agents are aliphatic ring hydroxylation by DBQ aromatic ring hydroxylation by MPH, S-oxidation by SCMC, and N-acetylation by sulfamethazine.

The results of phenotyping will be compared with the known carcinogenic susceptibilities of these monkeys.

Major Findings:

Preliminary results of phenotyping three monkeys from each of three species, rhesus, cynomolgus, and African green monkeys, are available. All of the monkeys tested rapidly metabolized DBQ, MPH, and SCMC. The metabolic ratio (concentration of parent drug to metabolite) was observed to range from 0.02 to 0.6 for DBQ, which is well below that reported for man. No differences between species were observed with the minimal sample size of three monkeys per group. MPH metabolism was extensive in these monkeys as well. From 6% to 52% of the administered dose of as excreted as 4 hydroxy-MPH in 24 hours, which is greater than that reported for man. There were no significant differences in the rate of MPH hydroxylation between species.

The rate of sulfoxidation of SCMC was also high in these monkeys. The metabolic ratio ranged between 0.8 and 3.8, but the rhesus and cynomolgus monkeys metabolized SCMC significantly ( $P < 0.10$ ) faster than the African green monkeys. The average SCMC sulfoxidation index per species was  $1.4 \pm 0.3$ ,  $1.2 \pm 0.3$ , and  $2.7 \pm 0.9$  for rhesus, cynomolgus, and African greens, respectively. These rates are more rapid than that reported for man. Rhesus monkeys were significantly ( $P < 0.01$ ) slower N-acetylators of sulfamethazine than African green monkeys, while the rate in the cynomolgus monkeys fell between the former two species. The average rate of N-acetylsulfamethazine formation was  $51 \pm 4\%$ ,  $65 \pm 12\%$ , and  $76 \pm 9\%$  for rhesus, cynomolgus, and African green monkeys, respectively. This rate of sulfamethazine acetylation in rhesus monkeys compares well with previously reported values. These rates also compare well with the human data, classifying the rhesus as poor N-acetylators, the African greens as extensive N-acetylators, and individual cynomolgus monkeys in both categories.

The rapid rate of enzymatic hydroxylation observed in these monkeys for DBQ and MPH would suggest that these monkeys would be susceptible to carcinogenesis upon exposure to aromatic hydrocarbon carcinogens. Aflatoxin B<sub>1</sub> and methylazoxy-methanol-acetate did produce tumors in these species (Adamson and Sieber in Langenbach, R., Nesnow, S. and Rice, J. M. (Eds.): Organ and Species Specificity in Chemical Carcinogenesis, New York, Plenum Press, 1983, pp. 129-140). However, several other carcinogens, including benzo[a]pyrene, 3-methylcholanthrene, and cigarette smoke condensate, did not induce tumors. Thus, there may be some correlation between the metabolic phenotype and chemical carcinogenesis susceptibility, but the data presently available are insufficient to draw firm conclusions.

#### Significance to Biomedical Research and the Program of the Institute:

A strong correlation between xenobiotic metabolism phenotypes and chemical carcinogenic susceptibilities in nonhuman primates would provide a firm basis for monitoring people for selective susceptible metabolic phenotypes. Individuals found to be extensive metabolizers of agents such as DBQ and MPH may be more susceptible to aromatic hydrocarbon carcinogens. Thus, these extensive metabolizers should not smoke and should avoid jobs that provide high risk of exposure to aromatic carcinogens. Evaluating the human population for individuals who may be at high risk of cancer from chemical carcinogens may, therefore, ultimately provide a prophylactic treatment of this disease.

#### Proposed Course:

The preliminary results from three monkeys from each of three species did not provide any polymorphisms in phenotypes within a given species. Several monkeys from one species will be phenotyped as described above in the search for metabolic polymorphisms. Since the suggested predictability of cancer susceptibility is based on polymorphisms within the human population, similar findings within the monkey colony would enhance these studies. Subsequent to further phenotyping of monkeys, selective monkeys will be sacrificed and organ and tissue culture studies initiated. The xenobiotic metabolizing and carcinogen-activating capabilities of these cultures will be used to compare with the in vivo phenotyping data and the site(s) of tumors in chemical carcinogenic susceptible monkeys.

#### Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05409-01 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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)

PI: Toru Masui Visting Fellow LHC NCI

Others: John F. Lechner Senior Staff Fellow LHC NCI  
 Curtis C. Harris Chief LHC NCI

COOPERATING UNITS (if any)

University of Maryland School of Medicine, Baltimore, MD (B. F. Trump);  
 Georgetown University 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 20205

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Defined methods to grow replicative cultures of normal human bronchial epithelial (NHBE) cells without serum have been developed. These cells can be subcultured several times; will undergo 35 population doublings; and have expected epithelial cell characteristics of keratin, desmosomes, and blood group antigens on their cell surface. NHBE cells inoculated at clonal density will multiply with an average generation time of 28 hr; the majority of the cells are small and migratory and have few tonofilaments. An autocrine growth factor was detected by measuring the growth rate as a function of cell density. This factor may be interleukin 1, since interleukin 1 was detected by immunoperoxidase staining, and highly purified interleukin 1 was found to increase the clonal growth rate. An unidentified autocrine squamous differentiation-inducing factor was also detected. Adding human blood-derived serum (BDS) depresses the clonal growth rate of NHBE cells in a dose-dependent fashion. In contrast, 10 representative lines of human lung carcinomas either replicate poorly or fail to grow at all when inoculated at clonal density in serum-free medium; their rates of multiplication increase in direct proportion to the amount of BDS added to the optimized medium. BDS reduces the clonal growth rate of NHBE cells by specifically inducing squamous differentiation. The differentiation-inducing activity was not present in plasma but was found in platelet lysates. An assay system based upon morphometric measurement of cell area by image analysis was developed to quantify fractionated BDS; differentiation-inducing activity was found to be approximately 50K daltons.

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

Toru Masui	Visiting Fellow	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To develop systems to study malignant transformation of human epithelial cells. These studies include the following: (1) develop defined media for replicative epithelial cell cultures from bronchial tissues, (2) identify and characterize an autocrine growth factor, (3) identify and characterize an autocrine squamous differentiation-inducing factor, (4) isolate and characterize a factor in BDS that induces squamous differentiation, (5) develop efficient assays to quantify the various squamous differentiation-inducing factors and to 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  $^3\text{H}$ -thymidine into acid precipitable material, and the labeling index by autoradiography. Squamous differentiation is determined by measuring the cell area, cell apposition index, and cross-linked envelopes.

Major Findings:

Human bronchial epithelial cell culture experiments have yielded the following results. (1) A method for routinely initiating replicative epithelial cell cultures of human bronchus was developed. Large pieces of bronchus tissue were initially set up as explant cultures and incubated in a rocking chamber for 7-10 days to facilitate reversal of ischemia. The explants were then cut into smaller pieces, explanted, and incubated in a serum-free medium optimized for growth of NHBE cells. This medium (LHC-9) permits rapid outgrowth of epithelium but retards growth of the fibroblastic cells. The medium is a modification of MCDB 152. The changes are as follows: the concentrations of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, choline, and serine are doubled; the

concentrations of  $MgCl_2 \cdot 6H_2O$  and  $CaCl_2 \cdot 2H_2O$  are increased 3.5 times; the concentrations of NaCl and HEPES buffer are reduced 20%; and the concentration of  $Na_2HCO_3$  is reduced 15%. LHC-9 is supplemented with insulin,  $8.7 \times 10^{-7}$  M; epidermal growth factor (EGF),  $8.25 \times 10^{-10}$  M; transferrin,  $1.25 \times 10^{-7}$  M; phosphoethanolamine,  $5 \times 10^{-7}$  M; ethanolamine,  $5 \times 10^{-7}$  M; hydrocortisone,  $2 \times 10^{-7}$  M; triiodothyronine,  $1 \times 10^{-9}$  M; epinephrine,  $1.6 \times 10^{-6}$  M; retinoic acid,  $3 \times 10^{-10}$  M; bovine pituitary extract, 35  $\mu$ g protein/ml; and gentamycin, 50  $\mu$ g/ml. (2) An autogenous growth factor was detected by measuring growth rate as a function of cell density. This factor is found in NHBE cell conditioned medium. NHBE cell conditioned medium will increase the growth rate of NHBE cells incubated in LHC-9 medium without epinephrine. In addition, the conditioned medium stimulates multiplication of normal human bronchial fibroblasts and mouse lymphocytes. (3) Interleukin 1 was found to increase the growth rate of NHBE cells. Further, interleukin 1 was detected in NHBE cells by immunoperoxidase staining. These observations suggest that the autocrine growth factor may be interleukin 1. (4) Cell density was found to influence the effect of  $Ca^{2+}$  on growth. Whereas optimal growth occurred at clonal densities in medium containing 1 mM  $Ca^{2+}$ , rapid squamous terminal differentiation occurred when the medium of dividing high-density cultures was changed from 0.1 to 1 mM  $Ca^{2+}$ . These observations suggest that the  $Ca^{2+}$  concentration influences the activity of an autocrine squamous differentiation-inducing factor. This autocrine differentiation-inducing factor may be interleukin 1, since interleukin 1 is thought to be an ionophore, and excess exogenous interleukin 1 induces NHBE cells to undergo squamous differentiation. (5) Supplementation of LHC-9 with as little as 0.25% fetal bovine blood-derived serum (BDS) resulted in a decrease in clonal growth rate; 8% supplementation completely inhibited growth by inducing terminal squamous cell differentiation. Human lung carcinoma lines were also incubated in LHC-9 medium without and with 8% BDS. The results showed that serum toxicity per se was not responsible for the observed inhibition of NHBE cell growth; all 10 carcinoma lines divided significantly more rapidly ( $p < 0.05$ ) in BDS-supplemented medium. Thus, the carcinoma cells have both increased requirements for BDS mitogens and a greatly reduced ability to respond to factors in BDS that induce the normal cells to undergo squamous differentiation. (6) Since cell areas and apposition index were found to increase in direct proportion to the concentration of BDS, these characteristics could be used as a quantitative assay for squamous differentiation-inducing activity. (7) Immunoperoxidase staining for involucrin clearly revealed that NHBE cells exposed to BDS were arranged in a multilayered fashion. The overlying cells were large and strongly involucrin positive, whereas the basal cell sheets were involucrin negative. (8) The serum factor that induces squamous differentiation was found by gel filtration to be approximately 50,000 daltons. In addition, the factor is resistant to beta mercaptoethanol, stable at pH 8.0, and soluble at low concentration of salt. (9) There was significantly less inhibition of NHBE cell growth with plasma-derived serum. On the other hand, platelet factors at concentrations that stimulated fibroblastic cell multiplication also inhibited DNA synthesis and stimulated terminal differentiation of NHBE cells.

#### Significance to Biomedical Research and the Program of the Institute:

Understanding those processes that control growth and differentiation of normal human epithelial cells and elucidating how these controlling mechanisms differ

in carcinoma cells are central to our understanding of carcinogenesis. Further, these differences in control processes may then be exploited both to identify premalignant cells and to design new and novel chemoprevention modalities.

Proposed Course:

The autocrine growth factor will be purified using FPLC columns and characterized. In addition, the mitogenic potency and specific binding of this factor will be determined for both NHBE and tumorigenic lung cells. The autocrine differentiation-inducing factor and the serum differentiation-inducing factor also will be purified and chemically characterized. The differentiation-inducing potencies and specific binding of these factors to NHBE and tumorigenic lung cells will also be determined. Finally, these factors will be used to help delineate the molecular mechanisms that control growth and squamous differentiation.

Publications:

Lechner, J. F.: Nutrient, hormone, growth factor and substrate interdependent regulation of epithelial cell growth. Fed. Proc. 43: 116-120, 1984.

Lechner, J. F., Haugen, A., McClendon, I. A. and Shamsuddin, A.: Control of growth and squamous differentiation of normal human bronchial epithelial cells. Differentiation 25: 229-237, 1984.

Lechner, J. F., Kaighn, M. E., Groden, J. and German, J.: Blooms syndrome cells: Abnormal serum growth response. Exp. Cell Res. 145: 381-388, 1983.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05410-01 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Hepatitis B Virus Carcinogenesis

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

PI:	George H. Yoakum	Senior Staff Fellow	LHC	NCI
Others:	Brent E. Korba	Staff Fellow	LHC	NCI
	John F. Lechner	Senior Staff Fellow	LHC	NCI
	Vincent Wilson	Senior Staff Fellow	LHC	NCI
	Dimitrios Boumpas	Visiting Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

## COOPERATING UNITS (if any)

Cancer Institute, Chinese Academy of Medical Science, Beijing, Peoples Republic of China (Sun Tsung-tang and Hsia Chu-chieh); Department of Pathology, University of Maryland, Baltimore, MD (B. F. Trump)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Carcinogen Macromolecular Interaction Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

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

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

The role of hepatitis B virus (HBV) in human cancer is being investigated by (1) transfection analysis of HBV and HBV genes into human cells and (2) nucleic acid hybridization analysis of peripheral blood lymphocytes (PBL) and lymph node tissues from various HBV-risk group patients. Since there is no in vitro system for HBV infection of human cells in culture, the application of viral transfection analysis provides the only available method to study the virus in vitro. HBV core (HBc) gene transfection into mucoepidermoid carcinoma cells (NCI-H292) and hepatocellular carcinoma cells (Malove) provides a model system to study the regulation and expression of a cytopathologic viral gene. Although the surface antigen gene (HBs) is constitutively expressed, the HBc gene is regulated by methylation of a HpaII site 276 base pairs from the AUG-start site of the HBc structural gene. The hormonal and nutritional requirements regulating the expression of the HBc gene are being studied. Although viable normal human hepatocyte cultures have been difficult to obtain, we are initiating experiments to transfect HBV into adult hepatocyte cultures for transient assays of viral gene products and studies of viral cytopathology associated with its role in liver carcinogenesis. Screening of nucleic acids from HBV risk group patients' PBL and lymph node tissues indicates the following: (1) HBV sequences, transcripts, and replicative DNA can be detected in serologically negative HBV patients' tissues; (2) approximately three of five chronically infected (CAH) patients' PBL nucleic acids contain viral transcripts and four CAH patients' PBL DNA samples contained replicative forms of HBV on Southern hybridization; (3) the slot-blot testing of larger groups of patients will provide information relevant to disease status that may be more sensitive than serologic evaluation since serologically negative patients can be screened for HBV-nucleic acid sequences. In addition, the mode of DNA replication may be determined from HBV DNA isolated from PBLs and lymph nodes.



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

George H. Yoakum	Senior Staff Fellow	LHC	NCI
Brent E. Korba	Staff Fellow	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
Vincent Wilson	Senior Staff Fellow	LHC	NCI
Dimitrios Boumpas	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The primary goals of this research project are the development and application of a genetic approach to problems of human carcinogenesis at the molecular level. This research program focuses on (1) the mechanisms of HBV pathology and its role in carcinogenesis and (2) the mechanism of biological responses involved in these carcinogenic processes with potential application to cancer epidemiology and disease prevention.

Methods Employed:

We have developed a method to genetically transfect a variety of human cell types, including normal fibroblastic and epithelial cells, carcinoma cells, transformed fibroblasts, and lymphoid cell lines. Introduction of hepatitis B virus (HBV) and HBV genes into human cells is essential to the development of effective research programs to study the role of HBV in human carcinogenesis at the genetic and molecular levels because there is no system available at this time to infect cells with HBV in tissue culture. The protoplast fusion transfection procedure permits transient expression of transferred genes in 70-90% of the recipient cell cultures for 6-12 days after the procedure. This permits the construction of human cell lines carrying HBV for in vitro characterization of virus pathology and carcinogenic potential. The protoplast fusion method of transfection for transfer of plasmids stably transfers genes into human cells at frequencies greater than  $10^{-3}$  units.

Standard nucleic acid hybridization analysis methods will be utilized to characterize the genetic organization of HBV transfected into human recombinant cell lines constructed for these studies. This includes slot-blot hybridization of DNA or RNA products to detect genomic DNA inserts and characterize transfected gene transcription products. Southern hybridization analysis of the genetic organization of HBV transfectants permits interpretation of gene expression experiments and testing of the methylation state of the transfected virus genome.

The survey of patient tissues for HBV DNA sequences and transcripts by slot-blot hybridization analysis and Southern hybridization requires coordination with the Family Epidemiology Section, NCI, to obtain appropriately classified human tissues from HBV-risk groups for this study. Selected samples of patient tissue DNA have been probed by Southern hybridization analysis to test for the presence of replicative HBV DNA.

Major Findings:

The primary areas of progress are (1) development of a method to transfect human cells at high frequency, (2) application of this method to transfection analysis of HBV gene expression and cytopathology in human epithelial and lymphoblastoid cell lines, and (3) initiation of projects to screen nucleic acids of patient PBL and lymph nodes to ascertain their relation to the early stages of virus infection and disease.

1. **Hepatitis B carcinogenesis.** Transfection of human cells with the pSV2gpt<sup>+</sup> plasmid constructed to carry a subgenomic fragment of HBV containing only the HBV core (HBc) gene expression immediately after the procedure. We isolated a human carcinoma cell line stably transfected with the HBc<sup>+</sup> gene fragment by selection of transfected cell cultures for gpt<sup>+</sup> expression. The gpt<sup>+</sup>/HBc<sup>+</sup> cell lines were used to determine that growth in serum-free medium and 5'-azacytidine treatment stimulate the production of Hbc gene product (HBcAg). Subsequently, we found that Hbc gene expression in a hepatocellular carcinoma cell line carrying the entire HBV genome is stimulated by these conditions. This cell line has carried the HBV genome since its isolation from a chronically infected patient. The temporal relationship of the cytopathologic response to Hbc gene expression is similar for both cell types, indicating a primary role for Hbc gene expression in the cytopathology of HBV-infected human liver. Transfection analysis has been extended to human T-cells capable of carrying and expressing HBV genes after transfection with a plasmid containing four genomic copies of HBV, and conditions are being established to transfect HBV into human hepatocytes for studies on the effects of HBV on liver cells.

2. Screening patient tissues for HBV nucleic acids has revealed that this sensitive assay can detect virus in infected patients that are serologically negative for HBV infection. In addition, the detection of HBV RNA in chronically infected patients indicates that virus expression status can be tested in patient samples. This survey has revealed that three acquired immune deficiency syndrome (AIDS) patients tested by Southern hybridization analysis carry HBV DNA in replicative forms consistent with the rolling circle model of replication in their lymph node tissue, detectable as monomeric and dimeric replicative intermediate sizes. To summarize these findings: AIDS patients were positive for HBV sequences (3/3), chronic active hepatitis (CAH) patients were positive for HBV sequences (5/5), patients recovered from acute infections were negative (5/5), and noninfected control patients were negative (12/12) for HBV sequences among the samples screened to date.

Significance to Biomedical Research and the Program of the Institute:

The development of a method to efficiently transfect a variety of human cell types is of general significance to biomedical research programs that employ human somatic cell genetics and molecular biology. The initial application of these procedures to problems related to various aspects of human carcinogenesis has produced a unique insight into the biological role of the core antigen gene of HBV. This information is immediately applicable to understanding the basis of Hbc gene expression and its association with chronic HBV infection and carcinogenesis. The gpt<sup>+</sup>/HBc<sup>+</sup> cell line produced by transfection analysis of the HBc<sup>+</sup> gene provides a unique opportunity to determine at the molecular level

the details of DNA methylation as a control factor for expression of a gene with proven biological significance. Screening HBV-risk group patients for HBV nucleic acids provides new insights into the role HBV plays in AIDS and the opportunity to investigate the significance of HBC gene-mediated cytopathology. In addition, studies of the hematopoietic system as an early target during the establishment of HBV infection will contribute to developing a complete picture of HBV pathology and the interaction of this system with the development of hepatitis and liver cancer.

#### Proposed Course:

1. The HBV carcinogenesis program will proceed as follows: (a) the effect of HBC<sup>+</sup> gene expression will be studied to determine the factors that regulate HBC<sup>+</sup> gene expression; (b) we will determine the role of the methylation site in HBC<sup>+</sup> promoter and structural genes in regulating HBC gene expression; and (c) we will transfect human liver cells and selected lymphocytes with tandem duplicates of the HBV genome for mechanistic carcinogenesis experiments. In addition, the role of HBC gene expression in lymphocytes and the utility of nucleic acid screening of HBV risk group patients will be determined.

2. Patient tissues will be screened and selected, immunocompetent and immunosuppressed patient samples will be used to test for gene-specific expression (HBs/HBC), and quantities of the replicative form of the virus will be isolated to determine the mode of DNA replication for HBV in patients.

#### Publications:

Yoakum, G. H., Korba, B. E., Lechner, J. F., Tokiwa, T., Gazdar, A. F., Seeley, T., Siegel, M., Leeman, L., Autrup, H. and Harris, C. C.: High frequency transfection and cytopathology of hepatitis B virus core antigen gene in human cells. Science 222: 385-389, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05411-01 LHC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Studies of Proliferative Response in Normal Human Bronchial Epithelial Cells

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

PI:	James C. Willey	Medical Staff Fellow	LHC	NCI
Others:	John F. Lechner	Senior Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Bruce M. Boman	Medical Staff Fellow	LHC	NCI
	Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
	George E. Mark, III	Expert	LHC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Normal human bronchial epithelial cells are cultured in a defined, serum-free medium. We investigated the mitogenic effects of compounds using clonal growth assays and colony-forming efficiency. We also measured ornithine decarboxylase activity and cAMP levels, since polyamine and cAMP metabolism are involved in cell proliferation in most systems. In the defined medium LHC-0, which already contains insulin, hydrocortisone, transferrin, phosphoethanolamine, and ethanolamine, the cells grow at a rate of 0.42 population doublings per day (PD/D). Many compounds were investigated, and most had no mitogenic effect, including human pituitary growth hormone, testosterone, estradiol, estrin, calcitonin, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and endothelial cell growth supplement (ECGS). EGF, bombesin, gastrin-releasing peptide amino acids 14-27 (GRP14-27), and human chorionic gonadotropin (HCG) each independently increase the clonal growth rate to 0.68, 0.65, 0.67, and 0.66, respectively. An aqueous extract of bovine pituitaries (PEX) has no effect by itself but does increase the clonal growth rate when EGF is also present. cAMP-enhancing agents are mitogenic only when both EGF and PEX are present. When bombesin, 0.1 micromolar is present, the concentration of PEX required to provide a half maximal growth response is decreased from 2.27 micrograms/ml to 0.4 micrograms/ml.

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

James C. Willey	Medical Staff Fellow	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Bruce M. Boman	Medical Staff Fellow	LHC	NCI
Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
George E. Mark, III	Expert	LHC	NCI

Objectives:

To assay relevant agents for mitogenic effects on normal human bronchial epithelial (NHBE) cells cultured in a defined, serum-free system. In the process, we hope to improve the defined medium and to better understand mechanisms involved in regulating cell proliferation. By comparing growth factor requirements for normal and malignant HBE cell proliferation, we hope to define important characteristics of malignantly transformed cells.

Methods Employed:

NHBE cells were dissolved with trypsin and inoculated onto coated (10 µg/ml fibronectin; 10 µg/ml bovine serum albumin [BSA]; 30 µg/ml vitrogen collagen) culture dishes. Cells were inoculated at 4000 cells per 60 mm dish for clonal growth assays, 100,000 cells per well on 24-well plates for ornithine decarboxylase (ODC) and epidermal growth factor (EGF) binding, and  $2 \times 10^6$  cells per 60 mm dish for cAMP assays.

The basic medium used in these experiments was LHC-0; this is LHC-8 medium without EGF, pituitary extract (PEX), or thyroxine (T3). The effects of medium additives on cell division were measured using a clonal growth rate assay. Four thousand cells were inoculated per 60 mm culture dish. Twenty-four hours later, medium was removed and replaced with medium containing the test compound(s). The medium was replaced with fresh medium after 4 days of incubation; after 7 days, the cells were fixed with 10% formalin and stained with 0.25% aqueous crystal violet. The mean number of cells per clone in 18 randomly selected colonies (9 per replicate dish) was determined for each condition tested. To derive the growth rate (population doublings per day [PD/D]), the  $\log_2$  of the average number of cells per clone was divided by the number of days of incubation. A computerized image analyzer (Artec 800) was programmed to count the number of cells per colony. Student's t-test was used to evaluate the significance of difference between experimental groups.

For the ODC assay, 100,000 cells/well were inoculated onto coated 24-well plates (16 mm wells) in 1 ml of LHC-0. Twenty-four hours later, media were removed and replaced with 250 µl of fresh media containing the test compounds. After a 6 hour incubation, media were removed and cells were quickly frozen on dry ice. After freezing and thawing 3 times to lyse the cells, ODC activity was quantified by measuring the release of  $^{14}\text{CO}_2$  from labeled ornithine during a 1 hour incubation.

For the measurement of cellular cAMP levels, 2 million cells were inoculated onto 60 mm culture dishes in LHC-0 medium. Twenty-four hours later, medium was removed and replaced with media containing the test compounds. After a 4 hour exposure, media were removed and the cells were exposed to 0.5 ml of cold trichloroacetic acid. After the cells were scraped off the dishes, the samples were centrifuged at 5000 g for 30 minutes. The resulting supernatant was run over a Dowex AX-80 ion exchange resin column, and the eluate was assayed for cAMP by radioimmunoassay.

Whole-cell EGF binding was measured by previously described methods. For these assays, 100,000 cells/well were inoculated onto 24-well plates containing LHC-0 medium. Twenty-four hours later, the medium was removed and replaced with fresh LHC-0 media containing the test compounds. After a further 6 hour incubation at 37<sup>o</sup>, the plates were placed on crushed ice. The media were removed and replaced with ice-cold fresh medium containing LHC-0 plus 0.2 nM <sup>125</sup>I-EGF. After a 4 hour incubation at 4<sup>o</sup>, the cells were washed 3 times with 1 ml of ice-cold Hank's buffered salt solution, solubilized with 1% SDS, 1% Triton X-100, and 0.1 N NaOH, and counted on an LKB gamma counter. Nonspecific binding was determined in the presence of 1 μM cold EGF.

#### Major Findings:

In LHC-0 medium, the PD/D of NHBE cells is 0.45. When varying concentrations of L-epinephrine are added to LHC-0 medium, there is no increase in PD/D. There is also no increase in PD/D when PEX alone (35 μg/ml) or in combination with L-epinephrine is added to LHC-0. EGF (1 μM) increases the PD/D to 0.65 PD/D, and the combination of PEX (300 μg/ml) and EGF increases the PD/D further to 0.95 PD/D. Addition of L-epinephrine to LHC-0 plus EGF alone has no effect. However, when varying concentrations of L-epinephrine are added to medium containing the combination of EGF and PEX, there is a further increase in PD/D reaching a maximum of 1.2 PD/D with an L-epinephrine concentration of 1.5 μM. This effect can be reproduced with the other cAMP-enhancing compounds, including isoproterenol, dibutryl-cAMP, 3-isobutyl-methylxanthine (IBMX), and cholera toxin (CT).

In LHC-0 medium, the ODC activity of NHBE cells is 0.5 ± 0.1 units/mg of cell protein. After a 6 hour incubation in medium containing EGF at a concentration that provides maximal stimulation of the clonal growth rate (1 μM), ODC activity is stimulated to 320% of control. Cells incubated for 6 hours in LHC-0 supplemented with 1.5 μM L-epinephrine also had ODC activity increased by 230%. When L-epinephrine and EGF are both added to LHC-0 medium, ODC activity in the cells is synergistically stimulated to 700% of control. In contrast, the optimal concentration of PEX (30 μg/ml) has no significant effect on ODC activity either when present alone or when present in combination with EGF and/or L-epinephrine. Isoproterenol, dibutryl-cAMP, CT, and IBMX also stimulate ODC in NHBE cells after a 6 hour incubation.

The concentration of cAMP in NHBE cells cultured in LHC-0 is 300 pM. Incubation with EGF (1 μM) or PEX (30 μg/ml) for up to 6 hours has no significant effect on the cAMP level. In contrast, incubation with 1.5 μM L-epinephrine causes an early 300% increase in the cAMP level that is sustained for at least 4 hours. Incubation with the combination of EGF and L-epinephrine leads to a cAMP level

higher than that obtained with L-epinephrine alone. However, when PEX is also present, there is a synergistic increase in cAMP.

Six hours of incubation with PEX either with or without L-epinephrine did not have any effect on EGF binding after a 6 hour incubation. In contrast, incubation with 0.8 mM EGF for 6 hours caused a marked decrease in EGF binding to 20% of control. This reduced level of binding was not altered by co-incubation with either PEX or L-epinephrine or the combination of both PEX and L-epinephrine. In competition assays, we also observed no change in maximum EGF binding under any of these conditions.

In clonal growth dose-response experiments, bombesin (0.1  $\mu$ M) and GRP14-27 (0.1  $\mu$ M) increase the clonal growth rate (Student's t-test,  $p < 0.05$ ) from  $0.42 \pm 0.02$  (mean  $\pm$  SD) to a maximum of  $0.70 \pm 0.03$  and  $0.68 \pm 0.03$  PD/D, respectively. In the presence of 0.8 mM EGF (a concentration previously found to provide maximal mitogenic effect), these peptides further increase the clonal growth rate from  $0.65 \pm 0.03$  to a maximum of  $0.80 \pm 0.03$  PD/D (bombesin, 0.1  $\mu$ M) and from  $0.72 \pm 0.03$  to  $0.91 \pm 0.03$  PD/D (GRP14-27, 0.1  $\mu$ M). In the absence of EGF, the colony-forming efficiency (CFE) of NHBE cells is  $0.8 \pm 0.2\%$ . Supplementing the medium with either bombesin (0.1  $\mu$ M) or GRP14-27 (0.1  $\mu$ M) increases ( $p < 0.05$ ) the CFE by 113% or 87%, respectively. In the presence of EGF (0.8 mM), the CFE of NHBE cells is  $1.5 \pm 0.1\%$ . The addition of bombesin (0.1  $\mu$ M) or GRP14-27 (0.1  $\mu$ M) to EGF-supplemented medium further increases the CFE by 101% or 74%, respectively.

Highly purified human chorionic gonadotropin increases the clonal growth rate to 0.65 PD/D.

#### Significance to Biomedical Research and the Program of the Institute:

We have established the mitogenic activity of several compounds for NHBE cells. This allows improved culture of these cells under defined conditions for use in transformation experiments and experiments aimed at understanding the processes involved in cell replication. We have demonstrated the efficacy of using defined culture conditions to investigate metabolic changes that take place during induction of proliferation. Furthermore, we have determined that two peptides often secreted by human bronchial carcinomas (gastrin-releasing peptide [GRP] and human chorionic gonadotropin) are mitogenic for normal human bronchial epithelial cells. Further investigation of these peptides may allow better understanding of the mechanisms by which neoplastically transformed cells grow in an uncontrolled fashion.

#### Proposed Course:

We plan to determine the mitogen present in PEX, characterize GRP binding, and compare proliferative responses in normal to those in malignant bronchial epithelial cells.

Publications:

Lechner, J. F., Stoner, G. D., Haugen, A., Autrup, H., Willey, J. C., Trump, B. F. and Harris, C. C.: In vitro human bronchial epithelial model systems for carcinogenesis studies. In Webber, M. and Sekely, L. (Eds.): In Vitro Models for Cancer Research. New York, CRC Press (In Press)

Willey, J. C., Lechner, J. F. and Harris, C. C.: Bombesin and the c-terminal portion of gastrin-releasing peptide GRP<sub>14-27</sub> are mitogenic for normal human bronchial epithelial cells. Exp. Cell Res. (In Press)



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05412-01 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Model System for the Investigation of Putative Tumor Promoters in NHBE Cells

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

PI: James C. Willey Medical Staff Fellow LHC NCI

Others: John F. Lechner Senior Staff Fellow 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 20205

## TOTAL MAN-YEARS:

0.8

## PROFESSIONAL:

0.8

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

Normal human bronchial epithelial (NHBE) cells and lung carcinoma cell lines were used to investigate the effects of putative human tumor promoters. Compounds were selected on the basis of known tumor-promoting activity in experimental animals or epidemiological evidence that they play a role in human cancer. We have previously reported that the mouse epidermal tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) induces terminal differentiation in NHBE cells; thus far, no resistant cells have grown from cultures of presumably normal, noninitiated bronchial epithelial cells after treatment with TPA. In contrast, all carcinoma cell lines are resistant to TPA-induced terminal differentiation. We have now investigated the effects of other putative tumor promoters on markers of terminal differentiation in NHBE cells. We find that alysiatoxin, debromoaplysiatoxin, teleocidin, cigarette smoke condensate (CSC), and fractions thereof increase cross-linked envelope formation and plasminogen activator activity and decrease epidermal growth factor (EGF) binding after a 12 to 24 hour incubation. We selected four fractions of CSC for further investigations based on their capacity to serve as tumor promoters in the mouse skin model, including insoluble basic fractions a and b (B1a, B1b), ethanol-extracted weakly acidic fraction (WAE), and methanol-extracted neutral fraction (Nmeoh). Of these, Nmeoh had the greatest capacity to inhibit growth, induce CLE and plasminogen activator, induce a squamous morphology, and decrease EGF binding.

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

James C. Willey	Medical Staff Fellow	LHC	NCI
John F. Lechner	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To evaluate biochemical, morphological, growth, and differentiation effects of cocarcinogens/tumor promoters on normal human bronchial epithelial (NHBE) cell cultures.

Methods Employed:

Bronchial epithelial cell outgrowths were dissociated with 0.02% trypsin, 0.1% EGTA and 0.1% PVP and inoculated onto coated (10  $\mu\text{g/ml}$  fibronectin; 10  $\mu\text{g/ml}$  bovine serum albumin; 30  $\mu\text{g/ml}$  vitrogen) plastic culture dishes. For the clonal growth and colony-forming efficiency assay, cells were inoculated at 200 cells/cm<sup>2</sup>. For plasminogen activator (PA), cross-linked envelopes (CLE), morphology, epidermal growth factor (EGF) binding, and phorbol dibutyrate binding assays cells were inoculated at 50,000 cells/cm<sup>2</sup>. PA activity was measured on 100,000 cells per well in 24-well plates by adding plasminogen to the medium (final concentration, 0.3 units/ml) and, after a further 1 hour incubation, assaying for plasmin using a <sup>14</sup>C-labeled synthetic substrate, glycine-proline-arginine-<sup>14</sup>C-anilide. For the CLE assay, 200,000 cells were inoculated onto each well of a 6-well plate. Twenty-four hours later, the number of cells per well was calculated using a grid, and the media were replaced with media containing the test compound plus 0.8% agar. After a 6 hour incubation, 2 ml of sodium dodecyl sulfate (4%) and dithiothreitol (20 mM) in dH<sub>2</sub>O was added over the agar. After a further 4 hour incubation at 37°C, the number of CLE per well and percentage of CLE per cell population were then calculated. EGF binding was conducted in whole cells, inoculated into a 24-well plate using <sup>125</sup>I-EGF.

Major Findings:

Aplysiatoxin and debromoaplysiatoxin inhibit the clonal growth rate of NHBE cells with 50% inhibitory doses (ID<sub>50</sub>) of  $3 \times 10^{-11}$  and  $10^{-10}$ , respectively, induce a squamoid morphology within 30 minutes after exposure, induce CLE formation from a control of 0.5% to 15% and 16%, respectively, after a 6 hour exposure, and stimulate PA secretion by 250% and 300%, respectively, at  $10^{-8}$  M. We conclude that, like TPA and teleocidin, these polyacetate compounds are inducing terminal differentiation in NHBE cells. The 50% inhibiting concentration (ID<sub>50</sub>) for cigarette smoke condensate (CSC), B1a, B1b, WAE, and Nmeoh after an initial 12 hour exposure was 10  $\mu\text{g/ml}$ , 10, 10, 13, T, respectively. Morphological changes in response to CSC became discernable after 12 to 24 hours of exposure, after which time the cells were observed to have migrated on top of one another, giving a multilayered appearance, and became elongated, often

assuming a dumbbell shape with marked thinning at the ends. Using the ID50 concentrations, after a 12 hour exposure, CSC, Nmeoh, B1a, B1b, and WAE induced CLE from a control of 0.5% to  $15 \pm 1$ ,  $12 \pm 2$ ,  $12 \pm 2$ ,  $20 \pm 3$ , and  $25 \pm 2$ , respectively; PA was induced significantly only by Nmeoh, to 220%. ODC was not significantly affected by any of the fractions.

#### Significance to Biomedical Research and the Program of the Institute:

The induction of terminal differentiation in NHBE cells by putative tumor promoters and the relative resistance of human lung carcinoma cell lines is consistent with the theory of tumor promotion that tumor promoters induce normal cells to terminally differentiate while initiated cells are resistant to this effect and are, therefore, able to selectively outgrow the normal cell population and present a larger target for additional transforming agents. Since there are compounds or metabolic precursors of compounds in CSC that have properties similar to the phorbol esters, teleocidin, and the polyacetates, it should be possible and worthwhile to further investigate CSC and fractions and isolate the compounds responsible. This would provide us with the most relevant compounds for investigating causes of human carcinogenesis.

#### Proposed Course:

Further investigate CSC and fractions thereof for compounds that may serve as tumor promoters in NHBE cells. Use such isolated compounds for carcinogenesis assays.

#### Publications:

Willey, J. C., Moser, C. E. and Harris, C. C.: Effects of aplysiatoxin and debromoaplysiatoxin on growth and differentiation of normal human bronchial epithelial cells. Cell Biol. Toxicol. (In Press)

Willey, J. C., Moser, C. E., Lechner, J. F. and Harris, C. C.: Differential effects of 12-O-tetradecanoylphorbol-13-acetate on cultured normal and neoplastic human bronchial epithelial cells. Cancer Res. (In Press)

Willey, J. C., Saladino, A. J., Ozanne, C., Lechner, J. F. and Harris, C. C.: Acute effects of 12-O-tetradecanoylphorbol-13-acetate, teleocidin B, or 2,3,7,8-tetrachlorodibenzo-p-dioxin on cultured normal human bronchial epithelial cells. Carcinogenesis 5: 209-215, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05413-01 LHC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Enzyme Immunoassay for Endogenous N-Nitrosation Products

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

PI: Glennwood E. Trivers Research Scientist LHC NCI

Others: Curtis C. Harris Chief LHC NCI

## COOPERATING UNITS (if any)

Department of Nutrition and Food Science, MIT, Cambridge, MA (S. Tannenbaum)

## LAB/BRANCH

Laboratory of Human Carcinogenesis

## SECTION

Biochemical Epidemiology Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

0.4

## PROFESSIONAL:

0.4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

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

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

Endogenous nitrosation of amino acids and related molecules is an important potential source of carcinogenic N-nitroso compounds in human populations. N-nitrosoproline (NPro), a noncarcinogenic, nonmutagenic substance has been established as a stable index for endogenous nitrosation. Ingested, preformed NPro is excreted unchanged in human and animal urine, and the concentration of NPro in human and animal urine has been shown to increase proportionally with increasing amounts of precursor nitrates and L-proline consumed, respectively, in food stuffs and drinking water. Moreover, preliminary studies in humans indicate that higher levels of NPro may be found in smokers as compared to nonsmokers. Similar results are found in individuals from geographical areas of high risk for stomach and esophageal cancer compared to those from areas of low risk for these diseases. Current knowledge and recent information of endogenous nitrosation was obtained using gas chromatography and terminal energy analyzer measurements of NPro in urine. This method detects a minimum of 0.2 ng NPro per ml which is insufficient for detection of serum levels of NPro. In this project, we intend to develop an enzyme immunoassay (EIA) and an ultrasensitive enzymatic radio-immunoassay (USERIA) with femtomole sensitivity and the possibility to use both urine and sera to measure NPro in vivo in human populations of interest to the study of human carcinogenesis.

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

Glennwood E. Trivers	Research Scientist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To develop simplified enzyme immunoassay (EIA) and ultrasensitive enzymatic radioimmunoassay (USERIA) procedures for detection and quantitation of N-nitrosopropoline (NPro) in human and animal tissues and fluid specimens; to study NPro levels in individuals and populations potentially at risk for developing related cancers; to study NPro in environmental and experimental conditions involving known and suspected chemical carcinogenesis.

Methods Employed:

Bovine serum albumin (BSA) coupled to NPro and keyhole limpet hemanocyanin (KLH) coupled to NPro are used as immunogens in Freund's complete adjuvant. Three rabbits, each given serial injections of each immunogen are bled periodically for serum to be tested for antibody activity. Antisera to BSA and KLH (Cappel) are used to verify the binding of conjugated NPro to polyvinyl microtiter plates, and BSA-NPro-coated plates are used to screen sera from KLH-NPro-injected rabbits and vice versa. Active sera are tested in noncompetitive EIAs to titer antibody and subsequently in competitive EIAs for the ability of the fluid phase conjugate to compete for the antibody.

Major Findings:

Each of three rabbits given intramuscular injections with the immunogens developed antisera with titers greater than 1:400,000 when tested in noncompetitive assays against 1 ng in the solid phase for EIA. Antisera reactions in homologous systems (BSA-NPro antisera vs. BSA-NPro and vice versa) compared to reactions in heterologous systems (BSA-NPro antisera vs. KLH-NPro and vice versa) demonstrated that 30% of the reacting antibodies in anti-BSA-NPro sera are directed against the hapten, 70% against the carrier; in anti-KLH-NPro sera, the ratio is 80% against the hapten and 20% against the carrier. Consequently, anti-KLH-NPro antiserum was used to establish conditions for a competitive EIA using 1 ng BSA-NPro as the solid phase immunosorbent. With 1:400,000 dilution of anti-KLH-NPro serum, the detection minimum is 0.1 picogram (pg) of BSA-NPro and 0.02 pg NPro, with 50% inhibition of 2.0 and 0.2 pg, respectively. However, to date, the ability of the antisera to detect uncoupled NPro in noncompetitive or competitive EIA and RIA systems is extremely low and, perhaps, impractical. Apparently, the major epitope in this initial attempt included a significant portion of the carrier, and the resulting antibodies do not recognize uncoupled NPro.

Significance to Biomedical Research and the Program of the Institute:

A simplified, more sensitive assay for NPro would economize and make more accessible the application of NPro assessment for clinical and research purposes. The ability to measure endogenous nitrosation will be an important addition to the technology for studies of human carcinogenesis. This important essential step will allow the possibly significant differentiation between cancer and noncancerous individuals and subgroups in the populations of heavy smokers; asbestos, coke oven, and aluminum workers at risk for malignancies of the esophagus, bronchus, and lung; and individuals in geographically endemic areas for high incidence of hepatoma associated with aflatoxin.

Proposed Course:

The failure of the current antisera to significantly detect uncoupled NPro is under investigation. The low concentration of anti-NPro antibodies could be related to the immunization procedure and to the size of NPro. NPro is an extremely small molecule (144.13 Nw), smaller even than the prostaglandins we have previously found difficult to use in the EIA system. To address those possibilities, a new set of animals will be injected by the subcutaneous and intradermal routes with different scheduling, and different molecules can be chosen as carriers. However, we are also attempting to enrich the anti-NPro antibody concentration by affinity purification.

Publications:

None

ANNUAL REPORT OF  
THE LABORATORY OF MOLECULAR CARCINOGENESIS  
NATIONAL CANCER INSTITUTE

October 1, 1983 to September 30, 1984

The Laboratory of Molecular Carcinogenesis (LMC) plans, develops, and conducts a research program designed to (1) clarify the molecular biology of carcinogenesis; (2) elucidate the fundamental nature of the interaction of carcinogenic agents, especially chemical, with biological systems in the induction of cancer; (3) identify those environmental and endogenous factors which relate to and modify the carcinogenic process; and (4) clarify the metabolic regulatory processes which are related to carcinogenesis.

The goal of the Laboratory of Molecular Carcinogenesis is to understand the molecular basis of carcinogenesis with the view toward identifying susceptible populations and preventing human cancer. The research program is designed to understand the molecular basis by which carcinogenic agents cause malignant transformation, and to identify and characterize those exogenous and endogenous factors involved in carcinogenesis. The Laboratory seeks to clarify the metabolic interaction of exogenous and endogenous agents in the living organism at the molecular, cellular and organism levels and seeks to understand the consequences of these interactions in terms of cell regulation and carcinogenesis. The processes are studied in biological preparations and cells from experimental animals and humans.

In the last several years, the course of the Laboratory research program has been markedly affected by the powerful new techniques of molecular biology and immunology. We have added younger staff highly experienced in DNA recombinant and related techniques, protein chemistry, and hybridoma technology. The power and precision of these techniques have had a highly positive influence on the progress of many of the projects of the Laboratory.

Cell Genetics Section - Studies (1) the cellular and molecular mechanisms of neoplastic transformation induced by chemical carcinogens; (2) isolation and characterization of human actin gene families; (3) the distribution and the possible biological implications of left-handed, helical Z-DNA; (4) clinical, cellular, and molecular abnormalities in xeroderma pigmentosum, dysplastic nevus syndrome, ataxia-telangiectasia and related diseases; (5) cellular and molecular effects of psoralens plus ultraviolet radiation; (6) generation of repeated sequence variants; and (7) isolation and characterization of opal suppressor tRNA genes in higher eukaryotes and the effects of template methylation on their expression.

The cellular macromolecules responsible for the expression of the neoplastic phenotype of chemically transformed human cells have been investigated. A new polypeptide recognized in a chemically transformed human cell line has been identified as a mutant of beta-actin which has aspartic acid at position 244 instead of glycine. Beta-actin genes were isolated from the transformed human cell line. Results on the determination of the structure and organization of

the beta-actin gene confirmed that the alteration of beta-actin in the transformed cells is ascribed to a point mutation (transition) in the structural gene of beta-actin. The synthesis of the mutated beta-actin was correlated with the expression of the transformed phenotype in variants of the transformed line and its hybrids with normal human fibroblasts. A mutation resulted in several defects in the function of beta-actin, such as increased instability, reduced incorporation into cytoskeletal elements, and decreased ability to polymerize in vitro. These defects in the beta-actin molecule were associated with the disruption and loss of the structural organization of the cytoskeleton, such as the actin cable network. The results suggest that a mutation in beta-actin leads the cells to express transformation by disrupting the cytoskeletal structure and its function.

Characterization of cell mutants of the Balb/3T3 cell line showing different susceptibility to chemically- or ultraviolet (UV)-induced transformation which have been isolated in our laboratory indicates that variants are different in the expression process. The resistant variant showed transformation only when cells treated with a carcinogen were further exposed to tumor promoters. The results of measurement of intercellular communication in the variant cells treated or untreated with tumor promoters suggest that these variants differ in the steps involved in the expression or promotion of transformation, and that inhibition of intercellular communication plays an important role in the promotion of transformation.

A family of human actin genes has been isolated by molecular cloning. Five of these genes (cardiac muscle actin, aorta-type smooth muscle actin, stomach-type smooth muscle actin, pseudo beta-actin and beta-actin), have been characterized by DNA sequencing. Although there are many pseudogenes for cytoplasmic actin (beta- and gamma-actin), the number of functional genes seems to be only one per haploid genome for each actin isoform. Comparison of the primary structure of actin genes, particularly the location of introns and the presence or absence of the cysteine codon following the initiation codon led to a hypothesis of the evolutionary pathways of actin gene families.

Purine-pyrimidine alternating sequences, such as (dC-dG) $n$ ·(dC-dG) $n$  and (dT-dG) $n$ ·(dC-dA) $n$ , take a right-handed helical conformation, mostly B-DNA, or a left-handed helical conformation called Z-DNA, depending on the buffer conditions. We have previously found evidence for the abundant existence of such Z-DNA-forming sequences in natural genomes by hybridization and by direct DNA sequencing. Z-DNA formation of the (dT-dG) $n$ ·(dC-dA) $n$  sequence was investigated by determining the sites which are sensitive to S<sub>1</sub>-nuclease in the plasmid containing the Pst 0.9 Kb fragment of the human cardiac actin gene including the (dT-dG)<sub>25</sub> sequences. Plasmid DNAs of five different supercoillings were separately obtained by using a nicking-closing enzyme in combination with different amounts of ethidium bromide. Both single strand scission and double strand scission were induced by S<sub>1</sub>-nuclease in plasmid DNA with superhelicity greater than 0.06, indicating that the sensitivity to S<sub>1</sub>-nuclease is dependent on supercoiling. Both single and double strand incisions occurred at 3 to 5 bases after the end of the (dT-dG)<sub>25</sub>·(dC-dA)<sub>25</sub> sequence. These results indicate that S<sub>1</sub>-nuclease incises the DNA strand near the junction between the B- and Z- forms of DNA, 3-5 bases outside of Z-DNA sequences.

In order to investigate the possible role of Z-DNA in the regulation of gene expression, the (dT-dG) $n$ ·(dC-dA) $n$  sequence was inserted into the pSV2-CAT



plasmid vector and into the c-Ha-ras 1 clone. pSV2-CAT is a recombinant plasmid containing a chloramphenicol acetyltransferase (CAT) gene. Insertion of the Z-DNA sequence resulted in a significant enhancement of expression of the CAT gene and the transforming potentials of the c-Ha-ras 1 gene when they were transfected into mammalian cells.

Human cancer-prone genetic diseases are being studied with a view toward identifying groups of people with an increased susceptibility to environmental carcinogenesis and understanding the mechanism of their cancer susceptibility. Patients with xeroderma pigmentosum (XP) and ataxia-telangiectasia (AT), diseases with UV and X-ray hypersensitivity, respectively, and patients with the dysplastic nevus syndrome of hereditary cutaneous melanoma (DNS) are being studied. Detailed examinations of the clinical features of affected individuals are being made. A large retrospective XP literature study (more than 800 cases) revealed a greater than 2000-fold increase in frequency of skin cancers, anterior eye cancers, and cancer of the tip of the tongue. Reported internal neoplasms (especially brain tumors) were also increased in frequency. A prospective registry of XP patients is under way. Field studies in Israel detected a possible new form of XP with defective DNA repair without neoplasia. Cell lines have been established for detection of XP heterozygotes. DNA transfection studies have demonstrated the suitability of SV40 virus-transformed XP cells as high efficiency recipients. A rapid host cell reactivation DNA repair assay is being developed that measures transient expression of CAT activity in XP and normal cells transfected with a UV-treated plasmid bearing the CAT gene. Collaborative clinical studies of 14 DNS kindreds have demonstrated: autosomal dominant inheritance of the melanoma trait, new melanomas only in family members with dysplastic nevi, and greater than 100-fold increased melanoma risk in family members with dysplastic nevi. Similar dysplastic nevi also occur in non-familial settings. A new classification of DNS was proposed. Laboratory studies demonstrated UV-induced hypermutability in DNS lymphoblastoid cell lines. AT is an autosomal recessive disorder with clinical and cellular x-ray hypersensitivity and a high incidence of neoplasms. AT is not an x-ray analogue of XP. Lymphoblastoid cell lines were established from 10 kindreds with AT. Bleomycin-induced chromosome breakage and delay in cell cycle progression were greater in AT homozygous than in AT heterozygous and normal lines. Histone protein levels and spontaneous and X-ray-induced histone synthesis were indistinguishable between AT and normal cells.

Psoralen plus long wavelength ultraviolet radiation (UV-A) is being investigated as a model system for clinically relevant photochemical carcinogenesis and as a probe for defective DNA repair. Used experimentally for treatment of psoriasis and mycosis fungoides, psoralen plus UV-A have been found to be mutagenic and carcinogenic. We have developed an in vitro assay to measure the effects of UV-A mediated psoralen-DNA binding on human lymphoid cells. Parameters monitored include the rate of DNA synthesis, induction of DNA-psoralen cross-links, induction of sister chromatid exchanges, alterations in the rate of cell proliferation and survival, and in immune reactivity. These studies indicate that the low doses of psoralen plus UV-A received by patients' leukocytes during therapy may result directly in decreased DNA synthesis in their circulating lymphoid cells. Cell survival was found to be markedly dependent on UV-A exposure and 8-MOP concentration, correlated with inhibition of DNA synthesis, and related to induction of DNA interstrand cross-links. There was a dose-dependent reduction in mixed leukocyte culture reactivity induced by

8-MOP plus UV-A treatment. Cells from a patient with Cockayne's syndrome had normal survival following 8-MOP plus UV-A treatment but reduced survival after treatment of UV-B. Thus repair of photosensitized 8-MOP damage involves at least one pathway that is different from that for UV-B damage.

A specific site of genome rearrangement has been detected by comparing members of a family of long repeated DNA sequences. About one-half of the members of this family are truncated at one end. The site of truncation is very specific and relates to a tandemly repeating structure internal to the long repeat element.

A cloned tRNA gene is being used to determine whether transcription by RNA polymerase III is sensitive to the presence of 5-methylcytosine in DNA. Cloned DNA containing specific methylation patterns is compared to unmethylated DNA by an *in vitro* transcription assay. The methylation status of this gene and its flanking sequences in genomic DNA is being investigated using methylation-sensitive restriction endonucleases. A collection of opal suppressor tRNA genes from higher eukaryotes has been isolated by molecular cloning. These genes encode serine tRNA species that suppress the termination codon, UGA. The genes are being characterized by DNA sequencing and by *in vitro* transcription. They have an unusual promoter structure that may be important in controlling the level of gene expression. They are strongly conserved evolutionarily. Although some genomes apparently contain inactive pseudogenes, we find that every higher eukaryotic genome tested contains at least one active gene. Thus the products of opal suppressor genes seem to play an indispensable intracellular role.

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) regulation, and structure of the genes for the enzymatic system primarily responsible for the metabolic activation and detoxification of PCH and other chemical carcinogens.

This section studies the molecular events of malignant transformation induced by chemical carcinogens, mainly those of the PCH class. The aim is to understand the enzymatic conversion of carcinogens to either detoxified forms, or to active carcinogenic forms. Higher organisms have systems for the detoxification and elimination of foreign chemical compounds, including carcinogens. These systems primarily involve microsomal cytochrome P-450 mixed-function oxygenases, but also include epoxide hydratase and conjugating enzymes. The vast majority of foreign compounds are processed by these enzyme systems. The mixed-function oxygenases are influenced by a variety of environmental factors such as drugs, pesticides and carcinogens, and are influenced by the nutritional and hormonal state of the animal. The age, sex and genetic makeup also determine enzyme activity. Work in this Laboratory provided the key studies which showed that this enzyme system was responsible for the activation of PCH procarcinogens to their ultimate carcinogenic forms. A primary goal is to define the enzymatic mechanism by which polycyclic hydrocarbons are activated either to carcinogenic forms or to detoxified products. As these enzymes are characterized and as sensitive methods are developed for their assay, it may be possible to characterize an individual's enzymatic makeup with respect to carcinogen metabolism and to understand the relationship between this metabolism and individual susceptibility to PCH-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 multileveled investigations of the carcinogen-metabolizing enzyme systems, continuing our use of HPLC to study carcinogen metabolites, using monoclonal antibodies (Mab) and enzyme inhibitors to study the properties of the enzymes and using recombinant DNA and other molecular biological techniques to study the structure and regulation of the genes for the enzymes of carcinogen-metabolizing systems.

The primary enzyme interface between environmental chemicals and higher organisms is the mixed-function oxidase system. The various forms of cytochrome P-450 constitute a major part of this system, and are the major receptors for a wide variety of drugs, carcinogens, and other environmental chemicals. Our goal is to identify and characterize these isozymes in different tissues, species, populations and individuals. A major and unique approach to this problem is to develop a library of MAbs which are highly specific for individual cytochromes P-450. We have found that the use of MAbs offers a powerful new dimension to numerous aspects of cytochrome P-450 research and believe their use will have a large impact. Thus far, we have developed panels of MAbs to five different cytochromes P-450: two rabbit forms, and three rat forms. The latter MAbs have been prepared to the major cytochrome P-450 in livers of rats treated with 3-methylcholanthrene (MC), phenobarbital (PB), and pregnenolone-16 $\beta$ -carbonitrile (PCN). The MAbs produced in each case are classified into three groups, based on their interaction with cytochromes P-450: (1) those that bind, precipitate, and inhibit enzymatic activity; (2) those that bind and precipitate, but do not inhibit activity; and (3) those that only bind, but do not precipitate or inhibit activity.

We have used inhibition of enzyme activity by the Mab 1-7-1, prepared to MC-induced rat liver cytochrome P-450, to detect the cytochromes P-450 recognized by this Mab. This Mab not only inhibited the aryl hydrocarbon hydroxylase (AHH) activity of MC-induced rat liver microsomes, but also the AHH activity of human placenta. Among placenta from different individuals, the extent of inhibition of AHH by this Mab was virtually complete. Inhibition of 7-ethoxycoumarin deethylase (ECD), however, exhibited a large degree of individual variation. Placentas from both dizygotic and dichorionic, monozygotic twins were examined for both absolute amounts of AHH and ECD, and for their degree of inhibition by Mab 1-7-1; high intra-pair concordances were observed, relative to that found in unrelated individuals. The Mab-sensitive activities were also found in human lymphocytes, but not in liver and monocytes. The cytochromes P-450 responsible for these activities in liver and monocytes are therefore antigenically distinct from the enzymes in placenta and lymphocytes.

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

MAb-sensitive activities in several animal tissues, species and strains were also examined as models for the study of cytochrome P-450 in humans. The AHH activities in liver from MC-treated rats, C57BL/6 mice, guinea pigs, and hamsters were all greatly inhibited. Hepatic ECD activity was inhibited in rats, partially inhibited in C57BL/6 mice, and unaffected in guinea pigs and hamsters. Neither hepatic AHH nor ECD was inhibited in the relatively non-responsive mouse strain, DBA/2. The pulmonary activities behaved differently from the hepatic activities in that both activities were inhibited by MAb in both C57BL/6 and DBA/2 mice. Studies of the types of cytochromes P-450 in different animal models may lead to a greater understanding of their multiplicity, genetic control, and relationship to drug and carcinogen metabolism.

We are also developing radioimmunoassays (RIAs) with MAbs to specific cytochromes P-450. An RIA based on the MAb, 1-7-1 has detected (1) the elevation in the level of MC-induced cytochrome P-450 in the livers of MC-treated rats, relative to untreated and PB-treated rats; (2) similar differences for guinea pigs and C57BL/6 mice but not for hamsters and DBA/2 mice; and (3) tissue-dependent differences in MC-induced rats, with liver having higher levels than lung or kidney. The RIA data are in general agreement with the enzyme inhibition results. The competitive RIA with MAb 1-7-1 was also applied to placenta from different individuals, and a large degree of individual variation was found. The method was compared to the enzyme inhibition method and was found to be more reliable in detection of cytochromes P-450 that readily denature but still maintain intact MAb-specific epitopes. Development of quantitative and sensitive RIAs based on several MAbs should greatly aid in the detection and phenotyping of cytochromes P-450 in tissues and individuals, and is a major goal of our laboratory.

Monoclonal antibodies have also proven to be new and powerful tools for immunopurification of cytochromes P-450. A Sepharose-MAb 1-7-1 immunoabsorbent tightly binds cytochrome P-450 from liver microsomes of MC-treated rats. The enzyme appears on SDS gel electrophoresis as two bands of MW 56,000 and 57,000. An immunoabsorbent based on MAb 1-31-2, however, only binds the MW 57,000 species. The latter, therefore, contains epitopes recognized by both MAb 1-7-1 and MAb 1-31-2, while the MW 56,000 species only has the MAb 1-7-1-specific epitope. In addition to the cytochrome P-450 purified from rat liver, various isozymes have been immunopurified from tissues of C57BL/6 and DBA/2 mice, guinea pigs, and hamsters. Such immunoabsorption experiments reveal epitope relatedness between cytochrome P-450 in different tissues, strains, and species. These results demonstrate the extraordinary efficacy of MAb-based immunoabsorbents for purification of individual cytochromes P-450. The various isozymes may then be analyzed structurally and enzymatically.

We have adopted recombinant DNA and related molecular biological techniques to assess, with greater precision, the molecular mechanisms of regulation of P-450 gene expression, the multiplicity of P-450s, and the structural-functional relationships among the P-450s. This work also has great potential for increasing our understanding of biochemical individuality in human carcinogenesis. We have characterized in much greater detail the structure of the P-450 gene whose isolation we reported last year. We have constructed subclones of the different domains of this gene and of adjacent sequences. Using these as hybridization probes, we have discovered repetitive DNA in the intervening sequences nearest the 5' and 3' ends of the gene. The repetitive

sequences in these two locations are different. We have also obtained preliminary evidence that the large intervening sequence nearest the 5' end of the gene contains sequences that code for a second mRNA that encodes a peptide of about 70 kilodaltons. We have also successfully cloned and characterized cDNAs corresponding in sequence to three different MC-induced cytochromes P-450 (MC-P-450s). Including the single cloned P-450 cDNA described last year, we now have recombinant plasmids containing cDNAs complementary to four distinct MC-P-450s. Two of the new recombinants correspond to the mRNAs for the two major forms of MC-P-450. The third corresponds to a minor form. Using these cloned cDNAs as hybridization probes, the two major MC-P-450 mRNAs were found to be 2000 and 2700 base pairs long, while the minor form mRNA was 2000 base pairs. We have found that both major MC-P-450 mRNAs are undetectable in liver from control animals but are strongly induced and are among the most abundant mRNAs found in MC-induced liver. The minor MC-P-450 mRNA is present in low levels in controls and is induced to ten-fold by MC. The kinetics of induction of these three MC-P-450 mRNAs are now being studied. By hybridization selection and translation, we find that these mRNAs encode 55 or 59 kilodalton peptides that are recognized by polyclonal antibodies to MC-P-450. These peptides are now being characterized further, using the P-450-specific monoclonal antibodies prepared in this laboratory. We have also used the three newly cloned MC-P-450 cDNAs as hybridization probes to isolate the corresponding genes from a library of the rat genome. At present two of these genes have been successfully isolated and are being characterized by restriction enzyme mapping and R-loop analysis; the isolation of the third gene is in progress.

Nucleic Acids Section - Studies (1) the interaction of chemical and physical carcinogens with nucleic acids and their actions on the functions of DNA, (2) the relationship between defects in repair of cellular DNA and human cancer, (3) chemically produced alteration of DNA and the repair of such alterations, and (4) the interaction of DNA with proteins believed necessary for cellular transformation.

The study of human cells defective in repairing damaged DNA was extended, with the rationale that DNA repair-deficient cells are more susceptible to the adverse effects of carcinogens than their repair-proficient counterparts. A group of human tumor (19) and SV40-transformed (7) strains deficient in a MW 22,000 protein responsible for repairing O<sup>6</sup>-methylguanine (O<sup>6</sup>-MeG, a modified DNA base made by certain methylating agents) was identified earlier in this project. Such strains are called Mer<sup>-</sup>. Viral transformation often produces Mer<sup>-</sup> strains.

Mer<sup>+</sup> strains are judged to contain on the average 60,000 repair protein molecules per cell. A group of 5 Mer<sup>+</sup> cell strains, somewhat sensitive to cell killing by MNNG, repairs approximately one-third as much O<sup>6</sup>-MeG as Mer<sup>+</sup> Rem<sup>+</sup> cell strains.

It was found that the ability of cell strains to repair O<sup>6</sup>-MeG, but not sensitivity to MNNG-produced cell killing, correlates with sensitivity to cell killing by chloroethyl-nitrosoureas, indicating different modes of cell killing by these agents. Mer<sup>-</sup> human tumor lines were determined to be more sensitive to cell killing by both  $\alpha$ - and  $\beta$ - human interferons than were Mer<sup>+</sup> human tumor cell lines, indicating a possible common origin of sensitivities to both agents.

The gene products of oncogenic viruses play a role in cellular transformation. A conclusive evaluation of the role of individual oncogene products has been

difficult because the purification of such proteins from tumor-bearing animals is an arduous task. Therefore, recombinant and monoclonal antibody techniques were employed to generate large quantities of proteins related to the middle tumor antigen of polyoma virus. In order to study the protein:DNA interactions responsible for metallothionein gene regulation, studies using the cloned human metallothionein gene and gene product are being carried out.

Protein Section - Conducts a diversified group of research projects for the general purpose of developing insight into how alterations in the normal biochemical flow of information from DNA into protein might be altered during carcinogenesis. Studies (1) protein-nucleic acid interactions which may regulate or coordinate the production of cellular proteins, (2) specific alterations in DNA sequences which may modify control functions or lead to altered levels of regulatory proteins, and (3) mechanisms which modify genetic information in DNA by post-transcriptional processing of RNA.

Because DNA transfection is currently a very important technique in carcinogenesis experiments, an understanding of the fate of the transfected DNA is essential. In studies with specially constructed shuttle vectors, it was found that such transfected DNA was susceptible to homologous recombination, leading to an unusually high frequency of spontaneous mutation. Analysis revealed that the mutations involved insertions of cellular DNA and deletions. The host cell DNA was not affected.

Continuing studies on the relation between the structure and function of chromatin indicate that the transcribable regions of chromatin are hyperacetylated. The same regions were also found to be 30-fold enriched in another minor chromatin protein, histone H<sub>1</sub><sup>o</sup>. Chromosomal proteins HMG-1 and HMG-2, which bind to DNA, bound preferentially to the Z form of DNA. This finding suggests that these proteins may bind selectively to particular regions of DNA, possibly playing some role in the function of the genome. Eleven monoclonal antibodies have been elicited against histone H-5. The epitopes of each of these was mapped. In other studies, the chromatin structure of the gene coding for a MC-induced P-450 enzyme has been studied. A DNase I hypersensitive region was found near the 5' terminal of the gene.

Phosphorylation of proteins has emerged as an important biochemical property of oncogenes. Another route to phosphorylation is the direct incorporation of a phosphorylated amino acid into the nascent protein chain. A transfer RNA having this property has been described in previous reports from this Laboratory. This tRNA belongs to the class of opal suppressors which function to permit translation to continue in the presence of the nonsense codon UGA. The chicken gene coding for this tRNA has now been characterized in great detail. It contains an unusual promoter region, and is transcribed poorly; the human genome contains a similar tRNA gene and an inactive pseudogene.

The complexity of the casein region on two-dimensional gels, the limited specificity of chymosin, and the lack of an antibody to human kappa casein, contribute to the problem of identifying human kappa casein on polyacrylamide gels. The protein from a human kappa enriched fraction was identified on SDS polyacrylamide gels by analogy with bovine kappa casein with respect to its staining properties with Ethyl Stains-all and its chymosin sensitivity.

Studies on the temporal sequence of replication and transcription have been started. The general intent of these studies is to explore methods of characterizing the genome by means of its dynamic properties. Although a good beginning has been made in exploring this somewhat novel concept, there are as yet no significant results to report.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04496-07 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

The Role of Chromosomal Proteins in the Structure and Function of Chromatin

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

PI:	Michael Bustin	Research Chemist	LMC	NCI
Others:	Leo Einck	Staff Fellow	LMC	NCI
	Ella Mendelson	Visiting Fellow	LMC	NCI
	Shulamith Druckmann	Visiting Fellow	LMC	NCI
	Hiroshi Hamada	Guest Researcher	LMC	NCI

## COOPERATING UNITS (if any)

Department of Biophysics, Kings College, London, U. K. (J. Allan); Department of Biochemistry, Georgetown University, Washington, D. C. (M. Smulson); Department of Membrane Biology, German Cancer Center, Heidelberg, Germany (W. Franke)

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Protein Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

3.7

## PROFESSIONAL:

3.1

## OTHER:

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 role of chromosomal proteins in maintaining the structure and regulating the function of chromatin is studied. Chromatin segments containing specific nuclear proteins have been isolated by immunoaffinity chromatography. The histones associated with polynucleosomes enriched in the protein, HMG-17, are hyperacetylated. The epitopes of monoclonal antibodies against histone H5 have been mapped. These monoclonals are used to study chromatin rearrangements and to isolate chromatin regions enriched in protein H1<sup>o</sup>. A survey of cells derived from higher and lower eukaryotes revealed that H1-like proteins are present only in higher eukaryotes. A plasmid-containing potential Z-DNA forming sequence has been used to study the binding of the chromosomal proteins, HMG-1 and HMG-2, to DNA in various conformations. The results indicate that HMG proteins can distinguish between various DNA structures and that they bind selectively to distinguishable single-stranded regions. The ability of HMG proteins to distinguish between various DNA structures may have functional implications in gene regulation.



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

Michael Bustin	Research Chemist	LMC	NCI
Leo Einck	Staff Fellow	LMC	NCI
Ella Mendelson	Visiting Fellow	LMC	NCI
Shulamith Druckmann	Visiting Fellow	LMC	NCI
Hiroshi Hamada	Guest Researcher	LMC	NCI

Objectives:

The objectives are 1) to understand the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes; and 2) to explore the possibility that neoplastic transformation is associated with defined alterations in either the type or the organization of chromosomal components.

Background Information and Research Strategy:

Because the chromatin fiber, which is the backbone of chromatin and chromosomes, is a dynamic structure, it is difficult to determine the structure-function relations of defined chromosomal components. This question was approached by purifying chromosomal proteins, eliciting specific antibodies to these, and using the antibodies as probes for studying the *in situ* arrangement of the defined chromosomal components at various stages of chromatin organization. So far, it has been possible to purify the antigens, elicit antibodies, and adapt and develop various immunochemical techniques for detecting and quantifying the binding of specific antibodies to chromatin and chromosomes. Immunoelectron microscopy and immunofluorescent techniques are used to study the organization of histones and defined nonhistone chromosomal proteins in the interphase, transcribing, and replicating chromatin fiber and in metaphase and polytene chromosomes.

An alternative approach is to purify proteins which bind to specific cloned DNA sequences. In this approach, cloned DNA sequences are labelled either by nick translation or by end-labelling. The retention of specific DNA fragments on nitrocellulose filters after addition of cellular protein extracts is an assay for the presence of proteins specific for particular DNA sequences. Their presence is verified by electrophoresis of the DNA fragments eluted from the nitrocellulose filters. The filter assay can be used to monitor purification of the DNA binding protein which is done by conventional techniques.

Currently, the research effort is concentrated in the following areas: 1) fractionation of chromatin by immunoaffinity chromatography, 2) studies on the interactions between nonhistone proteins and specific DNA conformations, and 3) defining antigenic determinants in the chromosomal protein, H1°.

Methods Employed:

Microinjection, enzyme linked immunoassays, immunofluorescence, immunoreplica, enzyme digestions, electrophoresis, centrifugation, chromatin preparation, monoclonal antibodies, autoradiography, DNA cloning, restriction analysis, nitrocellulose filter assays, radiolabelling and immunoaffinity chromatography.

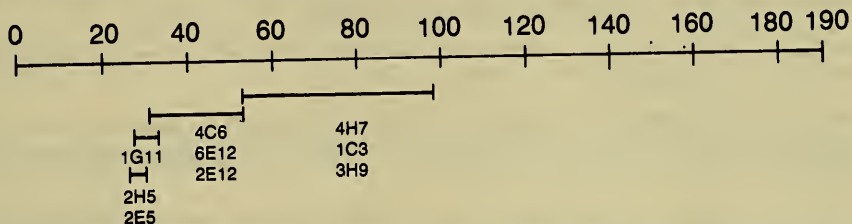
Major Findings:

1) Fractionation of chromatin by immunoaffinity chromatography. Superimposed on the repeated nucleosome structure of chromatin there is heterogeneity in chromatin structure and function. The presence of minor histone variants and small amounts of nonhistone chromosomal proteins indicates that there is compositional heterogeneity among various regions in chromatin. Antibodies against defined chromosomal components can be used to fractionate chromatin according to the antigenic composition of different regions. We have prepared an affinity column of antibodies to chromosomal protein HMG-17. This protein seems to be associated with transcribable regions in the genome. Polynucleosomes containing between three and ten nucleosomes were applied to these columns. About 10% of the input DNA bound to the columns. The proteins which were present in the bound nucleosomes containing, or in the vicinity of, protein HMG-17 were hyperacetylated. This finding supports the notion that transcribable chromatin regions are hyperacetylated. Similar approaches were used to isolate the chromatin regions containing chromosomal protein H<sub>1</sub><sup>o</sup>. The polynucleosomes bound to the affinity column were enriched 30-fold in H<sub>1</sub><sup>o</sup> as compared to the nucleosomes which were not bound. The DNA sequences associated with these specific chromosomal proteins are being studied.

2) Interaction between nonhistone proteins and specific DNA sequences. Plasmid Pst-09 is a pBR322 derivative containing a part of the human cardiac muscle actin gene. This element, under appropriate conditions, acquires the Z-DNA conformation. The plasmid is a pBR322 construct, contains a palindromic sequence at position 3065 and, under negative supercoiling, assumes a cruciform. Thus, this plasmid allows studies on the binding of specific proteins to various DNA conformations. The proteins tested were highly purified chromosomal proteins, HMG-1 and HMG-2. These proteins, which are ubiquitously distributed among various eukaryote kingdoms, may be involved in some regulatory aspects of chromatin structure. Our results clearly show that the proteins distinguish between various DNA conformations. They bound supercoiled DNA better than linear DNA. In form I DNA they can distinguish between various regions which are S1 sensitive (and therefore may have singlestranded characteristics). The binding to various single-stranded regions was detected by S1 protection and restriction analysis of the plasmid. Our results suggest that HMG proteins may recognize specific regions in chromatin. The binding of HMG to particular regions may have an effect on the function and regulation of the genome.

3) Definition of antigenic determinants in chromosomal proteins. Histone H<sub>5</sub> is an erythrocyte-specific histone which brings about condensation of the chromatin. A peptide region in this protein, named GH<sub>5</sub>, is responsible for stabilizing the basic nucleosome structure. In cells committed to differentiation, or in which DNA synthesis stops, a new histone called H<sub>1</sub><sup>o</sup> is induced. We have elicited antibodies to peptide GH<sub>5</sub> and demonstrated that it cross-reacts immunologically

with protein  $H_1^o$ . By trypsin digestion of  $H_1^o$ , an analogue of peptide GH5 was isolated from  $H_1^o$ . The antibody cross-reacted with this peptide. Thus, antisera which have been elicited against the chicken erythrocyte specific peptide, GH5, can be used to study the commitment of cells to differentiation. We have elicited 11 monoclonal antibodies against protein  $H_5$ . Cleavage of this molecule by various chemicals or proteolytic enzymes yielded a mixture of peptides which were used to map the epitopes of each of the monoclonal antibodies elicited. This was accomplished by fractionating the various peptides on polyacrylamide gels and identifying the peptide to which the various antibodies bound by immunoblotting. The location of the epitopes along the polypeptide chain is schematically diagramed below.



Immunofluorescence studies reveal that the monoclonal antibodies which cross-react with  $H_1^o$  stain the nuclei of somatic cells. Thus, the monoclonal antibodies will be suitable for studying the organization of  $H_1^o$  in chromatin. Indeed, the antibody binding is markedly dependent on the conformation of chromatin, which in turn is dependent on ionic strength. Thus, the availability of an antigenic site to antibody binding can reflect changes in chromatin structure associated with a particular protein segment. The antibodies will allow us to study various aspects of gene regulation in several diverse systems. We have surveyed a variety of organisms and found that the antibody recognized  $H_1^o$  like proteins in all higher eukaryote systems tested.

#### Significance to Biomedical Research and the Program of the Institute:

Understanding the mechanism of gene regulation and its relation to neoplasia requires knowledge of the structure of chromatin and chromosomes. The approach developed in this laboratory is presently the only approach in which specific probes for well-defined, purified, chromosomal components are used to study the organization of these components in intact chromatin and chromosomes. As such, a unique opportunity has developed whereby certain structural aspects of these nucleoproteins can be visualized and directly related to functional stages of the genome. The immunological techniques developed for the study of the in situ organization of proteins in chromatin and chromosomes are applicable to studies on damage and repair in the genome as a result of binding of carcinogens or X-ray and UV exposure.

#### Proposed Course:

Studies devoted to understanding the structure-function relation of chromosomal proteins will be continued. In the forthcoming year we will concentrate our

efforts on 1) understanding the role of protein H<sub>1</sub><sup>o</sup> in chromatin; 2) using immunoaffinity chromatography to fractionate chromatin; and 3) studying the interaction between specific chromosomal proteins and specific DNA sequences.

Publications:

Bustin, M.: Immunological studies on the structure and function of HMG proteins. In Bekhor, I. (Ed.): Recent Advances in Non-Histone Chromosomal Protein Research. West Palm Beach, CRC Press, Vol. 1 (In Press)

Dunn, B., Seidman, M. and Bustin, M.: Specific binding of alu sequences by HeLa nuclear extracts. Biochem. Biophys Res. Commun. 117: 378-384, 1983.

Einck, L. and Bustin, M.: Functional antibody fragments traverse the nuclear envelope. J. Cell Biol. 98: 205-213, 1984.

Einck, L. and Bustin, M.: Inhibition of transcription in somatic cells by microinjections of antibodies to chromosomal proteins. Proc. Nat. Acad. Sci. USA 80: 6735-6739, 1983.

Einck, L., Soares, N. and Bustin, M.: Localization of HMG chromosomal proteins in the nucleus and cytoplasm by microinjection of functional antibody fragment into living fibroblasts. Exp. Cell Res. (In Press)

Kleinschmidt, T. A., Scheer, U., Dabauvalle, M. C., Bustin, M. and Franke, W. W.: High mobility group proteins of amphibian oocytes: A large storage pool of soluble HMG-1-like proteins and involvement in transcriptional events. J. Cell Biol. 97: 838-848, 1983.

Kraemer, K., Soares, N., Waters, H. and Bustin, M.: Effect of X-radiation on DNA and histone synthesis in ataxia telangiectasia lymphoblastoid cells. Mutat. Res. 112: 359-367, 1983.

Malik, N., Smulson, M. and Bustin, M.: Enrichment of acetylated histones in polynucleosomes containing HMG-17 revealed by immuno affinity chromatography. J. Biol. Chem. 259: 699-702, 1984.

Mendelson, E. and Bustin, M.: Monoclonal antibodies against distinct determinants of histone H<sub>5</sub> bind to chromatin. Biochemistry (In Press)

Mendelson, E., Smith, B. J. and Bustin, M.: Mapping the binding of monoclonal antibodies to histone H<sub>5</sub>. Biochemistry (In Press)

Smith, B., Harris, M. R., Sigournay, C. M., Mayes, L. V. and Bustin, M.: A survey of H<sub>1</sub><sup>o</sup> and H<sub>5</sub> like protein structure and distribution in higher and lower eukaryotes. Eur. J. Biochem. 138: 309-317, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04516-08 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Cellular and Molecular Effects of Psoralen Plus Ultraviolet Light

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

PI: K. H. Kraemer Research Scientist LMC NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Cell Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

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

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

Psoralen plus long wavelength ultraviolet radiation (UV-A) is being investigated as a model system for clinically relevant photochemical carcinogenesis and as a probe for defective DNA repair. Used experimentally for treatment of psoriasis and mycosis fungoides, psoralen plus UV-A has been found to be mutagenic and carcinogenic. We have developed an in vitro assay to measure the effects of UV-A mediated psoralen-DNA binding on human lymphoid cells. Parameters monitored include the rate of DNA synthesis, induction of DNA-psoralen cross-links, induction of sister chromatid exchanges, alterations in the rate of cell proliferation and survival, and in immune reactivity. These studies indicate that the low doses of psoralen plus UV-A received by patients' leukocytes during therapy may result directly in decreased DNA synthesis in their circulating lymphoid cells. Cell survival was found to be markedly dependent on UV-A exposure and 8-MOP concentration, to be correlated with inhibition of DNA synthesis, and to be related to induction of DNA interstrand cross-links. There was a dose-dependent reduction in mixed leukocyte culture reactivity induced by 8-MOP plus UV-A treatment. Cells from a patient with Cockayne's syndrome had normal survival following 8-MOP plus UV-A treatment, but reduced survival after treatment with UV-B. Thus repair of photosensitized 8-MOP damage involves at least one pathway that is different from that for UV-B damage.

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

K. H. Kraemer	Research Scientist	LMC	NCI
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Objectives:

Humans are exposed to chemicals which may interact with ultraviolet radiation to become carcinogenic. A compound which is found in many plants, 8-methoxy-psoralen (8-MOP), plus high intensity long wavelength ultraviolet radiation (UV-A) is being used experimentally to induce remissions in psoriasis and in mycosis fungoides. The combination of 8-MOP plus UV-A produces DNA-8-MOP binding and has been shown to induce mutations in bacteria and in mammalian cells and to cause skin cancer in mice and in humans. Individuals with some cancer-prone genetic diseases may be at increased risk from this treatment. We are developing an in vitro model system to assess clinically relevant photochemical carcinogenesis and to explore photoactivated psoralens as a probe for DNA damage and repair in human genetic diseases.

Methods Employed and Major Findings:

We previously demonstrated that circulating lymphoid cells of some psoriasis patients receiving 8-MOP plus UV-A therapy had a significant reduction in DNA synthesis. We have developed an in vitro assay system to approximate some of the conditions of 8-MOP plus UV-A exposure of human lymphoid cells during in vivo therapy. The assay has been used with fresh lymphocytes and with long-term lymphoblastoid cell lines. These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells.

We have developed a simple microtiter assay to measure lymphoblastoid cell survival after treatment with 8-MOP plus UV-A or other DNA damaging agents, and have automated the end point analysis of this assay.

In the lymphoblastoid cells, as much as a 50% inhibition of DNA synthesis following 8-MOP plus UV-A treatment was associated with 100% survival. Greater inhibition of DNA synthesis resulted in an exponential decrease in cell survival. Similarly, measurements of 8-MOP-DNA cross-linking by the alkaline elution technique revealed a dose dependent increase in cross-link induction above a threshold of approximately 50% inhibition of DNA synthesis. The formation of detectable cross-links was also correlated with decreased cell survival. Thus, DNA-8-MOP interstrand cross-links may be responsible for inhibition of DNA synthesis and cell killing.

In vitro treatment of lymphocytes or lymphoblastoid cells with 8-MOP plus UV-A resulted in approximately a doubling in the number of sister chromatid exchanges per metaphase. Further increases in dosage of 8-MOP plus UV-A were toxic. Thus, it is likely that the doses of 8-MOP plus UV-A received by patients' lymphocytes are too low to permit routine detection of increased sister chromatid exchanges.

Mixed leukocyte reactivity of fresh human leukocytes was found to be inhibited in a dose dependent manner by 8-MOP plus UV-A *in vitro*. Stimulator and responder functions were both inhibited. This inhibition of immune reactivity may be exploited to provide immunosuppression.

Cultured cells from patients with Cockayne's syndrome (CS) are hypersensitive to the growth-inhibiting effects of sunlight (UV-B radiation). Cells from one CS patient are hypersensitive to UV-B but have a normal proliferative response to photosensitized 8-MOP. This implies that there is at least one human cellular recovery pathway that is different for UV-B and for photosensitized 8-MOP.

#### Significance to Biomedical Research and the Program of the Institute:

These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells. The fact that Cockayne's Syndrome cells are hypersensitive to UV-B but not to 8-MOP plus UV-A indicates that photosensitized psoralen damage may, in part, be handled by human cellular recovery pathways different from that for sunlight-induced damage.

#### Proposed Course:

Lymphoblastoid cell lines from patients with cancer-prone genetic diseases are being examined for evidence of hypersensitivity to psoralen plus UV-A-induced killing and mutagenesis. This may indicate populations at increased risk of toxicity from photochemotherapy and may provide clues as to the mechanism of carcinogenesis in these people.

#### Publications:

Kraemer, K. H.: Assessment of human lymphoid cell damage induced by therapeutic levels of 8-methoxypsoralen and long wavelength ultraviolet radiation *in vitro*. In Castellani, A. (Ed.): The Use of Human Cells for the Assessment of Risk from Physical and Chemical Agents. London, England, Plenum Press, 1983, pp. 141-154.

Kraemer, K. H. and Waters, H. L.: Effects of psoralens plus ultraviolet radiation on human lymphoid cells *in vitro*. JNCI (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1CE04517-08 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

## 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)

PI: K. H. Kraemer Research Scientist LMC NCI  
 Others: M. Protic-Sabljić V Fellow LMC NCI R. Day, III Sec. Head, LMC NCI  
 K. Um Guest Worker LMC NCI G. Peck, Sr. Invest. DB NCI  
 M. H. Greene Clin. Epidem. EEB NCI  
 J. Scotto Biometrician BB NCI  
 B. Howard Section Head LMB NCI  
 J. Fagan Sr. Staff Fellow LMC NCI  
 M. Bustin V Scientist LMC NCI

COOPERATING UNITS (if any) Dept. of Path., NJ Med. Sch., Newark, NJ (W. C. Lambert); Dept. of Derm., Columbia U., NY, NY (A. D. Andrews); NY Blood Ctr., NY, NY; (J. L. German); Dept. Derm., Hosp. U of Penn., Phila. PA (W. H. Clark); Tel Aviv U., Tel Aviv, Israel (H. Slor); Univ. of Chicago, Chicago, IL (B. Strauss)

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Cell Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

5.5

## PROFESSIONAL:

3.5

## OTHER:

2.0

## CHECK APPROPRIATE BOX(ES)

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

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

Patients with xeroderma pigmentosum (XP) and ataxia-telangiectasia (A-T), diseases with ultraviolet (UV) and x-ray hypersensitivity, respectively, and with the dysplastic nevus syndrome of hereditary cutaneous melanoma (DNS) are being studied. Detailed examinations of the clinical features of affected individuals are being made. A large retrospective XP literature study (more than 800 cases) revealed a greater than 2000-fold increase in frequency of skin cancers, anterior eye cancers and cancer of the tip of the tongue. Reported internal neoplasms (especially brain tumors) also increased in frequency. A prospective registry of XP patients is under way. Field studies in Israel detected a possible new form of XP with defective DNA repair without neoplasia. Cell lines have been established for detection of XP heterozygotes. DNA transfection studies have demonstrated the suitability of SV40 virus-transformed XP cells as high efficiency ( $3 \times 10^{-4}$ ) recipients. A rapid host cell reactivation DNA repair assay is being developed that measures transient expression of chloramphenicol acetyl transferase (CAT) activity in XP and normal cells transfected with a UV-treated plasmid bearing the CAT gene. Collaborative clinical studies of 14 DNS kindreds have demonstrated autosomal dominant inheritance of the melanoma trait, new melanomas only in family members with dysplastic nevi and >100-fold increased melanoma risk in family members with dysplastic nevi. Similar dysplastic nevi also occur in non-familial settings. A new classification of DNS was proposed. Laboratory studies demonstrated UV-induced hypermutability in DNS lymphoblastoid cell lines. A-T is an autosomal recessive disorder with clinical and cellular x-ray hypersensitivity and a high incidence of neoplasms. A-T is not an x-ray analogue of XP. Lymphoblastoid cell lines were established from 10 kindreds with A-T. Bleomycin-induced chromosome breakage and delay in cell cycle progression were greater in A-T homozygous than in A-T heterozygous and normal lines. Histone protein levels and spontaneous and x-ray-induced histone synthesis were indistinguishable between A-T and normal cells.



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

K. Kraemer	Research Scientist	LMC NCI
M. Protic-Sabljić	Visiting Fellow	LMC NCI
K. Um	Guest Worker	LMC NCI
M. H. Greene	Clinical Epidemiologist	EEB NCI
J. Scotto	Biometrician	BB NCI
B. Howard	Section Head	LMB NCI
J. Fagan	Sr. Staff Fellow	LMC NCI
M. Bustin	Visiting Scientist	LMC NCI
R. Day, III	Section Head	LMC NCI
G. Peck	Senior Investigator	DB NCI
R. Tarone	Matnematical Statistician	BB NCI
J. DiGiovanna	Expert	DB NCI
A. Moshell	Medical Officer	DAMSD NIADDK

Objectives:

Human cancer-prone genetic diseases are being studied with a view toward identifying groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting (1) to correlate such sensitivity with clinical abnormalities, (2) to determine if there is genetic diversity within such groups, (3) to understand the molecular basis of their cellular hypersensitivity, (4) to develop tests to identify persons at increased risk of neoplasia, (5) to explore methods of cancer prevention in these patients, and (6) to educate the medical community as to the importance of early recognition and diagnosis of these disorders.

Methods Employed:

Patients are examined with particular emphasis on cutaneous abnormalities, and cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory analysis. Patients with xeroderma pigmentosum (XP), ataxia telangiectasia (A-T), and dysplastic nevus syndrome of familial cutaneous malignant melanoma (DNS) have been studied clinically. The English language medical literature on XP is being reviewed comprehensively, and information on individual patients abstracted and entered into a computer for analysis. Physicians treating patients with XP are encouraged to fill out a Xeroderma Pigmentosum Registry questionnaire about their patients. New clinical forms of XP are investigated in depth. Clinical diagnostic features of DNS are being refined. Pigmented lesions in XP are cultured and studied for DNA repair. Cultured XP, AT and DNS cells are being examined for the effects of DNA-damaging agents (UV, x-ray, bleomycin) on cell survival, mutagenesis, DNA synthesis and repair, histone synthesis, and chromosome integrity. DNA mediated gene transfer experiments are being utilized to attempt to isolate the portion of DNA responsible for the UV hypersensitivity of the XP cells and to develop a host cell reactivation assay. XP patients with multiple cutaneous neoplasms are being treated with 13-cis retinoic acid to attempt to reduce the rate of tumor formation.

Major Findings:

XP is an autosomal recessive cancer-prone disease with clinical UV hypersensitivity, accompanied by cutaneous and neurological abnormalities. Cultured cells from XP patients have cellular UV sensitivity and defective DNA repair. We have compiled the most comprehensive review of the world literature to date on XP including both clinical and laboratory observations. Data on more than 800 XP patients described in the literature have been entered into the computer. In this retrospective study, we have documented reduction of 50 years in the age of onset of skin neoplasms in XP in comparison to the U.S. population. There is a 2000-fold increase in all three major types of skin neoplasms: basal cell carcinomas, squamous cell carcinomas, and melanomas. The XP melanoma site distribution does not correspond to areas of greatest sun exposure implying that factors other than UV may be important in melanoma. Oral cavity neoplasms are increased, possibly due to UV exposure of the anterior tongue and/or to dietary carcinogens. We found lymphoblastoid cell lines from XP patients to be hypersensitive to killing by tryptophane pyrolysis products (carcinogens produced in charbroiled foods). Internal neoplasms, particularly sarcoma of brain, were found at increased frequency. A registry of XP patients is being established. In a collaborative study with H. Slor of the Tel Aviv University in Israel, we identified kindreds with a form of XP that has not been previously recognized. Clinically, facial lesions predominate. One family member has reduced DNA repair with minimal clinical symptoms while a brother with equally reduced DNA repair has numerous neoplasms. This suggests that other, presently unmeasured, factors may be crucial to the development of neoplasms in XP. In our attempts to clone the genes responsible for UV hypersensitivity in XP cells, we have collaborated with the Laboratory of Molecular Biology (see Project Number Z01CB08719-05 LMB) and developed a protocol that gives high efficiency (about  $10^{-3}$ ) transfection of cloned selectable genes into XP cells. We found that SV40-transformed XP cells, but not primary fibroblasts, are suitable recipients of cloned genes. We developed a simple, rapid, thin layer chromatography assay for measuring xanthine phosphoribosyl activity in transferase (XPRT) activity transfected cells, and demonstrated stable transfection of XPRT activity into XP cells. XP cells were transfected with a plasmid containing two genes: chloramphenicol acetyl transferase (CAT) and XPRT. Eighty to 90% of cell clones selected for ability to express XPRT also expressed CAT. A host cell reactivation assay demonstrated reduced repair of UV-damaged plasmid in XP cells.

A-T, an autosomal recessive cancer-prone disease with cutaneous, neurological, and immunological abnormalities, has x-ray sensitivity. We are studying the ability of cultured cells from A-T patients and their parents to survive DNA damage induced by the chemotherapeutic agent, bleomycin. This agent also induced an abnormally large increase in chromosome breakage (but not in sister chromatid exchanges) in A-T homozygous lymphoblastoid cells but not in heterozygous cells. DNA synthesis in A-T homozygotes, but not heterozygotes, was found to be resistant to treatment by x-ray or bleomycin in comparison to the response of normal cells. A-T cells did not differ from normal cells in major histone or non-histone protein levels or in histone synthetic rates following x-ray treatment.

A newly recognized clinical disease, familial malignant melanoma with a characteristic precursor lesion, the dysplastic nevus, is being examined in a collaboration with the Environmental Epidemiology Branch, NCI (Z01CE04410-08 EEB). This

laboratory is contributing dermatological expertise to the clinical definition of the syndrome in a study of more than 400 family members in 14 kindreds with an average 6 year followup. DNS family members with these distinctive nevi were found to have several hundred-fold increased risk of developing cutaneous melanoma. Other findings include autosomal dominant inheritance of the melanoma trait, new melanomas only in family members with dysplastic nevi, a young age at diagnosis of melanoma, and a high frequency of multiple primary melanomas. We estimated that, in the U.S., about 32,000 people have familial DNS representing about 6% of the melanomas. Similar dysplastic nevi also occur in non-familial settings involving an estimated several million people in the U.S. A new classification of DNS was proposed, emphasizing that the most numerous group (sporadic DNS without melanoma) is probably at lowest melanoma risk. Lymphoblastoid cell lines from selected DNS patients are being examined for evidence of sensitivity to DNA agents as measured by cell survival and mutagenesis and examined for possible DNA repair defects. We found lymphoblastoid cell lines from DNS patients to be hypermutable by UV. This indicates that DNS is a generalized disorder and is the second hypermutable human disease to be found.

#### Significance to Biomedical Research and the Program of the Institute:

These studies may identify persons with increased risk of cancer, may be useful in revealing the mechanism of cancer induction, and may suggest modes of cancer prophylaxis. In addition, these diseases serve as models for studies of human environmental carcinogenesis.

#### Proposed Course:

This project will be continued along the lines indicated above.

#### Publications:

Greene, M. H., Goldin, L. R., Clark, W., Lourien, E., Kraemer, K. H., Tucker, M. A., Elder, D. E., Fraser, M. C. and Rowe, S.: Familial cutaneous malignant melanoma - an autosomal dominant trait possibly linked to the Rh locus. Proc. Natl. Acad. Sci. USA 80: 6071-6075, 1983.

Kraemer, K. H.: Cellular hypersensitivity and DNA repair. In Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, M. M. and Austen, K. F. (Eds.): Dermatology in General Medicine. New York, McGraw-Hill (In Press)

Kraemer, K. H.: Cellular hypersensitivity to physical and chemical agents in patients with ataxia-telangiectasia: Disorders in cell growth and chromosomal integrity. Ann. Intern. Med. 99: 367-379, 1983.

Kraemer, K. H.: Dysplastic nevi as precursors to hereditary melanoma. J. Dermatol. Surg. Oncol. 9: 619-623, 1983.

Kraemer, K. H.: Heritable diseases with increased sensitivity to cellular injury. In Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, I. M. and Austen, K. F. (Eds.): Dermatology in General Medicine. New York, McGraw Hill (In Press)

Kraemer, K. H.: Use of human lymphoblastoid cell lines to determine cellular hypersensitivity to physical and chemical agents. In Castellani, A. (Ed.): The Use of Human Cells for the Assessment of Risk from Physical and Chemical Agents. London, England, Plenum Press, 1983, pp. 275-283.

Kraemer, K. H.: Xeroderma pigmentosum. In Provost, T. T. and Farmer, E. R. (Eds.): Current Therapy in Dermatology. Philadelphia, B. C. Decker, Inc. (In Press)

Kraemer, K. H., Greene, M. H., Tarone, R. E., Elder, D. E., Clark, W. H., Jr. and Guerry, D., IV: Dysplastic naevi and cutaneous melanoma risk. Lancet 2: 1076-1077, 1983.

Kraemer, K. H., Lee, M. M. and Scotto, J.: DNA repair protects against cutaneous and internal neoplasia: Evidence from xeroderma pigmentosum. Carcinogenesis 5: 511-514, 1984.

Kraemer, K. H., Robinson, R. C., Tarone, R. E., Protic-Sabljić, M. and Gelboin, H. V.: Alterations in leukocyte aryl hydrocarbon hydroxylase activity associated with treatment and age in psoriasis patients and normal individuals. Arch. Dermatol. Res. 276: 105-110, 1984.

Kraemer, K. H., Soares, N., Waters, H. L. and Bustin, M.: Effect of X-radiation on DNA and histone synthesis in ataxia-telangiectasia and normal lymphoblastoid cells. Mutat. Res. 112: 359-367, 1983.

Protic-Sabljić, M., Whyte, D., Fagan, J. and Kraemer, K. H.: Transfection of xeroderma pigmentosum cells with cloned DNA. In Friedberg, E. and Bridges B. (Eds.): Cellular Responses to DNA Damage, ICN-UCLA Symposia on Molecular and Cellular Biology, New Series. New York, Alan R. Liss, 1983, Vol. II, pp. 647-656.

Tucker, M. A., Greene, M. H., Clark, W. H., Kraemer, K. H., Fraser, M. C. and Elder, D. E.: Dysplastic nevi on the scalp of prepubertal children from melanoma-prone families. J. Pediatr. 103: 65-69, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE04525-12 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

**Studies on Electrophoretic Techniques for Protein, RNA, and DNA**

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

PI:	Andrew C. Peacock	Chief, Protein Section	LMC	NCI
Others:	Sylvia L. Bunting	Research Chemist	LMC	NCI
	Michael Seidman	Senior Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

2.1

PROFESSIONAL:

1.1

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.)

In earlier work, we developed a two-dimensional method for the display of DNA restriction fragments generated by the sequential action of two or more restriction enzymes. The method was found to yield new information about certain sequences repeated many times in the mammalian genome. This two-dimensional analysis of DNA has been extended by the use of cloned probes. Cloned DNA sequences coding for "30 S" RNA were studied. These DNA sequences are homologous to the DNA of Harvey rat sarcoma virus. The degree of repetition of these sequences was found to be significantly greater than the 30-fold expected. The high copy number made further analysis difficult. The technique was applied to a study of the DNA of the slime mold *Physarum polycephalum*. Electrophoretic characterization of DNA prepared from this organism revealed the presence of a co-purifying contaminant, and permitted the identification of the impurity as an acidic polysaccharide. These observations formed the basis of a methodology for preparing DNA free of the contaminant. The purified DNA was readily cleaved with restriction enzymes, and contained many repeated sequences.

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

Andrew C. Peacock	Chief	LMC	NCI
Michael Seidman	Senior Staff Fellow	LMC	NCI
Sylvia L. Bunting	Research Chemist	LMC	NCI

Objectives:

Current efforts are directed towards finding useful ways of examining whether, during carcinogenesis, there are changes in the genomic location of identifiable DNA sequences. For present purposes, the DNA sequences are defined on the basis of lengths between sites cleaved by selected restriction enzymes, and by ability to hybridize to specified probes. The immediate objective is to devise and evaluate electrophoretic methods that will give maximum information about the location of DNA sequences of low multiplicity. A longer range goal is to use time order of replication as an additional parameter to characterize DNA sequences. Sequences which have changed genomic location might be replicated during a different interval in the DNA-synthesis period; the validity of this assumption will be investigated.

Methods Employed:

Tissue cultures of rodent cells, assay of radioactivity, isolation and electrophoresis of RNA and DNA, preparative electrophoresis and ultracentrifugation, hybridization, DNA restriction, and cloning.

Major Findings:

A cloned cDNA homologous to a member of the "30 S" RNA found in abundance in rats bearing the Harvey sarcoma virus was used to detect DNA sequences by a two-dimensional technique described earlier (see Project Number Z01CP04525-11 LMC). Some difficulties in effecting electrophoretic transfer from the gel to the nitrocellulose membranes used for annealing, stemming from the unusual thickness of the gel, were overcome. There were very many bands as zones on the blot which annealed to the portion of the probe which contained the long terminal repeat, and a smaller, but still very large, number of bands corresponding to the portion of the probe believed to be unique. The number of spots and bands was too great to be analyzed by the technique employed, and the problem was set aside for the present.

In preparation for a study on the time order of replication, methods suitable for the culture of the acellular slime mold, Physarum polycephalum, were studied. Under appropriate conditions, this organism grows naturally in almost complete mitotic synchrony. Because the experimental protocols involve preparation of a genomic library, it is important that the DNA to be cloned be of high purity. Electrophoretic analyses of the DNA prepared from this organism by a variety of standard methods showed that methods previously employed failed to remove an

acidic polysaccharide. Although the chemical and physical properties of this contaminating polysaccharide and the DNA are very similar, their electrophoretic and staining properties permit easy distinction between them. Repeated cycles of isopycnic ultracentrifugation ultimately yielded a product free of the contaminant. Analysis of the DNA by the two-dimensional method revealed that the genome contained a much larger fraction of repeated DNA sequences than had been observed in mammalian cells studied earlier. These repeated sequences may interfere with construction of the genomic library, but are themselves interesting to study.

Significance to Biomedical Research and the Program of the Institute:

There is a widely-held (but disputed) belief that genomic rearrangements are important in the conversion of a cell from a normal to a malignant state. These studies are designed to provide suitable new technologies for investigating this problem, and to arrive at some firm conclusions as to the validity of the hypothesis.

Proposed Course:

- 1) Continue development of two-dimensional gel techniques, with special reference to the study of the time order of replication of repeated DNA sequences.
- 2) Study the time ordering of transcription of repeated and single copy DNA sequences.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04555-10 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Multiple Steps Involved in Neoplastic Transformation by Chemical Carcinogens

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

PI: T. Kakunaga Head, Cell Genetics Section LMC NCI

Others: M. Mullinix Biologist LMC NCI

## COOPERATING UNITS (if any)

International Agency for Research on Cancer, Lyon, France (H. Yamasaki)

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Cell Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

0.8

## PROFESSIONAL:

0.3

## OTHER:

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 purpose of this work is to define the cellular and molecular basis for the steps involved in the process of cell transformation by chemical carcinogens and radiation. Cell mutants of the Balb/3T3 cell line showing different susceptibility to chemically- or ultraviolet (UV)-induced transformation have been isolated in our laboratory. Characterization of these mutants indicates that they are different in the expression process of transformation. The resistant variant showed transformation only when the cells treated with carcinogen were exposed to tumor promoters. Measurement of intercellular communication in the variant cells treated or untreated with tumor promoters suggests that these variants differ in the steps involved in the expression or promotion of transformation, and that inhibition of intercellular communication plays an important role in the promotion of transformation.



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

T. Kakunaga	Head, Cell Genetics Section	LMC NCI
M. Mullinix	Biologist	LMC NCI

Objectives:

To clarify the cellular and molecular mechanisms of neoplastic cell transformation by chemical and physical carcinogens by defining and identifying the steps involved and the genetic and physiological factors affecting the transformation process, and to devise an optimal system for studying the mechanism of cell transformation.

Methods Employed:

In order to study the complex mechanisms of cell transformation, it is obviously of great importance and advantage to isolate and characterize cell mutants which are affected in the process of cell transformation. This laboratory has isolated and characterized Balb/3T3 cell mutants showing differential susceptibility to transformation by ultraviolet light and chemical carcinogens. Previous studies have shown that the mutants are not altered in the initiation step, but are affected in the late stage of transformation, i.e., the expression of the transformed phenotypes. A number of laboratory techniques are employed to pursue the objectives. A quantitative assay system for malignant transformation by chemical carcinogens and its enhancement by tumor promoters has been developed in our laboratory using the Balb/3T3 cell line. Morphology is followed by light and fluorescence microscopy. Cell to cell communication is monitored by measuring the intercellular transfer of a microinjected fluorescent dye, Lucifer Yellow CH, and by measuring ionic coupling. The transfer of dye from the injected cells to surrounding cells is monitored under a phase contrast-fluorescence microscope.

Major Findings:

The pursuit of this project has led to major new findings in five pertinent areas: 1) development of an improved assay system for transformation, 2) enhancement of model systems for assaying tumor promoters in culture, 3) genetic evidence for the promotion step in neoplastic cell transformation, 4) identification of covalent adducts responsible for initiation of transformation, and 5) new information on the mechanism of tumor promotion.

During the past year, both our understanding of the fundamental mechanism of tumor promotion and the usefulness of our mutant cells for carcinogenesis studies have been greatly enhanced.

When the variant cells were treated with 3-methylcholanthrene (3-MC), significant numbers of transformed foci were induced in the highly and intermediately susceptible variants, but no detectable foci were found in the resistant variants. However, if teleocidin B, a potent tumor-promoting agent, was added to the culture medium after 3 MC-treatment, transformed foci appeared in the resistant variant (A31-115); the transformation frequency was also enhanced by teleocidin B in both the highly and the intermediately sensitive variants (A31-1-13 and A31-1-1). Without pretreatment with 3-MC, teleocidin B did not induce transformation. These results clearly indicate that 3-MC treatment resulted in the fixation of transformation in the resistant variants, supporting our hypothesis that the differential susceptibilities of variant cells are to be ascribed to their different potentials for expression of the transformed phenotype, the step which follows the fixation of transformation resulting from treatment with chemicals or UV. The numbers and the binding abilities of the receptors for phorbol ester were similar among the three classes of variant cells. The highly susceptible variant seems to produce a high level of endogenous promoters, which stimulates the expression of the transformed phenotype. The resistant variant does not seem to produce promoters.

There were striking differences between variant cells in the intercellular communication at confluence; the intercellular communication was very low in the highly susceptible clone, A31-1-13, and high in the resistant clone, A31-1-15. Interestingly, the intercellular communication between A31-1-13 cells is high for several days after they reach confluence.

The low level of intercellular communication of A31-1-13 cells at later than the sixth day after reaching confluence corresponds to the level of A31-1-15 cells treated with tumor promoters. The most effective period for treatment with tumor promoters to enhance cell transformation is several days after the cells reach confluence. Thus, these observations are consistent with our working hypothesis that variant cells differ in the expression of transformation, the step which tumor promoters enhance.

#### Significance to Biomedical Research and the Program of the Institute:

There is ample evidence implicating the etiological importance of the promotion stage in carcinogenesis. However, very few genetic studies have examined this late stage of cellular neoplastic transformation. Our study has shown that the late stage of transformation can be the limiting factor controlling susceptibility to chemically-induced transformation. This study has also developed a promoter-sensitive system for genetic study of expression of the transformed state. Our data raise the possibility that abnormalities in tumor promotion may be present in some individuals genetically predisposed to cancer.

#### Proposed Course:

The mechanisms of the reduction of intercellular communication at the confluent state in the highly susceptible A31-1-13 clone will be explored. The reduction of communication in A31-1-13 cells occurs at the time when the cell-cell junctions are formed in other cells and when the promoters are the most effective in enhancing transformation and reducing the communication. Cell-cell junctions of A31-1-13 cells will be examined at various times after reaching confluence by electron microscopy and will be compared with those of A31-1-15 cells. The extracellular

matrix and cytoskeleton will also be examined by using antibodies against their components.

The possibility that the highly susceptible A31-1-13 clone produces higher amounts of more effective endogenous promoter-like substances will be tested by attempting to detect the promoter-like activity in the supernatant of the culture of A31-1-13 cells or the extracts from the cells. Production of various growth factors including transforming growth factors will also be examined.

The transfer of transformation sensitivity or resistance will be attempted by DNA transfection with or without co-transfection with a selective marker, in order to isolate the presumptive genes which control the susceptibility to chemically-induced transformation.

#### Publications:

Elmore, E., Kakunaga, T. and Barrett, J. C.: Comparison of spontaneous mutation rates of normal and chemically transformed human skin fibroblasts. Cancer Res. 43: 1650-1655, 1983.

Kakunaga, T., Hirakawa, T., Shimomura, K., Mullinix, M. and Sugimura, T.: Promoter-induced cellular responses closely correlated with the enhancement of cell transformation. In Hecker, E., Moore, R. E., Weinstein, I. B., Fujiki, T. and Sugimura, T. (Eds.): Cellular Interactions by Environmental Tumor Promoters. Japan Sci. Soc. Press, Tokyo/Univ. Park Press, Baltimore (In Press)

Shimomura, K., Mullinix, M., Kakunaga, T., Fujiki, T. and Sugimura, T.: A bromine residue at hydrophilic region exerts marked influence on biological activity of a new tumor promoter, aplysiatoxin. Science 222: 1242-1244, 1983.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04578-08 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Role of Molecular and Functional Alterations of Actin in Neoplastic Transformation

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

PI:	T. Kakunaga	Head, Cell Genetics Section	LMC	NCI
Others:	N. Battula	Expert	LMC	NCI
	S. Taniguchi	Visiting Fellow	LMC	NCI
	S. Iijima	Visiting Fellow	LMC	NCI
	H. Ueyama	Visiting Fellow	LMC	NCI

## COOPERATING UNITS (if any)

Laboratory of Cell Biology, National Heart, Lung and Blood Institute, NIH  
(E. D. Korn)

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Cell Genetics Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

4.3

## PROFESSIONAL:

3.8

## OTHER:

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 cellular macromolecules responsible for the expression of the neoplastic phenotype of chemically transformed human cells have been investigated. A new polypeptide found in a chemically transformed human cell line has been identified as a mutant of beta-actin which has aspartic acid at position 244 instead of glycine. Determination of the structure and organization of this beta-actin gene confirmed that the alteration of beta-actin in the transformed cells is due to a point mutation (transition) in the structural gene of beta-actin. The synthesis of the mutant beta-actin was correlated with the expression of the transformed phenotype in both variants of the transformed line and its hybrids with normal human fibroblasts. This mutation resulted in several defects in the function of beta-actin, such as increased instability, reduced incorporation into cytoskeletal elements, and decreased ability to polymerize *in vitro*. These defects in the beta-actin molecule were associated with the disruption and loss of some of the structural elements of the cytoskeleton, such as the actin cable network. The results suggest that a mutation in beta-actin leads the cells to express transformation by disrupting the cytoskeletal structure and its function.

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

T. Kakunaga	Head, Cell Genetics Section	LMC	NCI
N. Battula	Expert	LMC	NCI
S. Taniguchi	Visiting Fellow	LMC	NCI
S. Iijima	Visiting Fellow	LMC	NCI
H. Ueyama	Visiting Fellow	LMC	NCI

Objectives:

The overall goal is to understand the role of cytoskeleton alterations in neoplastic transformation. Specific aims are to 1) discover if altered cytoplasmic actins are present in neoplastic cells; 2) identify the molecular changes occurring in the altered actins at the DNA, RNA and protein levels; 3) investigate the relationship between the expression of altered actins and the transformed phenotype; and 4) characterize the altered actin in vitro.

Methods Employed:

The most common biological characteristics of neoplastic cells involve alterations in cell morphology and motility. Since the cytoskeleton plays a major role in maintaining and controlling cell morphology, motility and membrane fluidity, it is conceivable that the structure and function of the cytoskeleton is altered in transformed cells. Actin cables (or stress-fibers) are seen to be disorganized in transformed cells when the cells are stained with a fluorescent antibody against actin. Diffusion of actin cable structure generally correlates with the expression of transformed phenotypes. However, the mechanisms by which the structure and function of the actin cables are disrupted in transformed cells and what role this disruption plays in neoplastic transformation are poorly understood. Thus, it was of great importance to analyze the molecular changes in the cytoskeletal proteins in the transformed cells. We have found an altered beta-actin that was co-expressed with normal actins in a chemically transformed human fibroblast line. The human beta-actin gene was cloned from DNA of the cells. The DNA sequences and organization of the beta-actin gene was determined. The biochemical properties of mutated beta-actin were examined in vitro. A number of techniques of molecular biology were required to clone and characterize the beta-actin gene, including various one-dimensional and two-dimensional gel electrophoresis, in vitro translation, ultracentrifugation, DNA-DNA and DNA-RNA hybridization, DNA sequencing, autoradiography, cutting and ligating DNA fragments by using various enzymes, and column chromatography. Beta-actins were extracted from the transformed human cells and purified by using DEAE cellulose columns, Sephacryl columns, and polymerization techniques. Purified, polymerized and unpolymerized actins were analyzed by isoelectric focusing and/or SDS-polyacrylamide gel electrophoresis and by electron microscopic observation. Microfilaments were isolated by immunoprecipitation with monoclonal antibody against tropomyosin.

Major Findings:

The pursuit of this project has led to major new findings in four pertinent areas: 1) the first evidence for a point mutation as a cause for the altered proteins in the transformed cells, 2) new information concerning the molecular mechanisms for the alteration of cytoskeletal structure in transformed cells, 3) increased understanding of the mechanism of the expression of the transformed phenotype, and 4) new information on the structure and organization of the human actin gene family.

During the past year our understanding of both the functional defects of the mutated beta-actin and its role in the expression of the transformed phenotype has been greatly enhanced. Our knowledge of the structure and organization of the human beta-actin gene has also increased. One amino acid substitution at position 244, glycine replaced by aspartic acid, reduces the efficiency of beta-actin polymerization, particularly at the initiation step of polymerization. The deficiency of the mutated beta-actin in polymerization is profound in a low ionic concentration, indicating the altered response of the mutated actin to the changes in ionic environment.

The human beta-actin gene consists of five exons and four introns including one intron in the 5'-untranslated region. Strong conservation of DNA sequences in introns and in the 3'-and 5'- untranslated regions as well as in the coding region of the beta-actin gene among different species indicate that these untranslated regions have some role in the function of the gene, such as regulation of gene expression. Hybridization studies using a specific probe for beta-actin indicate that the human genome contains only one pair of functional beta-actin genes, supporting further a point mutation as the cause of alteration of beta-actin in the transformed cells.

Significance to Biomedical Research and the Program of the Institute:

Elucidation of the molecular basis for alterations of cell morphology and motility in neoplastic cells is crucial to our understanding of the mechanisms of cell transformation, as well as to our understanding of the transformed phenotype. Since actin is a major component of the cytoskeleton that controls cell morphology and motility, the demonstration of a mutated beta-actin in a transformed line has provided an important clue for investigating the mechanisms by which morphology and motility are altered in neoplastic cells. This study was also the first to show that alteration of at least one of the polypeptides present in the transformed cells could be ascribed to the mutation of a structural gene. Recently it was found that activation of the ras oncogene was associated with a point mutation of a guanine base in the proto-oncogene. The protein encoded by the mutated ras gene, P21, migrates on SDS gel electrophoresis at much lower rates than the normal P21 encoded by the unmutated ras gene. These observations exactly parallel our findings with mutated beta-actin. Furthermore, a recent discovery by K. Robbins et al. (Science 223: 63-66, 1983) that the fgr-oncogene contains a part of the gamma-actin gene gives further support for the hypothesis of this study: 1) alteration of cytoplasmic actins may play an important role in neoplastic transformation, and 2) actin genes may serve as proto-oncogenes which may be activated into oncogenes by a point mutation.

Proposed Course:

In order to examine further the role of a mutated beta-actin in the expression of the transformed phenotype, four approaches are being taken. First, the mutated and normal beta-actin genes are being isolated from a DNA library prepared from DNA of HuT-14 cells and their structure is also being determined. Since there are many pseudo beta-actin genes in the human genome, it was difficult to isolate functional beta-actin genes. However, by using a synthetic polynucleotide which can recognize one nucleotide difference as a hybridization probe, the isolation of the normal beta-actin gene is assured and isolation of the mutated beta-actin gene should be successful.

The relationship between expression of mutated actin and changes in the phenotype will be examined by introducing the mutated gene into various recipient cells, including NIH/3T3 cells. The transformants will be selected by cotransfecting selective markers, e.g., gpt, or by observing alteration of morphology and growth patterns. Furthermore, the structure-activity relationship of actin genes will be examined for induction of phenotypic changes by transfecting actin genes whose DNA sequence is modified by site-specific mutagenesis.

Second, DNA extracted from HuT-14 and its sublines will be tested for transforming ability by transfecting them into various cells, including NIH/3T3 cells, with or without cotransfection with the *myc*-gene. If transformation is induced by them, the expression of mutated actin will be examined in the primary and secondary transformants. If the expression of mutated actin is associated with transformation through the serial propagation of transforming DNA, it is likely that the mutated actin gene serves as the transforming gene. In this case, the transforming gene will be isolated from the transformant and its structure will be determined.

Third, other variant cells, highly tumorigenic or less tumorigenic, will be isolated from HuT-14 cells. More cell hybrids between normal cells and HuT-14 cells will also be isolated. The relationship between the expression of the mutated actin and the transformed phenotype will be examined with these variant cells and hybrid cells. An attempt will be made to identify the chromosomes whose presence is associated with the expression of the mutated actin and/or the expression of the transformed phenotype.

Fourth, the properties of the mutated actin molecule will be further investigated in vitro. For example, the interaction of the mutated actin with actin associating proteins will be compared with that of normal actin in vitro. The actin-associating proteins including myosin, tropomyosin, filamin, actinogelin, alpha-actinin, beta-actinin, gelsolin, profilin, DNase I and caldesmon. Interactions will be measured by physico-chemical binding, morphology observed by electron microscopy, alteration of physico-chemical properties of actin complexes, and enzyme activity. These studies will provide useful information on the mechanism of dynamic regulation of microfilament structure and function, the effects of a point mutation in actin on its activity, and the mechanisms by which a point mutated actin causes disorganization of actin cable and possibly alteration of morphology and other phenotypes.

Publications:

Hamada, H. and Kakunaga, T.: Expression of mutated actin gene associated with malignant transformation. In Conn, W. E. (Ed.): Progress in Nucleic Acid Research and Molecular Biology. New York, Academic Press, 1983, Vol. 29, pp. 259-262.

Hirakawa, T. and Kakunaga, T.: Introduction of genetic markers into human diploid cells. Prot. Nucleic Acid and Enzymes. (In press)

Kakunaga, T.: Involvement of a point mutation in the neoplastic transformation of human fibroblasts. Fed. Proc. (In Press)

Kakunaga, T.: Mutations associated with neoplastic transformation by chemical carcinogens. In Omenn, G. S. and Gelboin, H. (Eds.): Banbury Report 16, The Role of Genetic Predisposition in Responses to Chemical Exposure. New York, Cold Spring Harbor Laboratory, 1984, pp. 257-274.

Kakunaga, T.: Mutations in actin-related genes and neoplastic transformation. Metabolism. (In Press)

Kakunaga, T., Crow, J. D., Leavitt, J. and Hamada, H.: Cellular and molecular mechanisms of neoplastic transformation of human cells. In Proc. 13th Int. Cancer Congress Symposium, Part B, Biology of Cancer. New York, Alan R. Liss, 1983, pp. 127-140.

Kakunaga, T., Crow, J. D., Leavitt, H., Hamada, H. and Hirakawa, T.: Mechanisms of neoplastic transformation of human cells by chemical carcinogens. In Harris, C. C. and Autrup, H. N. (Eds.): Human Carcinogenesis. New York, Academic Press, 1983, pp. 259-262.

Kakunaga, T., Leavitt, J., Hamada, H. and Hirakawa, T.: A point mutation in  $\beta$ -actin gene and neoplastic transformation. In Weinstein, I. B. and Vogel, H. J. (Eds.): Genes and Proteins in Oncogenesis. New York, Academic Press, 1983, pp. 351-367.

Kakunaga, T., Taniguchi, S., Leavitt, J. and Hamada, H.: A point mutation and other changes in cytoplasmic actins associated with the expression of transformed phenotypes. In Levine, A., Topp, W., Vande Woude, G. and Watson, J. D. (Eds.): Cancer Cell. New York, Cold Spring Harbor Laboratory. (In Press)



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04782-14 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Hormones and Breast Tissue Interactions

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

PI: M. R. Green Research Chemist LMC NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Protein Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

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

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

The purpose of this project is to contribute to the understanding of hormone action on metabolic processes in normal breast tissue and to understand the aberrant response to hormones in hyperplastic or neoplastic breast tissue. Modified proteins in mouse and human breast tissues and milk are investigated to determine changes that may occur during differentiation. Explants from mammary tissue of mice are incubated in a defined synthetic medium in the presence or absence of peptide and steroid hormones and labeled with appropriate radioisotopes to identify phosphorylated and glycosylated proteins by autoradiography following electrophoresis on polyacrylamide gels. Prolactin induced the phosphorylation of caseins and a protein of approximately 60,000 molecular weight. The latter corresponded to a protein present in colostrum but not in mature milk. To investigate changes in the modified proteins of human milk that may occur throughout lactation, proteins in colostrum and milk were separated by electrophoresis on polyacrylamide gels. Using the metachromatic cationic carbocyanine dye, Ethyl Stains-all (ESA), some phosphorus- and sialic acid-containing proteins were found in colostrum but not mature milk. Proteins that migrated at the same molecular weight on SDS polyacrylamide gels could be distinguished from one another by two-dimensional electrophoresis and by distinctive staining with ESA. Many laboratories have experienced problems in identifying and characterizing the kappa casein of human milk. This protein was identified by analogy with bovine kappa casein with respect to its staining properties with ESA and its chymosin sensitivity.

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

M. R. Green            Research Chemist            LMC            NCI

Objectives:

The objective is to gain a clearer understanding of the interaction of hormones with breast tissue. The topics of interest are the interactions of hormones such as insulin, estrogens, hydrocortisone, and prolactin with breast tissue, and the metabolic effects of these interactions. Responses by breast tissue in culture to prolactin, carcinogens, and steroids in terms of nucleic acid synthesis and synthesis of macromolecules such as phosphoproteins, sialoglycoproteins, glycosaminoglycans, and proteins are under investigation. Modulation of these responses in rodent tissues following carcinogen treatment or in tissue bearing the mouse mammary tumor virus is investigated. Human milk and pathologic breast specimens from humans are also studied.

Methods Employed:

Methods employed include culture of mammary tissue explants in vitro using defined medium, isolation and characterization of nucleic acids, separation of nucleic acids by electrophoresis on agarose-acrylamide gels and ultracentrifugation in CsCl gradients, radioactive tracer techniques, isolation and purification of proteins from milk and explants, measurement of enzymatic activities, polyacrylamide gel electrophoresis, histochemical analysis of gels and tissue sections, autoradiography and binding of hormones to macromolecules, and immunochemical procedures.

Major Findings:

Proteins of human colostrum and mature milk were separated by one- and two-dimensional electrophoresis on polyacrylamide gels and examined for the presence of phosphoproteins, glycoproteins and sialic acid-rich glycoproteins using staining, enzymatic, and immunochemical procedures. By use of the metachromatic cationic carbocyanine dye, Ethyl Stains-all (ESA), several proteins modified by acidic groups were detected. In colostrum, a periodic acid-Schiff positive protein that precipitated at pH 4.6 and stained blue with ESA migrated between the 200,000 molecular weight marker and the unreduced secretory IgA on 5% polyacrylamide gels containing SDS. This protein was not seen in mature milk. Other ESA blue-staining proteins of lower molecular weight than the major casein of milk were also observed in colostrum but not in mature milk.

Migrating more rapidly than the major phosphorylated casein in both colostrum and mature milk was a band that stained green. Experiments to determine whether this protein might be the sialic acid-containing kappa casein of human milk were performed. The staining properties and chymosin sensitivity of a human casein fraction were compared to bovine caseins.

Bovine beta casein stained blue (phosphorus) and bovine kappa casein stained green (sialic acid). Chymosin treatment of these bovine proteins caused the breakdown of the latter to a glycomacropeptide and para-kappa-casein. Beta casein was not affected. Human beta casein stained blue and the lower molecular weight casein stained green and was chymosin sensitive, showing that the latter is the kappa casein of human milk.

On separation of skim milk proteins by two-dimensional electrophoresis and staining with Coomassie Blue, a complex pattern of spots was observed in the casein region. Staining with ESA revealed that green-staining proteins with similar isoelectric points migrated at the same molecular weight as the caseins. These have not been identified.

The identity of some of the neuraminidase sensitive, green ESA-staining material, in fixed pathologic specimens of human breast cancer was determined. The sections were tested by an immunoperoxidase method using antibodies against several antigens which are known to be neuraminidase sensitive sialic acid-rich glycoproteins. In serial sections, reactions for Human Chorionic Gonadotropin (HCG) and Carcino Embryonic Antigen (CEA) were positive. The positive reactions for each corresponded to areas of the section that stained blue-green.

In explants from mammary tissue of mice, proteins modified by phosphorylation in response to prolactin were identified by fluorography of polyacrylamide gels. Prolactin induced the phosphorylation of caseins and a protein of approximately 60,000 molecular weight. The latter corresponded to a protein present in colostrum but not in mature milk.

#### Significance to Biomedical Research and the Program of the Institute:

Difficulties in identifying the kappa casein of human milk stem from the fact that, unlike bovine kappa casein, the human protein has not been clearly separable on alkaline urea polyacrylamide gels from the major phosphorylated casein of milk. The initial action of chymosin is restricted to the cleavage of the peptide bond between phenylalanine and methionine. Caseins other than K-caseins are split, given sufficient time. It is difficult to distinguish the protease activity from specific chymosin activity on Coomassie blue stained gels. By use of ESA staining, the relative activities of chymosin toward kappa versus beta casein were determined. Previous claims that human kappa casein could serve as a marker for breast cancer were not borne out by subsequent studies which showed that the antibodies used were reactive against a number of proteins other than caseins.

Studies of modified proteins in milk and breast tissue at various stages of development in the human may reveal the source of certain proteins made by tumor tissue. While the sialic acid containing CEA and HCG were related to the blue-green ESA-staining regions (sialic acid-containing) breast tumor sections, the two proteins did not account for all the sialic acid-containing regions observed.

Conditions for preparation of  $^{32}\text{P}$ -labeled explant proteins that reflect in vivo processes have been established for proteins from mammary tissue of pregnant

mice incubated in defined medium. This has been assessed by co-migration on two-dimensional polyacrylamide gels of unlabeled stainable milk proteins with labeled proteins from explants. These studies are a necessary prerequisite for future studies of prolactin effects on modification of proteins in the mammary gland and the regulation of the prolactin receptor.

Proposed Course:

The nature of the modifying groups in the human colostrum proteins will be assessed by use of enzymatic and chemical procedures followed by electrophoretic and staining analyses on gels. Antisera to milk or serum proteins will be used in an attempt to identify some of the proteins. The possibility that there may be proteins in early milk or in fetal tissue that may be re-expressed in breast tumor tissue will be explored.

The feasibility of studying modified receptors isolated from human tissue will be examined. Hormone binding to receptor and subsequent transduction of the signal may depend on modulation of more than one acidic group on either the hormone or the receptor. Changes in responsiveness of target tissue to hormones take place during development and the carcinogenic process.

Using explants from mammary glands of mice, prolactin effects on protein synthesis and modification at times earlier than 24 hours and in cell compartments other than the soluble 10,000 x g supernatant fraction will be examined. It should be possible to separate effects that may relate to cell division and those that relate to milk protein synthesis in this explant system. Following a wave of cell division that occurs during the first two days of incubation, the epithelial cells respond to prolactin by increased protein synthesis. In explants from mice bearing the mouse mammary tumor virus this may not be the case. A comparison of events following hormone stimulation in these types of explants may help to elucidate the role of the virus on mammary tumor formation. A collaboration with B. K. Vonderhaar, Laboratory of Pathophysiology, National Cancer Institute, to study regulation of the prolactin receptor by prolactin and other hormones in mouse mammary explants and to determine whether phosphorylation or glycosylation reactions are involved in this regulation has begun.

Publications:

None

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE04785-14 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

DNA Repair Studies on Human and Mouse Normal, Tumor, and Transformed Cells

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

PI: R. S. Day, III Research Physical Scientist LMC NCI

Others: D. B. Yarosh Staff Fellow LMC NCI

T. Yagi Visiting Fellow LMC NCI

M. Babich Staff Fellow LMC NCI

A. J. Fornace, Jr. Cancer Expert DCT NCI

COOPERATING UNITS (if any) Chemical Carcinogenesis Program, Litton Bionetics, Inc., Frederick, MD (D. Scudiero); Laboratory of Molecular Genetics, NICHD, NIH Bethesda, MD (H. Okayama).

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Nucleic Acids Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

4.3

## PROFESSIONAL:

3.3

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

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

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

The study of human cells defective in repairing damaged DNA was extended, with the rationale that DNA repair-deficient cells are more susceptible to the adverse effects of carcinogens (cell killing, mutagenesis, sister chromatid exchange, and malignant transformation) than their repair-proficient counterparts. A group of human tumor (19) and SV40-transformed (7) strains deficient in the repair of O-6-methylguanine (O-6-MeG, a modified DNA base made by certain methylating agents) was identified earlier in this project. Such strains are called Mer-. Transformation by SV40, Rous sarcoma virus, adenovirus, or Epstein-Barr virus produces Mer- strains. A 22,000 MW protein, present in 13 Mer+ strains but not in 16 Mer- strains, demethylates O-6-MeG in DNA, thereby repairing this damaged base and producing guanine. After the reaction, the methyl group remains bound to protein, presumably to the O-6-MeG-DNA methyltransferase itself. Using methyl group transfer as a stoichiometric measure, Mer+ strains contain on the average 60,000 methyltransferase molecules per cell. A group of 5 Mer+ cell strains, sensitive to cell killing by MNNG, was termed Mer+ Rem-. This group was found able to repair approximately one-third as much O-6-MeG as Mer+ Rem+ cell strains. Two groups of Mer- Rem+ cells were identified. Such strains fail to repair O-6-MeG but are resistant to cellular inactivation by MNNG. This shows that O-6-MeG (if lethal to Mer- Rem- strains) is not lethal to Mer- Rem+ strains. Mer- Rem- and Mer- Rem+ strains are equally sensitive to inactivation by chloroethylnitrosoureas, indicating that while repair of O-6-MeG may not be necessary for survival in Mer- Rem+ strains, repair of O-6-chloroethylguanine (an adduct that leads to DNA:DNA crosslinks) probably is necessary. Finally, data obtained so far indicate that human interferons  $\alpha$  and  $\beta$  inactivate Mer- Rem- tumor strains while Mer+ Rem+ human tumor strains are more resistant to such treatment, indicating an association between defective repair of O-6-MeG and sensitivity to interferons.

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

R. S. Day, III	Research Physical Scientist	LMC	NCI
D. B. Yarosh	Staff Fellow	LMC	NCI
T. Yagi	Visiting Fellow	LMC	NCI
M. Babich	Staff Fellow	LMC	NCI
A. J. Fornace, Jr.	Cancer Expert	DCT	NCI

Objectives:

To learn more about DNA repair mechanisms in human and other mammalian cells and about their role in carcinogenesis. In particular, to determine the nature of the DNA repair defects both in human tumor cells and in cells from persons who are genetically predisposed to cancer. In addition, to use human cell strains with characterized defects to study the mechanisms of action of carcinogens, or suspect carcinogens, chemotherapeutic agents, and other chemicals, in altering either DNA or the repair of damaged DNA.

Methods Employed:

1. Plaque assay: An adenovirus-host cell reactivation assay, developed previously in this project, was used to quantitate the deleterious effects of various chemical and physical treatments on the ability of the virus to initiate and sustain infection. The method involves establishing monolayer cell cultures which are infected with treated or non-treated adenovirus. The infected cells are then incubated 12-14 days with feeding by means of periodic overlaying with a nutrient agar. Non-treated virions or those treated ones which have been "reactivated" by cellular repair mechanisms form plaques of dead, lysed cells which are then counted.
2. Cellular extracts and partially purified fractions were assayed for O<sup>6</sup>-methyl-guanine-DNA methyltransferase repair activity using as a substrate either DNA methylated by [<sup>3</sup>H-methyl]-methylnitrosourea (MNU) or a synthetic double-stranded polymer (kindly supplied by Dr. S. Mitra, Oak Ridge National Laboratory) containing [<sup>3</sup>Hguanine]-O<sup>6</sup>-methylguanine. Reaction mixtures were incubated at 37°C, then heated in acid to remove purines, which were separated by high pressure liquid chromatography (HPLC) and quantified by liquid scintillation counting. A reduction of the O<sup>6</sup>-methylguanine to guanine ratio or a conversion of O<sup>6</sup>-methylguanine to guanine was interpreted to mean that repair of O<sup>6</sup>-methylguanine had occurred.
3. Discontinuous SDS polyacrylamide gel electrophoresis was used to measure the size of O<sup>6</sup>-methylguanine-DNA methyltransferase. After cellular extracts had been incubated with a substrate (prepared by treating DNA with [<sup>3</sup>H-methyl]-MNU), and the labeled methyl group of O<sup>6</sup>-methylguanine was transferred to the O<sup>6</sup>-methyl-guanine-DNA methyltransferase, the proteins were precipitated, resuspended in buffer, boiled with SDS, and subjected to electrophoresis.
4. DNA repair synthesis was measured by incorporation of <sup>3</sup>H-thymidine into TCA-insoluble-double stranded DNA either according to the method of Scudiero et al.

(BND-cellulose assay) or according to the method of Pettijohn and Hanawalt (CsCl/BUDR assay).

5. The survival of cells treated with ultraviolet light or chemicals was assayed by growth of the cells into colonies of at least 50 cells.

6. Plasmid production and purification together with transfer to *E. coli* and human cells, and assays for their presence followed published protocols.

7. Sister chromatid exchanges were assayed essentially by the method of Latt as modified by Perry and Wolfe. N-methyl-N'-nitro-N-nitrosoguanidine-treated (MNNG) and non-treated cultured cells are grown for two rounds of DNA synthesis in the presence of BUDR, a manipulation that labels (with BUDR) one chromatid of each chromosome in both DNA strands and the other chromatid in one DNA strand. After dye photosensitized alteration of the DNA that contains BUDR, DNA without BUDR is more heavily stained by Giemsa, a fact that affords differential visualization of the two sister chromatids of each chromosome. Any somatic recombination that occurred between sister chromatids is visualized as a reciprocal exchange of heavily for lightly Giemsa-stained material.

8. The production and repair of methylated purines in DNA was followed using HPLC techniques, with either carbon-18 or strong cation exchange columns to separate both methylated bases and methylated deoxyribonucleosides.

#### Major Findings:

A major part of this year's research concerned the mechanisms involved in repair of DNA damage produced by alkylating agents (principally methylating agents), including chloroethylnitrosoureas (CNU's). Earlier in this project, we identified a group of 19 (of 93) human tumor cell strains that is unable to repair adenovirus damaged by MNNG. This repair-deficient phenotype we have termed Mer<sup>-</sup>. Human tumor cells having the Mer phenotype fail to repair O<sup>6</sup>-methylguanine (O<sup>6</sup>MeG), lack a 22,000 MW protein called O<sup>6</sup>MeG-DNA methyltransferase (O<sup>6</sup>MT), and are extremely sensitive to MNNG in terms of both post-MNNG colony-forming ability and post-MNNG sister chromatid exchange (SCE) production.

Normal-appearing fibroblasts of two patients whose tumors gave rise to Mer<sup>-</sup> strains were determined to be Mer<sup>+</sup>. Moreover, Mer<sup>-</sup> human tumor cells show more post-MNNG DNA repair synthesis but less post-MNNG semiconservative DNA synthesis than do human fibroblasts, and fail to restore control tertiary structure to their DNA after MNNG treatment.

We have also identified a group of five Mer<sup>+</sup> strains, that, while more resistant than Mer<sup>-</sup> strains to MNNG in terms of post-treatment colony-forming ability, are about three-fold (slope difference) more MNNG-sensitive than are 13 human fibroblast strains or other Mer<sup>+</sup> tumor strains. Rem<sup>+</sup>, for resistance to MNNG, is the phenotype we have assigned to human fibroblasts, so that the MNNG-sensitive Mer<sup>+</sup> strains are Mer<sup>+</sup> Rem<sup>-</sup>. Mer<sup>+</sup> Rem<sup>-</sup> cells behave as if they contained about one-third of the O<sup>6</sup>MT complement present in Mer<sup>+</sup> Rem<sup>+</sup> cells.

Mer<sup>-</sup> strains are produced from Mer<sup>+</sup> strains by SV40 transformation, but these strains are Rem<sup>+</sup>. Further, transformation by other viruses, adenovirus, Rous sarcoma, and Epstein-Barr virus, also produces Mer<sup>-</sup> strains.

Thus, in human tumor lines, Mer function (i.e., O6MT) may be shut off by oncogene activity or is produced in them after infection by an adventitious virus, also present in the tumor biopsy.

1. Among 93 human tumor strains studied, 19 were Mer<sup>-</sup> and 74 were Mer<sup>+</sup>. Forty of 40 normal human strains were all Mer<sup>+</sup>; none were Mer<sup>-</sup>. It was suggested to us by the laboratory of Dr. M.C. Paterson, Atomic Energy of Canada, Ltd., Chalk River, Ontario, that we test a fetal fibroblast strain GM11, a strain they found to be increasingly more sensitive to inactivation by MNU with increasing passage number. Indeed, fibroblasts from GM11 (high passage) were Mer<sup>-</sup>, whereas GM11 (early passage) cells showed a large Mer<sup>+</sup> component. We will soon test another fibroblast strain GM3314 from a Gardiner's syndrome patient found both by Dr. Paterson's laboratory and by the laboratory of Drs. V. Maher and J.J. McCormick, Carcinogenesis Laboratory, Michigan State University, to be highly sensitive to killing by agents that produce O<sup>6</sup>MeG. Because we have found previously that fibroblasts from patients whose tumors gave rise to Mer<sup>-</sup> cell strains, were Mer<sup>+</sup>, the finding of a Mer<sup>-</sup> fibroblast strain is highly interesting. Because transformation by viruses (SV40 adenovirus, Rous sarcoma virus, Epstein-Barr virus) often converts Mer<sup>+</sup> cultures to Mer<sup>-</sup>, we suspect that GM11 and GM3314 may have become Mer<sup>-</sup> due to infection by an adventitious virus or activation of an oncogene.

2. We have continued to study O6MT activity in extracts from Mer<sup>-</sup> and Mer<sup>+</sup> cells. To date, extracts of 13 Mer<sup>+</sup> cell lines remove methyl groups from O<sup>6</sup>MeG in DNA, while extracts from 16 Mer<sup>-</sup> cultures fail to do so. Because the protein transfers the methyl group to one of its own amino acids, the protein becomes labelled if DNA containing [<sup>3</sup>H-methyl]-O<sup>6</sup>MeG is used as a substrate. The molecular weight of O6MT has been found to be 22,000 ± 2,000 both in SDS polyacrylamide gels and in a protein-seiving HPLC column. The same molecular weight is found for O6MT extracted from human placenta, human liver, or from Mer<sup>+</sup> Rem<sup>+</sup> or Mer<sup>+</sup> Rem<sup>-</sup> cultured human tumor cells.

When log phase Mer<sup>+</sup> cells were treated with 0.5 μM of MNNG, the level of extractable O6MT was decreased to 30-70% of its control value. Within 48 hours of post-treatment incubation, the O6MT level was restored to 100-150 percent of its original value, and had a MW of 22,000 both in Mer<sup>+</sup> Rem<sup>+</sup> and in Mer<sup>+</sup> Rem<sup>-</sup> cultures. We suggest that the Mer<sup>+</sup> Rem<sup>-</sup> phenotype is not due to an inability to resynthesize O6MT.

3. We have identified two groups of human cells having the Mer<sup>-</sup> Rem<sup>+</sup> phenotype, i.e., they fail to repair MNNG-damaged adenovirus (and fail to repair O<sup>6</sup>MeG), but are as resistant in terms of post-MNNG colony-forming ability as are normal human fibroblasts. The groups include four Mer<sup>-</sup> cell strains obtained by SV40 transformation and a number of MNNG-resistant revertants of the A1235 Mer<sup>-</sup> human astrocytoma cell strain. However, strains of the Mer<sup>-</sup> Rem<sup>+</sup> phenotype are as sensitive as Mer<sup>-</sup> Rem<sup>-</sup> to inactivation by CNU or 3-hydroxyethyl-1-chloroethyl-1-nitrosourea (HECNU), believed to be lethal due to O<sup>6</sup>-chloroethylguanine production and (if not repaired) consequent DNA:DNA crosslink formation. Thus the Mer<sup>-</sup> phenotype



correlates with lack of  $O^6$ MeG repair activity, increased susceptibility to MNNG-produced SCEs, and sensitivity to killing by CNU or HECNU, but not with sensitivity to killing by MNNG.

4. Several human tumor strains were reported in the literature to be sensitive to killing by human interferon- $\alpha$  (IFN) or human IFN- $\beta$  that we had found to be Mer<sup>-</sup>. In pursuing this possible association, we have found that the Mer<sup>-</sup> human tumor strains are indeed more sensitive than the Mer<sup>+</sup> strains, that we have so far studied, to inactivation by both  $\alpha$  and  $\beta$  IFNs. We have obtained evidence that little, if any,  $O^6$ MeG is produced by the IFN treatment. Apparently the mechanisms for resistance to IFNs- $\alpha$  and  $\beta$  and for production of O6MT are often coordinately shut down during tumorigenesis.

5. There are probably at least two genes that modulate the lethal effects of MNNG. One gene (Mer), correlating with the presence of O6MT, presumably is involved in controlling the expression or specifying the structure of that gene product. The nature of the product of the other gene is not known, but it provides high post-MNNG cellular survival in the absence of repair of  $O^6$ -MeG. Strategies for cloning the O6MT gene on the basis of its putative biological activity require 1) knowledge of whether the gene product is active in hybrids of Mer<sup>+</sup> and Mer<sup>-</sup> cells and 2) having a Mer<sup>-</sup> strain with sufficiently high ability to be transfected. In initial transfection experiments with the plasmids pSV2gpt and pSV2neo, 10 Mer<sup>-</sup> strains were assayed for transfectability and one was selected for further work. Hybrids selected from fusions to other Mer<sup>-</sup> strains gave no evidence of complementation. Hybrids selected from fusions of the Mer<sup>-</sup> Rem<sup>-</sup> strain to three different Mer<sup>+</sup> Rem<sup>-</sup> strains were Mer<sup>+</sup> Rem<sup>+</sup>, Mer<sup>+</sup> Rem<sup>-</sup>, and Mer<sup>-</sup> Rem<sup>-</sup>, showing the Mer<sup>+</sup> product to be dominant in some cases, and the situation to be more complex than originally suspected. Fifteen thousand clones were selected by treating the transfectable Mer<sup>-</sup> strain with pSV2neo plus DNA from a Mer<sup>+</sup> Rem<sup>+</sup> strain, and selecting for the neomycin resistance marker. Of these, 14 were selected as resistant to MNNG: one was Mer<sup>+</sup>, and contained O6MT. Control experiments testing the possibility that this neo resistant transfectant may be a revertant have been negative. Similar transfection strategies are being employed using, as a vector, a cDNA library from human fibroblasts, and as recipients Mer<sup>-</sup> human cells or rodent cells that have been characterized as deficient in repair of  $O^6$ MeG, and other agents that select for cells containing O6MT more specifically than does MNNG.

Another approach to cloning the Mer gene is based upon the assumption that Mer<sup>-</sup> strains fail to produce the mRNA specifying the Mer<sup>+</sup> product. A cDNA library enriched in sequences specifying the Mer<sup>+</sup> message was enriched by competitive hybridization of mRNA from Mer<sup>-</sup> cells to cDNA (from mRNA) of Mer<sup>+</sup> cells. Probes (prepared from library clones) hybridizing to mRNA of Mer<sup>+</sup> strains, but not to that of Mer<sup>-</sup> strains, likely carry information related to genes identical to (or controlled coordinately with) the Mer gene.

6. Work on mouse cell lines has also progressed. We have identified 4 of 19 mouse lines defective in repair of  $O^6$ MeG and lacking O6MT. These include SV-T2, 34I, clone M3, and C57BL-CL.

The mouse strains that have increased sensitivity to MNNG in terms of sister chromatid exchanges and colony forming ability lack O6MT. However, the 34I cell

line shows neither hypersensitivity.

Significance to Biomedical Research and the Program of the Institute:

The results of this project suggest the possibility that a fraction, one-fifth, of all human tumors is composed of repair-defective cells. Certain bifunctional alkylating agents 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)] are known to be effective against some individual human tumors and to be relatively ineffective against others, also indicating the possibility that the molecular basis for the success of alkylation chemotherapy may be specific to a tumor or a group of tumors. Our studies of the mechanisms by which repair occurs and upon the inhibition of such repair are designed, in part, to understand ways by which tumors might be more successfully treated and ways by which tumors might arise. Physical, chemical, and viral carcinogens are all known to alter the structural integrity of the cellular genetic apparatus. An evaluation of the role of DNA repair and/or related mechanisms in conferring resistance or susceptibility to mutagenesis and carcinogenesis is an important facet in any overall program having as its goal the understanding of the molecular pathways which, when perturbed, give rise to carcinogenesis in humans. It is the long-range goal of this project to determine whether or not the elucidation of repair mechanisms is important to the understanding of carcinogenesis. It is expected that an understanding of human repair mechanisms, in general, will benefit many areas of biomedical research.

Proposed Course:

1) To continue surveying human strains for Mer<sup>-</sup> strains using the MNNG-treated adenovirus plaque assay and the O6MT activity assay; 2) to clone the O<sup>6</sup>-methyltransferase gene as described; 3) to purify the O6MT from human tissue, including placenta and liver; and 4) to pursue the relationship of interferon sensitivity to the Mer<sup>-</sup> condition.

Publications:

Day, R. S., III, Yarosh, D. B. and Ziolkowski, C. H. J.: Relationship of methyl purines produced by MNNG in adenovirus 5 DNA to viral inactivation in repair deficient (Mer<sup>-</sup>) human tumor cell strains. Mutat. Res. 131: 45-52, 1984.

Day, R. S., III and Ziolkowski, C. H. J.: Induced reversion using human adenovirus. In Kilbey, B. J., Legator, M., Nichols, W. and Ramel, C. (Eds.): Handbook of Mutagenicity Test Procedures. Amsterdam, Elsevier, North Holland, Inc., 1984, pp. 83-91.

Scudiero, D. A., Meyer, S. A., Clatterbuck, B. E., Mattern, M. R., Ziolkowski, C. H. J. and Day, R. S., III: Relationship of DNA repair phenotypes of human fibroblast and tumor strains to killing by N-methyl-N'-nitro-N-nitrosoguanidine. Cancer Res. 44: 961-969, 1984.

Scudiero, D. A., Meyer, S. A., Clatterbuck, B. E., Mattern, M. R., Ziolkowski, C. H. J. and Day, R. S., III: Sensitivity of human cell strains having different abilities to repair O<sup>6</sup>-methylguanine in DNA to inactivation by alkylation agents including chloroethylnitrosoureas. Cancer Res. (In Press)

Teo, I. A., Broughton, B. C., Day, R. S., III, James, M. R., Karran, P. L., Mayne, V. and Lehmann, A. R.: A biochemical defect in the repair of alkylated DNA in cells from an immunodeficient patient (46BR). Carcinogenesis 4: 559-564, 1983.

Yagi, T., Yarosh, D. B. and Day, R. S., III: Comparison of repair of O<sup>6</sup>-methylguanine produced by N-methyl-N<sup>1</sup>-nitro-N-nitrosoguanidine in mouse and human cells. Carcinogenesis 5: 593-600, 1984.

Yarosh D. B., Mattern, M. R., Scudiero, D. A. and Day, R. S., III: The Mer phenotype: Human tumor cell strains defective in repair of alkylation damage. In Castellani, A. (Ed.): The Use of Human Cells for the Evaluation of Risk from Physical and Chemical Agents. New York, Plenum Press, 1983, pp. 731-734.

Yarosh, D. B., Rice, M., Day, R. S., III; Foote, R. S. and Mitra, S.: O<sup>6</sup>-methylguanine-DNA methyltransferase in human cells. Mutat. Res. 131: 27-36, 1984.

Yarosh, D. B., Rice, M., Ziolkowski, C. H. J., Day, R. S., III, Scudiero, D. A., Foote R. S. and Mitra S.: O<sup>6</sup>-methylguanine methyltransferase in human tumor cells. In Friedberg, E. C. and Bridges, B. A. (Eds.): Cellular Responses to DNA Damage. New York, Alan R. Liss, Inc., 1983, pp. 261-270.

Yarosh, D. B., Scudiero, D. A., Ziolkowski, C. H. J., Rhim, J. S. and Day, R. S., III: Hybrids between human tumor cell strains differing in repair of MNNG-produced DNA damage. Carcinogenesis 5: 627-633, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05086-06 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Monoclonal Antibodies to Human Carcinogen Metabolizing Enzymes

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

PI: S. S. Park Senior Staff Fellow LMC NCI  
Others: H. V. Gelboin Chief LMC NCI  
H. Miller Biological Lab Technician LMC NCI

COOPERATING UNITS (if any)

Vanderbilt University School of Medicine, Nashville, TN (F. P. Guengerich)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

Benzo(a)pyrene (BP) may be metabolized to nontoxic products or to the carcinogen, BP-7,8-diol-9,10-epoxide. The direction of BP metabolism depends on the presence of specific forms of enzymes in individuals exposed to chemical carcinogens. Monoclonal antibodies (MAbs) are specific probes for particular antigenic determinants and are useful tools for identification of particular isoenzymes. We prepared MAbs to cytochrome P-450 and epoxide hydrolase (EH), which are two key components of the system responsible for the metabolism of BP. The MAbs bind to human liver cytochrome P-450 but do not immunoprecipitate or inhibit enzymatic activity; the last observation suggests that the MAbs were raised against an antigenic determinant whose binding does not interfere with the functioning of the catalytic site. The MAbs against EH bind and precipitate only the form which was used as antigen but do not interact with other forms.

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

S. S. Park	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI
H. Miller	Biological Lab Technician	LMC	NCI

Objectives:

Benzo(a)pyrene (BP) is metabolized by mixed function oxidases to carcinogenic products and nontoxic water soluble compounds. Many cytochrome P-450 forms have been reported but specific forms appear to predominate following treatment with specific inducers. Specific carcinogen-metabolizing enzymes might predominate in individuals who smoke heavily, or who are exposed to carcinogenic environmental chemicals. Microsomal preparations of placenta from women who smoke heavily possess high levels of mixed function oxidases, as do human monocytes, lymphocytes, and lung cell lines induced with benzo(a)anthracene. The objective of this work is to prepare monoclonal antibodies (MAbs) to enzymes involved in BP metabolism, and to use these MAbs as a tool to study enzyme multiplicity and identify the predominant isozymes induced by carcinogens. A long range goal is to identify patterns of cytochromes P-450 that are responsible for differences in carcinogen susceptibility.

Methods Employed:

Balb/c female mice were immunized with cytochrome P-450 derived from human liver and placental microsomes, and with human liver microsomal epoxide hydrolase (EH). The primed spleen cells were isolated and fused with myeloma cells using polyethylene glycol. The hybrid cells were grown in a selective medium (HAT) and screened by radioimmunoassay for hybridomas producing MAbs to their respective immunogens. The effect of MAbs on enzyme activities was measured by fluorometric and HPLC assays. Ouchterlony double immunodiffusion analyses were performed to assay for precipitin reactions.

Major Findings:

Four IgG1 MAbs were obtained to human liver cytochrome P-450. Mouse serum to the cytochrome P-450 precipitated the antigen but the MAbs did not. Direct inhibition of aryl hydrocarbon hydroxylase activity was not observed but activity could be removed by precipitating the MAb-enzyme complex from solution with protein-A bound to Sepharose 4B. We also observed that the hybridomas gradually lost their capacity to produce MAbs. Since it is not possible to obtain an induced form of human liver cytochrome P-450, cytochrome P-450 from different individuals was used for immunization of mice. Three additional hybridomas were prepared and tested for enzyme inhibitory activity with a newly developed progesterone hydroxylation assay. Four MAbs were obtained against EH. These MAbs bound and precipitated the form of EH that was used as antigen, but not other forms.

Significance to Biomedical Research and the Program of the Institute:

Cytochrome P-450 and epoxide hydrolase are two key enzymes leading to the formation of the carcinogenic metabolite, BP-7,8-diol-9,10-epoxide. Both of these enzymes are present in multiple forms. Preparation of MAbs to specific forms of these enzymes is essential for the study of the activation and regulation of BP metabolism. Provided with a series of MAbs to enzymes in BP metabolism, not only can we better understand chemical carcinogenesis, but we will be able to analyze the relationship between enzyme content and differences in individual susceptibility to cancer. We can thus develop techniques for screening populations to identify individuals at high risk for cancer. The MAbs to carcinogen-metabolizing enzymes will also be useful for the study of chemotherapeutic agents which require activation during cancer treatments.

Proposed Course:

1) Additional MAbs to the cytochromes P-450 and EH isoenzymes from different human sources will be prepared. 2) Competitive radioimmunoassays directed by MAbs are being developed. 3) The cross-reactivity of MAbs to human enzymes with enzymes from different species will be assessed.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05109-05 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Changes in Chromatin Structure During Carcinogenesis

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

PI:	Michael Bustin	Research Chemist	LMC	NCI
Others:	Leo Einck	Staff Fellow	LMC	NCI
	John Fagan	Senior Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)

Department of Human Genetics, Tel Aviv University, Israel (H. Slor)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.6

PROFESSIONAL:

1.2

OTHER:

0.4

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.)

A gene coding for a 3-methylcholanthrene-induced P-450 enzyme has been subcloned into pBR322. Various parts of the gene have been further subcloned to yield 14 overlapping gene regions. These clones are used to study the chromatin structure of the P-450 gene in control and carcinogen treated rats. Nuclease digestions of nuclei isolated from the liver of these rats indicate that the gene is organized in a nucleosomal conformation, that the DNA of the gene is more susceptible to DNase I digestion than bulk chromatin, and that a DNase I hypersensitive region is present near the 5' terminal of the gene.

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

Michael Bustin	Research Chemist	LMC	NCI
Leo Einck	Staff Fellow	LMC	NCI
John Fagan	Senior Staff Fellow	LMC	NCI

Objectives:

To elucidate changes in the chromatin structure of particular genes associated with carcinogenesis.

Methods Employed:

Restriction analysis of DNA, chromatin isolation, gene cloning, radioactive labelling of nucleic acids, chromatin digestion and analysis.

Major Findings:

In earlier work (Project Number Z01CE05196-04), a gene coding for a 3-methylcholanthrene (3-MC) induced P-450 enzyme was selected from a rat library and cloned. The gene has been cloned into pBR322. In addition, various regions of the gene have been subcloned to give a set of 14 subclones overlapping the entire gene. It has been found that intervening sequences of this gene contain at least two distinct classes of repetitive DNA. Furthermore, the gene codes for two messages. One message is for the P-450 inducible enzyme. The second message is larger and non-inducible and the gene is only a part of this larger gene. The second gene seems to be, in part, an intron of the first gene. Two distinct mRNA molecules hybridize to the genomic clone. Careful analysis using the set of 14 subclones and immunoprecipitation of translation products has demonstrated that only the smaller message codes for the P-450 inducible enzyme. The second message hybridizes to two introns of the P-450 gene and is non-inducible by 3-MC. The details of this unusual gene arrangement and especially the control mechanisms are being examined. To study the chromatin structure of this gene, nuclei were isolated from the liver of normal and carcinogen treated rats. The nuclei were digested with: a) micrococcal nuclease, b) DNase I under conditions which allow detection of hypersensitive regions and c) S1 nuclease. The DNA from the digested nuclei was isolated and analyzed by slot blot hybridization and by electrophoresis after digestion with restriction enzymes. The separated fragments were transferred to nitrocellulose and analyzed with nick-translated subclones of the P-450 gene. In control, untreated rats, the DNase I sensitivity of the gene was similar to that of the bulk chromatin. A DNase I hypersensitive region, close to the 5' terminal of the gene, has been identified. Interestingly, the gene in carcinogen treated rats was more resistant to DNase I digestion than the gene in control rats, yet the hypersensitive region was more rapidly digested than in control rats. The S1 sensitive sites were mapped in a plasmid containing the entire gene. As controls for the structure of a transcribed gene, rat albumin was used; for a non-transcribed gene, the chromatin structure of insulin was used.



Significance to Biomedical Research and the Program of the Institute:

Understanding the processes involved in the interaction of chemical carcinogens with the genome of a target cell may help to elucidate various aspects of the mechanism of carcinogenesis. Study of the chromatin structure of P-450 genes may help explain various aspects of the manner in which cells respond after exposure to carcinogens and mutagens.

Proposed Course:

Studies aimed at accomplishing the goals of this project will be continued using the experimental approaches described above. In the next year, the research will center on elucidation of changes in the chromatin structure upon induction of the P-450 gene described.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05125-04 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Preparation of Monoclonal Antibodies to Rat Liver Cytochromes P-450

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

PI:	S. S. Park	Senior Staff Fellow	LMC	NCI
Others:	H. V. Gelboin	Chief	LMC	NCI
	S. S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (if any) Vanderbilt Univ. School of Med., Nashville, TN (F. P. Guengerich); Univ. of Connecticut, Farmington, CT (J. B. Schenkman); Woods Hole Oceanographic Institute, Woods Hole, MA (J. B. Schenkman); PA State Univ., Hershey, PA (E. S. Vesell); Laboratory of Chemical Pharmacology, NHLBI, NIH (J. R. Gillette)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

Cytochrome P-450 plays a key role in the metabolism, activation, and detoxification of drugs, endogenous steroids, xenobiotics, and environmental carcinogens. Cytochromes P-450 were purified from the livers of rats treated with phenobarbital (PB-P-450), 3-methylcholanthrene (MC-P-450),  $\beta$ -naphthoflavone (BNF-P-450), pregnenolone 16- $\alpha$ -carbonitril (PCN-P-450), and the environmentally induced marine fish (Scup-P-450); monoclonal antibody-producing hybridomas were prepared to these enzymes. Among twelve hybrid clones producing MABs active toward PB-P-450, seven were IgG1 and five were IgM. Three classes of MABs were produced: one class bound but neither precipitated the PB-P-450 nor inhibited its aryl hydrocarbon hydroxylase (AHH) activity; a second class bound and immunoprecipitated, but did not inhibit enzyme activity; and a third class comprising a single clone, MAB 2-66-3, bound, immunoprecipitated and completely inhibited the AHH of PB-P-450. MAB 2-66-3 did not inhibit the AHH activities of MC-P-450, BNF-P-450, or PCN-P-450. The MAB 2-66-3 inhibited the AHH, ethoxycoumarin deethylase, and benzphetamine demethylase of liver microsomes from PB-treated rats, but did not inhibit these activities in microsomes from control, BNF-, or MC-treated rats. The MAB 2-66-3 showed high cross-reactivity in binding, immunoprecipitation, and inhibition of enzyme activity of PB-induced cytochrome P-450 from rabbit liver. Monoclonal antibodies to PCN-P-450 and Scup-P-450 are in the process of characterization. Monoclonal antibodies to different cytochromes P-450 will be extraordinarily useful for a variety of studies, including phenotyping, genetic analysis, and purification of cytochromes P-450.

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

S. S. Park	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

Benzo(a)pyrene (BP) is activated by aryl hydrocarbon hydroxylase (AHH) and epoxide hydrolase, both of which exist in multiple forms. Metabolism of BP by these enzymes leads to a number of products: to the formation of nontoxic, water soluble, excretable conjugates, and/or to the formation of active carcinogenic forms. Exposure of individuals to environmental chemical carcinogens influences metabolism through induction of specific isozymes. We previously observed that monoclonal antibodies (MAbs) to 3-methylcholanthrene-induced rat liver microsomal cytochrome P-450 (MC-P-450) were cross-reactive with placental microsomes of women who smoked heavily. We used these MAbs to identify and characterize human carcinogen-metabolizing enzymes. This report describes similar experiments with MAbs to cytochrome P-450s of rats treated with phenobarbital (PB-P-450) and the environmentally induced marine fish, scup (Stenotomus chrysops) (Scup-P-450).

Methods Employed:

Balb/c female mice were immunized with purified cytochrome P-450 of rats which were treated with phenobarbital or other inducers. The primed spleen cells were isolated and fused with myeloma cells, using polyethylene glycol. The hybrid cells were grown in a selective medium (HAT) and screened by radioimmunoassay to identify hybridomas producing MAbs to PB-P-450 and Scup-P-450. The effect of MAbs on enzymatic activity was measured both fluorometrically and with HPLC. Ouchterlony double immunodiffusion analysis and competitive radioimmunoassay were also carried out for the characterization of MAbs.

Major Findings:

1) Twelve hybridomas producing MAbs to PB-P-450 were obtained: seven were IgG1 and five were IgM. 2) Based on the interaction with PB-P-450, these MAbs can be classified into three groups: binding but not precipitating; binding, precipitating but not inhibiting; and binding, precipitating, and inhibiting. The MAb 2-66-3 bound, precipitated, and completely inhibited the AHH activity of PB-P-450 but did not inhibit the AHH activities of cytochrome P-450 of rats treated with 3-methylcholanthrene (MC-P-450),  $\beta$ -naphthoflavone (BNF-P-450) or pregnenolone-16- $\alpha$ -carbonitril (PCN-P-450). 3) The MAb 2-66-3 inhibited the AHH, ethoxycoumarin deethylase (ECD) and benzphetamine demethylase of liver microsomes from PB-treated rats by 22-38%. However, this MAb did not inhibit these activities in microsomes from control, BNF-, or MC-treated rats, and had no effect on ethylmorphine demethylase. 4) The MAb 2-66-3 also inhibited the formation of BP metabolites by PB-P-450 by more than 85%. 5) The MAb 2-66-3

showed high cross-reactivity in binding, immunoprecipitation, and inhibition of enzyme activity of PB-P-450 from rabbit liver.

Nine independent hybrid clones produced MABs to Scup-P-450 and they were IgG1 and IgHG2b types. Three of the MABs inhibited AHH and ECD activities of Scup-P-450 by 87-90% and 89-96%, respectively. One of the scup MABs (1-12-3) did bind and inhibit the AHH activity of BNF-P-450 by 40%. Further characterization of Scup-MABs is in progress.

#### Significance to Biomedical Research and the Program of the Institute:

The various cytochromes P-450 display stereoselectivity for both substrate and product formation. Therefore, the balance of BP metabolism between detoxification and carcinogen formation is dependent on the types and amounts of isozymes present. Each isozyme of cytochrome P-450 possesses specific antigenic determinants and the MABs prepared to PB-P-450 are specific to PB-P-450, whereas previously prepared MABs to MC-P-450 are active toward both MC-P-450 and BNF-P-450. Therefore, antigenically related cytochromes P-450 can be induced by different chemicals. MABs originally prepared to rat or rabbit enzymes that also react with human cytochrome P-450 would be useful in phenotyping isozyme patterns of individuals and perhaps relating these to individual differences in carcinogen sensitivity. Monoclonal antibodies to the environmentally induced marine fish, Scup-P-450, bound to MC-P-450 and BNF-P-450 would be useful for monitoring environmental pollution and for phylogenetic studies. The monoclonal antibody technique is also a powerful new tool for numerous studies on the genetics of these crucial enzymes of carcinogen metabolism.

#### Proposed Course:

To gain more insight into the catalytic mechanisms of cytochromes P-450, we will examine the influence of different MABs on the spectrum of metabolites obtained from the action of cytochromes P-450 on various substrates (e.g., 2-acetylaminofluorene, imipramine, propranolol). We will prepare additional MABs using a variety of P-450 preparations from rats and other animals. These additional probes for different cytochromes P-450 will make feasible a more extensive characterization and phylogenetic studies of the isozymes in various sources including human tissues.

#### Publications:

Friedman, F. K., Robinson, R. C., Park, S. S., and Gelboin, H. V.: Monoclonal antibody-directed immunopurification and identification of cytochromes P-450. Biochem. Biophys. Res. Commun. 116: 859-865, 1983.

Gelboin, H. V., Park, S. S., Fujino, T., Song, B. J., Robinson, R. C., and Friedman, F. K.: Monoclonal antibody directed phenotyping, radioimmunoassay and purification of cytochromes P-450 that metabolize drugs and carcinogens. In Singer, T. P., Mansour, T. E. and Ondarza, R. N. (Eds.): International Symposium on the Biochemical Basis of Drug Action. New York, Academic Press, 1983, pp. 259-275.

Gelboin, H. V., Fujino, T., Song, B. J., Park, S. S., West, D., Robinson, R., Miller, H., and Friedman, F. K.: Monoclonal antibody directed phenotyping of Cytochromes P-450 by enzyme inhibition, immunopurification and radioimmunoassay. In Omenn, G. and Gelboin, H. V. (Eds.): The Role of Genetic Predisposition in Response to Chemical Exposure, Banbury Report. New York, Cold Spring Harbor Laboratory, 1984, pp. 65-84.

Park, S. S., Fujino, T., Miller, H., Guengerich, F. P., and Gelboin, H. V.: Monoclonal antibodies to phenobarbital induced rat liver cytochrome P-450. Biochem. Pharmacol. (In Press)

Park, S. S., Fagan, J. B., Fujino, T., Friedman, F., Song, B. J., Park, K. H., Miller, H., West, D., Pastewka, J., Robinson, R., and Gelboin, H. V.: The use of monoclonal antibodies in identification of mRNA translation products and cloned cDNA. In Han, M. H. (Ed.): Symp. on Genetic Eng. and Biotech. Seoul, Korea, KAIST (In Press)

Song, B. J., Fujino, T., Park, S. S., Friedman, F. K., and Gelboin, H. V.: Radioimmunoassay of monoclonal antibody-specific cytochrome P-450. J. Biol. Chem. 259: 1394-1397, 1984.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05196-04 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Cloning, Structure, and Regulation of the Genes for the Cytochromes P-450

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

PI:	J. B. Fagan	Senior Staff Fellow	LMC	NCI
Others:	H. V. Gelboin	Chief	LMC	NCI
	S. C. Chalberg	Guest Researcher	LMC	NCI
	J. Pastewka	Chemist	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

2.8

PROFESSIONAL:

1.8

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Recombinant DNA and related techniques have been used to study the molecular biology of the cytochromes P-450 (P-450s), a family of enzymes central to the activation and detoxification of carcinogens and to the metabolism of xenobiotics and drugs. The goal is to understand the structure and regulation of P-450 genes, to elucidate the molecular basis for the multiplicity of the P-450s and for the structural-functional relationships among them, and to determine the extent to which individual susceptibility of humans to cancer depends on the regulation of expression of specific P-450s. This year we have further characterized the P-450-M gene, further elucidating the finding that this gene is interdigitated with another independently regulated gene whose gene product is immunologically unrelated to P-450-M. We have used cloned cDNAs to P-450-BNF-B and P-450-ISI-G to show that the mRNAs for the P-450s have unique and common regions and to show that these mRNAs are regulated independently. We have continued our efforts to isolate the complete P-450-BNF-B and P-450-ISI-G genes.

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

J. B. Fagan	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI
S. C. Chalberg	Guest Researcher	LMC	NCI
J. Pastewka	Chemist	LMC	NCI

Objectives:

- 1) The primary, long range objective of this project is to elucidate the structure and regulation of expression of the genes for the cytochromes P-450.
- 2) Analyze the structure of the P-450 genes and compare both the regulatory and structural sequences of the genes for different P-450s. Specific objectives are: a) electron microscopic analysis of heteroduplexes between P-450 mRNA and cloned P-450 genes will be used to determine the location and size of intervening sequences and other gross structural characteristics of the P-450 genes; b) cloned P-450 genes will be used as hybridization probes against Southern blots of other cloned P-450 genes in order to identify regions of homology within the genes of this family; c) the organization of the P-450 gene family within the rat genome and the copy number of these genes will be determined by Southern hybridization and other mapping techniques; d) regulatory regions and other interesting regions of the P-450 genes will be analyzed by DNA sequencing; and e) the functional properties of regulatory sequences will be studied by cloning these sequences into eukaryotic expression vectors. The latter and derivatives thereof that have been generated by site-specific mutagenesis will then be introduced into cells in culture by DNA mediated gene transfer, and the regulatory properties of these sequences assessed in cell culture.
- 3) Study the regulation of P-450 gene expression at the mRNA level and at the gene level. Specific objectives are: a) to quantitate intracellular levels of P-450 mRNAs under various induction conditions; b) to measure transcription rates in isolated nuclei in vitro; and c) to determine transcription rates and mRNA stability in cells in culture by measuring <sup>3</sup>H-uridine incorporation.
- 4) Correlate regulatory data for the purpose of identifying relationships between gene structure and regulation.
- 5) Complete isolation of the native genes for methylcholanthrene (MC)-inducible cytochromes P-450 from recombinant bacteriophage libraries of the rat and other eukaryotic genomes, and isolate other P-450 genes, as appropriate. As necessary, construct, identify and characterize more clones carrying cDNA complementary to the mRNAs for the cytochromes P-450 induced by MC, phenobarbital (PB) and other inducers.
- 6) Use the P-450 cDNA and genomic clones to assess a) the multiplicity of P-450s expressed in different tissues; b) the relatedness of P-450s expressed

in different tissues; and c) the relatedness of P-450 genes in different eukaryotes. Both the regulatory and structural sequences will be assessed.

#### Methods Employed:

RNA isolation, fractionation and characterization have been modified for use with rat liver. These include RNA extraction and isolation with guanidine-HCl, RNA size fractionation by methylmercuric hydroxide agarose gel electrophoresis and by sucrose gradient centrifugation, in vitro translation of mRNA, immunoprecipitation of translation products with P-450-specific IgG, and SDS polyacrylamide gel electrophoretic analysis of translation products and immunoprecipitates. Levels and rates of RNA synthesis are studied by Northern blot hybridization and solution hybridization to P-450 cDNAs. DNA complementary to P-450 mRNA is synthesized and cloned in the bacterial plasmids. Clones of the complete, native P-450 genes are isolated from libraries of rat genomic DNA ligated into phage lambda and screened by standard plaque hybridization techniques. Cloned cDNAs and genes are prepared in large amounts by standard microbiological and biochemical procedures. Cloned cDNAs and genes are characterized and studied by a wide range of techniques including electron microscopic analysis of RNA-DNA heteroduplexes, hybridization selected translation and immunoprecipitation, colony and plaque hybridization, restriction endonuclease mapping, DNA sequencing, Southern hybridization, Northern hybridization, solution hybridization, and in vitro transcription. Regulatory sequences are characterized by subcloning into eukaryotic expression vectors, introducing these recombinants into cells in culture by the calcium phosphate precipitation technique for DNA mediated gene transfer, and analysis of the response of such cells to P-450 inducers. Site-specific mutagenesis is used to alter regulatory sequences cloned into these expression vectors to identify specific nucleotides essential to the activity of these sequences.

#### Major Findings:

Characterization of cytochrome P-450 mRNAs by in vitro translation and immunoprecipitation. Using in vitro translation and immunoprecipitation with P-450-specific antibodies we have shown that (1) there are multiple P-450 mRNAs and these encode multiple immunologically related P-450 peptides; (2) many P-450 mRNAs are strongly induced by polycyclic aromatic hydrocarbons (PAH) and highly abundant in induced liver; (3) there are two major size classes of PAH-induced P-450 mRNA, which can be fully resolved by methyl mercuric hydroxide-agarose gel electrophoresis; (4) each major size class of PAH-induced P-450 mRNA contains multiple species that can be partially resolved electrophoretically; and (5) it is likely that each individual P-450 is encoded by a separate mRNA distinguishable by size from other P-450 mRNAs.

Isolation and characterization of recombinant plasmids containing P-450 cDNAs. Recombinant plasmid libraries containing cDNAs representing the total mRNA population from the livers of rats treated with MC or pregnenolone carbonitrile (PCN) were constructed and screened by hybridization selected translation and immunoprecipitation using P-450-specific antibodies. In this way recombinant plasmids containing cDNAs derived from the most abundant PAH-induced P-450s, designated P-450-B and P-450-G, as well as cDNAs for a minor MC-induced P-450,



designated P-450-M, and for P-450-E have been isolated and characterized to various degrees.

The mRNAs encoding the major PAH-induced P-450s, P-450-B and P-450-G. Three recombinant plasmids have been used as hybridization probes to characterize the P-450-B and P-450-G mRNAs.

By hybridization-selected translation and Northern hybridization analysis it has been shown that the P-450-B mRNA is 2900 bases long and encodes a 55-kilodalton peptide, while the P-450-G mRNA is 2100 bases long and encodes a 52-kilodalton peptide. These findings suggest that P-450-B and P-450-G mRNAs have regions of partial homology and also regions whose sequences are very different. These findings are quite different from those reported for the PB-family of P-450s. The mRNAs for this subfamily of the P-450s seem to differ in only a small number of isolated amino acids or nucleotides.

Other Northern hybridization experiments using poly A+ RNA revealed 5200- and 3500-base precursors to the P-450-B mRNA and a 3100-base precursor to the P-450-G mRNA.

The kinetics of induction of P-450-B and P-450-G mRNAs have also been studied. It has been found that the kinetics of induction of P-450-B and P-450-G differed from each other and both of these differed from the kinetics of induction of aryl hydrocarbon hydroxylase (AHH) activity. These findings suggest that (1) there are significant differences in either the rates of transcription or in the stabilities, or both, of the P-450-B and P-450-G mRNAs. Thus, although their induction is triggered by the same signal, they may well not be regulated coordinately; (2) since AHH activity remains high while P-450 mRNA levels dropped, the AHH enzyme is more stable than the mRNA encoding it and the continued presence of P-450 mRNA is not required to maintain AHH activity.

Isolation and characterization of the P-450-M gene. We have isolated the complete native gene for P-450-M from a rat genomic library using as the hybridization probe the recombinant plasmid containing DNA derived from the P-450-M mRNA. This gene has been characterized by heteroduplex analysis, restriction mapping, and Southern and Northern hybridization-selected translation. This gene is 6800 bases long, contains 7 intervening sequences, ranging in size from 150 to 2500 bases, and contains 8 exons ranging in size from 100 to 350 bases.

Detailed mapping of the P-450-M gene revealed that there are coding sequences for a second gene within at least one intron near the 5' end of the P-450-M gene and at a second location, as well, near the 3' end of the P-450-M gene. The exons of this second gene do not overlap those of the P-450-M gene but seem to be "interdigitated" with the P-450-M exons. The protein encoded by the second gene is about 62 kd and is immunologically unrelated to P-450-M and to all other P-450s for which we have antibodies. Furthermore, this second gene is regulated independently of P-450-M and its point of transcription initiation probably does not coincide with that of P-450-M.

Isolation and characterization of the P-450-B and P-450-G genes. The isolation of the P-450-B and P-450-G genes is in progress. Presently 3800 bases of the P-450-B gene have been isolated and extensively characterized by Northern

hybridization, Southern hybridization, hybridization-selected translation and heteroduplex analysis. We have constructed a new genomic library and are in the process of isolating the remaining 1200 bases of the P-450-B gene and the P-450-G gene from this library. Our preliminary findings with the P-450-B gene are quite interesting. This gene is clearly complementary to two sizes of P-450 mRNA and primary transcripts of this gene may be processed by alternative routes to yield multiple related mRNAs.

Significance to Biomedical Research and the Program of the Institute:

The P-450s, as part of the mixed-function oxidase system, are central to the activation and detoxification of carcinogens, to the detoxification of xenobiotics and to drug metabolism. There is a wealth of evidence indicating that these enzymes are highly inducible and that they exist in multiple forms. The source of the diversity of P-450s and the mechanisms of their regulation have not yet been elucidated at the molecular level. The significance of the diversity and regulation of the P-450s in determining the response of individuals to specific carcinogens, drugs and xenobiotics has not been assessed. Accomplishment of the objectives described above should result in new insights into the molecular basis for the diversity of P-450s and into the molecular mechanisms of regulation of P-450 gene expression. This will result in a better understanding of the balance between activation and detoxification of carcinogens, of the influence of this system on drug action and of other questions in carcinogenesis, toxicology and pharmacology. Besides being of practical interest in the fields of cancer biology and toxicology, the P-450 system is also interesting on a more fundamental level as a system for studying the regulation of expression of a family of genes. Thus, the work with this system should contribute to the basic understanding of eukaryotic gene structure and regulation.

Proposed Course:

- 1) Continue analysis of the structure of the P-450-M gene. Those regions of the P-450-M gene containing coding sequences for a second mRNA will be sequenced. The 5' end of the transcribed region of the P-450-M gene will be mapped by primer extension and S-1 mapping. The stretch of DNA upstream of the of the 5' end of the P-450 gene will be sequenced as the first step in locating possible regulatory sequences associated with this gene.
- 2) The cloning and initial characterization of the -naphthoflavone induced P-450 (P-450-BNF-B) and isosafrole induced P-450 (P-450-ISF-G) genes will be completed.
- 3) The structure of these genes will be analyzed in detail. This will include sequence analysis of selected regions and analysis of gene organization and copy number by hybridization of subcloned fragments of these genes to Southern transfers of genomic DNA and Northern transfers of RNA isolated from control and MC-treated rats.
- 4) The three P-450 genes will be compared by Southern transfer and hybridization. This will provide evidence concerning the existence and extent of homology among these genes and provide an indication of the relatedness of these genes.

5) The rates of transcription of P-450 genes in livers from control and MC-treated rats will be determined by measuring  $^{32}\text{P}$  incorporation into nascent mRNA transcripts in vitro in isolated nuclei.

6) In order to functionally identify and to characterize the properties of regulatory (especially promoter) regions associated with the three P-450 genes that we have isolated we will a) identify possible regulatory regions of these genes based on sequence information and on their location relative to structural regions of the P-450 genes; b) introduce these putative regulatory regions into the pSV0-CAT vector of Gorman, Moffat and Howard in such a way that these regions can control chloramphenicol acetyl transferase (CAT) gene expression; and c) introduce these recombinants into cells in culture by the calcium phosphate precipitate technique of DNA mediated gene transfer and test the ability of these putative regulatory sequences to modulate the expression of CAT. During the upcoming year we will construct these recombinants and carry out preliminary expression experiments. In depth regulatory studies with these recombinants, including site-specific mutagenesis experiments to delineate detailed correlations between regulatory activity and specific bases within the putative P-450 regulatory regions, will be carried out in the subsequent year.

#### Publications:

Gozukara, E. M., Fagan, J. B., Pastewka, J. V., Guengerich, P. F. and Gelboin, H. V.: Induction of cytochrome P-450 mRNAs quantitated by in vitro translation and immunoprecipitation. Arch. Biochem. Biophys. (In Press)

Park, S. S., Fagan, J. B., Fujino, T., Friedman, F., Song, B. J., Park, K.H., Miller, H., West, D., Pastewka, J., Robinson, R. C., and Gelboin, H. V.: The use of monoclonal antibodies in identification of mRNA translation products and cloned cDNA. In Han, M. H. (Ed.): Symp. on Genetic Eng. and Biotech. Seoul, Korea, KAIST (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CE05204-04 LMC

PERIOD COVERED

October 1, 1983 through September 30, 1984

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

DNA Sequence Alterations In Vivo Following DNA Modification by Carcinogens

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

PI: Michael Seidman Senior Staff Fellow LMC NCI  
 Other: Sekhar Chakrabarti Visiting Fellow LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Bacterial plasmids have been used to study repair and mutagenesis of benzopyrene diol epoxide (BPDE)-damaged DNA in *E. coli*. In "nontargeted" experiments, the plasmids were randomly modified by BPDE and introduced into *E. coli* strains which differed in their capacity for repair and mutagenesis. By measuring both the survival of bacteria containing plasmids and the mutagenesis of a plasmid gene, it was possible to identify host cell functions for error-free repair and for mutagenesis. It was found that repair functions can be distinguished temporally from mutagenic activities after induction of the inducible repair response (SOS). In "targeted" experiments, a specific fragment of the plasmid from a nonessential marker gene was modified with BPDE and ligated back into the plasmid. The survival curves of these constructs were virtually identical to those of the randomly modified plasmids, suggesting that the principal determinant for survival of BPDE-damaged DNA is the simple presence of the carcinogen, rather than secondary mutational events in essential functions. Furthermore, plasmid survival was a function of the adduct/plasmid, not the adduct/nucleotide, ratio. Mutants were found in the targeted regions but not in another nontargeted gene, indicating that mutagenesis is targeted. A collection of these mutants has been sequenced and transitions, transversions, and frame shift mutations have been identified.

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

Michael Seidman	Senior Staff Fellow	LMC	NCI
Sekhar Chakrabarti	Visiting Fellow	LMC	NCI

Objectives:

To study repair and mutagenesis of carcinogen modified DNA, to distinguish direct from indirect effects, and to identify the cellular processes involved.

Methods Employed:

Bacterial plasmids were constructed which have marker genes (chloramphenicol resistance, galactokinase, tetracycline resistance) as well as functions required for replication and ampicillin resistance. In the targeted experiments, fragments from either the tetracycline gene, in one case, or galactokinase in another, were covalently modified with benzopyrene diol epoxide (BPDE) and then ligated back into the remainder of the unmodified plasmid. The randomly modified and target modified plasmids were used to transform *E. coli* strains which are defective in in specific repair and/or mutagenesis gene functions. The number of bacteria which survived on ampicillin medium were counted and the number of mutants at the appropriate locus were determined. These data were plotted as a function of the amount of BPDE on the plasmid. Selected mutants were sequenced.

Major Findings:

1) Mutagenesis in this system is targeted. Mutations arose in the targeted regions, not in other undamaged regions of the plasmids. The mutations generated by BPDE damage of DNA include transitions, transversions, and frame shifts. 2) After inducible repair response (SOS) induction (by UV light) of recipient bacteria, functions for survival (error-free repair) and mutagenesis were induced above constitutive levels. The uvrA gene-dependent activity rose and fell in the first 30 minutes after induction, while the mutagenesis activity was fully induced only 60 minutes after induction. Thus, the two functions could be distinguished temporally. 3) Survival of BPDE-modified plasmids was largely a function of the presence of the carcinogen and not due to secondary effects such as mutations in essential functions. The survival of BPDE plasmids of different sizes was found to be a function of the adduct/ plasmid, not the adduct/nucleotide ratio. Since plasmid mortality is due to interference of replication by the BPDE adducts, this finding indicates that once a modified plasmid begins replication the probability of the replication fork reaching an adduct is much greater than the probability of adduct repair.

Significance to Biomedical Research and the Program of the Institute:

The study provides a direct estimate of the consequences of covalent modification of DNA by chemical carcinogenesis. The cellular processes involved in carcinogen-induced mutagenesis could be further characterized with this approach.

Proposed Course:

This project will not be continued.

Publications:

Chakrabarti, S., Mizusawa, H. and Seidman, M.: Survival and mutagenesis of bacterial plasmids with localized carcinogen adducts. Mutat. Res. 126: 127-137, 1984.

Mizusawa, H., Chakrabarti, S. and Seidman, M.: Temporal distinction between repair and mutagenesis of benzo pyrene (a) adducts after SOS induction in Escherichia coli. J. Bacteriol. 156: 926-930, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05208-04 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Phenotyping of Cytochrome P-450 in Human Tissues Using Monoclonal Antibodies

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

PI:	Tadahiko Fujino	Expert	LMC	NCI
Others:	Sang S. Park	Senior Staff Fellow	LMC	NCI
	Harry V. Gelboin	Chief	LMC	NCI
	Byung-Joon Song	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

Roswell Park Memorial Institute, Buffalo, N.Y. (H. L. Gurtoo); University of Colorado, Denver, CO. (K. Gottlieb); University of Colorado, Denver, CO. (D. Manchester); Hebrew University, Jerusalem, Israel (C. Kapitulnik); University of Tel Aviv, Tel Aviv, Israel (H. Slor)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

Monoclonal antibodies (MABs) were used to study the diversity of cytochromes P-450 in human tissues such as placenta and lymphocytes. The MABs were used to study P-450 dependent aryl hydrocarbon hydroxylase (AHH) and ethoxycoumarin deethylase (ECD) and determine the contribution of epitope specific P-450s to each reaction in each tissue studied. This approach can be used as the basis for a general method for phenotyping the diversity of P-450s in human tissues.

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

Tadahiko Fujino	Expert	LMC	NCI
Sang S. Park	Senior Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Byung-Joon Song	Visiting Fellow	LMC	NCI

Objectives:

Differences in the profile of cytochromes P-450 present in different tissues may account for differences in metabolism that result in either the carcinogenic activation, or the detoxification of drugs, mutagens, and potential carcinogens. We are using cytochrome P-450-specific monoclonal antibodies (MAbs) to investigate the diversity and multiplicity of these enzymes by 1) development of a phenotypic description of the number and quantity of cytochromes P-450 in tissues and individuals, 2) determination of their role in the detoxification or activation of specific xenobiotics, 3) determination of their role in individual variations in drug and carcinogen responsiveness, and 4) examination of the complex interactions and influence of hereditary and environmental factors through an investigation of twin placentas.

Methods Employed:

MAbs were obtained from hybridomas made by the fusion of myeloma cells and spleen cells derived from BALB/c mice that had been immunized with methylcholanthrene (MC)-induced rat liver cytochrome P-450. Human monocytes and lymphocytes were isolated from peripheral blood and treated with benzanthracene. Human placental microsomes were prepared from single birth and twin birth placentas from women who are smokers and non-smokers. Aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (ECD) activities were measured by a fluorometric assay after incubation with MAbs. The effects of MAbs on the metabolism of benzo(a)pyrene by cytochrome P-450 were also studied by high pressure liquid chromatography (HPLC).

Major Findings:

Cytochromes P-450 are a family of enzymes largely responsible for the metabolism of xenobiotics such as drugs, carcinogens and environmental pollutants as well as physiological compounds including steroids, prostaglandins and fatty acids. We prepared a monoclonal antibody (Mab 1-7-1) to a polycyclic hydrocarbon-induced rat cytochrome P-450 that antigenically defines and inhibits a type of cytochrome P-450 responsible for aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (ECD) activity in human placenta. We examined the placentas from single and twin births from mothers who smoked cigarettes and from non-smokers. The Mab 1-7-1 inhibited the smoking-induced AHH activity of essentially the entire population of placentas by 70-95%. Thus, up to 95% of the AHH in a population of human placentas is catalyzed by a type of cytochrome P-450 that contains an antigenic site recognized by Mab 1-7-1. A second type of cytochrome P-450, which is insensitive to Mab 1-7-1, is responsible for the ECD activity in the placentas of



non-smokers. In the placentas from smokers, both types of P-450 contribute to ECD activity. Their ratios can be determined by the amount of inhibition by MAb 1-7-1 which ranges from 0-70%. The placentas from both dizygotic and dichorionic monozygotic twins showed extraordinarily high intratwin concordance for both the absolute amounts of AHH and ECD and their inhibition by MAb 1-7-1 compared to unrelated individuals, indicating that interindividual differences in these parameters of biological activity are not due to random variability or experimental error. Our results show that the content of antigenically unique types of cytochrome P-450 responsible for different drug and carcinogen reactions can be measured in different individuals by the amount of their inhibition by highly specific MAbs. These findings may have general application to studies on the relationship of cytochrome P-450 phenotype to population differences in drug and carcinogen biotransformation.

#### Significance to Biomedical Research and the Program of the Institute:

Monoclonal antibodies provide us with the ability to analyze the complicated phenomenon of polycyclic hydrocarbon metabolism from a new perspective. Use of monoclonal antibodies to cytochrome P-450 should identify those P-450 species involved in carcinogen activation and clarify the metabolic pathways responsible for chemical carcinogenesis. Knowing the enzymatic steps leading to the activation of the carcinogen will be useful for the detection and prevention of chemical carcinogenesis.

#### Proposed Course:

Additional MAbs will be developed as a tool for the study of human cytochromes P-450. These might recognize the different antigenic determinants of the multiple forms of cytochrome P-450. MAbs will help define catalytic specificities of the various cytochromes P-450 by studying their inhibitory effects on metabolism of substrates other than benzo(a)pyrene and 7-ethoxycoumarin. MAbs may be utilized to phenotype cytochromes P-450 in tissues, organs and individuals. Such information is useful in determining the relationship between cytochrome P-450, drug activation, and susceptibility to chemical carcinogenesis.

#### Publications:

Fujino, T., Gottlieb, K., Manchester, D. K., Park, S. S., West, D., Gurtoo, H. L., and Gelboin, H. V.: Monoclonal antibody phenotyping of inter-individual differences in cytochrome P-450 dependent reactions of single and twin human placenta. Cancer Res. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05240-03 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Rearrangement of a Plasmid Sequence in Mammalian Cells

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

PI:	Michael Seidman	Senior Staff Fellow	LMC	NCI
Others:	Abdur Razzaque	Visiting Fellow	LMC	NCI
	Sekhar Chakrabarti	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

A shuttle vector plasmid has been constructed which can be used to study gene stability, rearrangement, and recombination in mammalian cells. The plasmid contains sequences derived from a bacterial plasmid, from SV40 virus, and a marker gene, galactokinase, which can be scored in the appropriate bacterial host. This construct replicates in mammalian cells and bacteria. An experimental protocol was designed in which mammalian cells were infected with the plasmid, replication permitted and then the plasmid DNA extracted from the cells. After purification and elimination of residual infectious DNA, the plasmid was introduced into a bacterial host which permitted the detection of the presence or absence of a functional galactokinase gene. With this assay it was possible to assess quantitatively the stability of the plasmid in the mammalian cells. It was found that approximately 1% of the progeny plasmids had lost a functional marker gene. The defective plasmids contained point mutations, deletions, and insertions of cell DNA. The hypermutagenesis was found with different methods of DNA infection. Further studies showed that the mutagenesis was a property of the plasmid sequences, and was not shared by cellular genes. The plasmid mutagenesis occurs largely before plasmid replication begins, although there is some postreplication mutagenesis.

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

Michael Seidman	Senior Staff Fellow	LMC	NCI
Abdur Razaque	Visiting Fellow	LMC	NCI
Sekhar Chakrabarti	Visiting Fellow	LMC	NCI

Objectives:

To study DNA sequence rearrangement in mammalian cells and to identify DNA sequences and gene functions involved in DNA rearrangement and recombination.

Methods Employed:

A shuttle vector was constructed which consisted of sequences from the bacterial plasmid, pBR322, which confer ampicillin resistance and a bacterial origin of replication, the early genes and origin of replication from SV40 virus which permit replication in permissive mammalian cells, and a marker gene for the enzyme galactokinase, which can be scored in the appropriate *E. coli* strain. After DNA infection and replication in the mammalian cells, the plasmid DNA is extracted from the cells and purified. It is then treated with a restriction enzyme which cleaves only the infectious DNA so that only the plasmid molecules which replicated in the mammalian cells are introduced into the bacterial strain, which is galactokinase negative. A comparison of the total bacterial colonies and those which are galactokinase negative is made and a mutation frequency for the galactokinase gene in mammalian cells is calculated. By using constructs with direct repeated sequences flanking the marker, it is possible to study homologous recombination by screening for plasmids with the precise deletion of the intervening sequence.

Major Findings:

There was a significant level of spontaneous mutagenesis of the plasmid. The majority of the mutagenesis occurred before the replication of the plasmid, although it is clear that mutations may arise at any time during the replication period. The defective plasmids contained point mutations, deletions, and insertions of cell DNA. The mutagenesis appears to be restricted to the transfected DNA, as cell genes were not mutagenized. Plasmids were constructed to study recombination between direct repeated sequences flanking the marker gene. Recombination produced mutant plasmids with specific deletions which could be readily detected in the general mutant population. When these experiments were done in an SV40 transformed monkey cell line 1% of the progeny plasmids were recombinant molecules. The frequency of these molecules in the plasmid population harvested from normal monkey cells was five-fold lower.

Significance to Biomedical Research and the Program of the Institute:

DNA transfection techniques are widely used in current molecular and cell biology programs, including studies designed to detect and characterize oncogenes. Our

results indicate that spontaneous mutagenesis of the DNA used in these experiments does occur at high frequency and must be considered when interpreting data from transfection experiments.

Proposed Course:

Most of the spontaneous deletion-insertion mutagenesis of the shuttle vector occurs before replication of the plasmid. A vector which permits the survival of only those deletions which occur on replicating plasmids and their progeny has been constructed. This vector will be used to study the influence of DNA damaging agents and special DNA sequences (Z-DNA, etc) on the rearrangement of replicating and progeny plasmid mini-chromosomes. Similar experiments will be performed with vectors designed to study recombination on replicating and progeny plasmid minichromosomes.

Publications:

Razzaque, A., Chakrabarti, S., Joffe, S. and Seidman, M.: Mutagenesis of a shuttle vector plasmid in mammalian cells. Mol. Cell. Biol. 4: 435-441, 1984.

Razzaque, A., Mizusawa, H. and Seidman, M.: Rearrangement and mutagenesis of a shuttle vector plasmid after passage in mammalian cells. Proc. Natl. Acad. Sci. USA 80: 3010-3014, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05241-03 LMC

PERIOD COVERED  
 October 1, 1983 to September 30, 1984

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

**Role of Left-Handed Helical DNA in Carcinogenesis**

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

PI: T. Kakunaga Head, Cell Genetics Section LMC NCI

Others: H. Ueyama Visiting Fellow LMC NCI  
 H. Hamada Guest Researcher LMC NCI  
 S. Taniguchi Visiting Fellow LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.9

PROFESSIONAL:

1.6

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Purine-pyrimidine alternating sequences, such as (dC-dG)n·(dC-dG)n and (dT-dG)n·(dC-dA)n, take a right-handed helical conformation, mostly B-DNA, or a left-handed helical conformation called Z-DNA, depending on the buffer conditions. We have previously found evidence for the abundant existence of such Z-DNA-forming sequences in the natural genome by hybridization and by direct DNA sequencing. Z-DNA formation by the (dT-dG)n·(dC-dA)n sequence was investigated by determining the sites which are sensitive to S1-nuclease in a plasmid containing the Pst 0.9 Kb fragment of the human cardiac actin gene (including the (dT-dG)25·(dC-dA)25 sequences. Plasmid DNAs of five different supercoilings were separately obtained by using a nicking-closing enzyme in combination with different amounts of ethidium bromide. Both single strand scission and double strand scission were induced by S1-nuclease in plasmids with superhelicity greater than 0.06, indicating that the sensitivity to S1-nuclease is supercoiling dependent. Both single and double strand incisions occurred at 3 to 5 bases from the end of the (dT-dG)25·(dC-dA)25 sequence. These results indicate that S1-nuclease incises the DNA strand near the junction between the B- and Z- forms of DNA, 3-5 bases outside of Z-DNA sequences.

In order to investigate the possible role of Z-DNA in the regulation of gene expression, the (dT-dG)n·(dC-dA)n sequence was inserted into a pSV2-CAT plasmid vector and a c-Ha-ras-1 clone. pSV2-CAT is a recombinant plasmid containing the chloramphenicol acetyltransferase (CAT) gene. Insertion of the Z-DNA sequence resulted in a significant enhancement of expression of the CAT gene and the trans-forming potentials of the c-Ha-ras-1 gene when they were transfected into mammalian cells.

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

T. Kakunaga	Section Head	LMC	NCI
H. Ueyama	Visiting Fellow	LMC	NCI
H. Hamada	Guest Researcher	LMC	NCI
S. Taniguchi	Visiting Fellow		

Objectives:

To determine whether the  $(dT-dG)_{25} \cdot (dC-dA)_{25}$  sequence which was found in nature takes the Z-conformation and to investigate the role of Z-DNA sequences in the regulation of gene expression, in cell differentiation, and in carcinogenesis.

Methods Employed:

Singleton et al. (Nature 299: 272-316, 1982) reported that the regions around the poly(dG-dC) sequence in negatively supercoiled plasmids are sensitive to  $S_1$ -nuclease, and suggested that the poly(dG-dC) sequence forms a Z-structure in negatively supercoiled plasmids. However, since the poly(dG-dC) sequence may adopt a cruciform structure which is also sensitive to  $S_1$ -nuclease, it was necessary to determine whether the  $S_1$ -nuclease cuts at the boundary region near the poly(dT-dG) sequence which does not adopt the cruciform configuration. pPst 0.9 DNA, a plasmid DNA containing the Pst 0.9-kb fragment of the human cardiac actin gene (including the  $(dT-dG)_{25}$  sequence), was treated with a nicking-closing enzyme in combination with different amounts of ethidium bromide, and DNAs of five different supercoillings (0, -0.03, -0.06, -0.01 and -0.02) were separately obtained. To determine the cleavage sites, the  $S_1$ -nuclease-cut DNAs were digested with both EcoRI and BamHI, and analyzed on an agarose gel (for double-strand cleavage sites) and an alkaline agarose gel (for single-strand cleavage sites).

For examination of the possible role of Z-DNA in the regulation of gene expression, the  $(dT-dG)_n \cdot (dC-dA)_n$  sequence or human DNA fragments containing the  $(dT-dG)_n \cdot (dC-dA)_n$  sequence were inserted into the pSV2-CAT plasmid vector and the human c-Ha-ras-1 clone. pSV2-CAT is a recombinant plasmid consisting of an ampicillin resistance gene and a replication origin from pBR322, a replication origin and poly(A)-addition signal sequence from SV-40 and the chloramphenicol acetyltransferase (CAT) gene from Tn 5 (Gorman et al., Proc. Natl. Acad. Sci. USA 79: 6777-6781, 1982). The potential Z-DNA sequences with or without flanking sequences were inserted at a BamHI site of pSV2-CAT in both orientations and designated pSV2-CAT Z. The expression of CAT activity was measured when the recombinant DNA constructs were transfected into monkey cells. The human c-Ha-ras-1 clones used contained a normal human gene homologous to the ras oncogene of Harvey murine sarcoma virus with or without LTR sequences derived from Harvey murine sarcoma virus. The potential Z-DNA sequences with or without flanking sequences were inserted at a BamHI site of the c-Ha-ras-1 clones in both orientations. The stable transformation frequencies were measured when the recombinant DNA constructs were transfected into NIH/3T3 cells.

Major Findings:

The pursuit of this project has led to major new findings in three pertinent areas: 1) demonstration of the presence of a Z-DNA sequence,  $(dT-dG)_n \cdot (dC-dA)_n$ , as a moderately repeated element in eukaryote genomes; 2) new information concerning the Z-conformation of the  $(dT-dG)_n \cdot (dC-dA)_n$  sequence; 3) demonstration of the ability of the  $(dT-dG)_n \cdot (dC-dA)_n$  sequence to enhance gene expression in mammalian cells.

Both single strand scission and double strand scission were induced by  $S_1$ -nuclease in plasmids with superhelicity greater than 0.06, indicating that the sensitivity to  $S_1$ -nuclease is supercoiling dependent. The site of scission was determined to be 3 to 5 bases from the end of the  $(dT-dG)_{25}$  sequence. These results provide evidence that the  $(dT-dG)_n$  sequence takes a Z-form in negatively supercoiled DNA under physiological salt conditions.

All pSV2-CAT Z DNAs produced increased (2.5 - 4 times) CAT activity in the transfected cells in comparison to pSV2-CAT DNA. There were no significant differences in the amount or topological status of the incorporated plasmid DNA in cells transfected with pSV2-CAT or with pSV2-CAT Z. On the other hand, the insertion of a 1.0 - 1.5 Kb BamHI DNA fragment, randomly isolated from the human genome but without a  $(dT-dG)_n$  sequence, into the same site of pSV2-CAT did not result in increased CAT activity in the transfected cells. Similar results were obtained whether the recipient cells were CV-1 or COS-1 cells. COS-1 is a SV40-transformed CV-1 cell line in which the transfected pSV2-CAT DNA can replicate. These results clearly indicate that the insertion of the  $(dT-dG)_n$  sequence into pSV2-CAT resulted in enhanced expression of the CAT gene. This enhancement of expression by the  $(dT-dG)_n$  sequence is similar to the increased transcription produced by viral enhancers, since 1) it works at a location distant from the promoter and 2) the orientation of the  $(dT-dG)_n$  sequence is not critical to its enhancement.

Furthermore, insertion of  $(dT-dG)_{25}$  into human c-Ha-ras-1 clones resulted in an increased transformation frequency after transfection into NIH/3T3 cells. In contrast to the expression of CAT gene, the orientation of the  $(dT-dG)_{25}$  sequence affected its enhancement.

Significance to Biomedical Research and the Program of the Institute:

It was of great importance to know whether the stretches of alternating purine and pyrimidine nucleotides (the sole possible Z-DNA sequences) occur naturally, and how frequently and how widely they exist. This study is the first to show that one of the potential Z-DNA sequences,  $(dT-dG)_n$ , is randomly dispersed in all eukaryotic genomes examined. Until this study, the research on Z-DNA was concentrated on the use of synthetic poly(dG-dC)·poly(dG-dC). This study has shown that  $(dG-dC)_n$  sequences exist in eukaryotic cells but at a much lower frequency than  $(dT-dG)_n$  sequences and that a few  $(dG-dC)_n$  sequences are present in some eukaryotic cells. Thus this study has provided evidence suggesting the greater importance of  $(dT-dG)_n$  sequences as possible Z-DNA sequences than that of  $(dG-dC)_n$ .

Another important question which has been raised concerning Z-DNA is its biological significance. Although the potential Z-DNA conformation has been suggested for short stretches of purine-pyrimidine alternating sequences in viral enhancers, there was no constructive evidence for the enhancer activity of the potential Z-DNA sequences. The clear demonstration of enhancing effects of the  $(dT-dG)_n$  sequence on expression of CAT activity and transforming activity of the human c-Ha-ras obtained in this study provides an important clue for clarifying the role of DNA conformation in the regulation of expression of genes, including oncogenes. The clarification of the role of DNA conformation in the regulation of gene expression has been a crucial step forward in understanding mechanisms of carcinogenesis.

Proposed Course:

The course, as outlined in the previous sections, will be pursued and the results published.

Publications:

Kakunaga, T.: Z-DNA sequences. J. Mod. Chem. (In Press)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05242-03 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Monoclonal Antibody Mapping of Cytochromes P-450 in Different Species and Tissues

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

PI:	Tadahiko Fujino	Expert	LMC	NCI
Others:	Harry V. Gelboin	Chief	LMC	NCI
	Sang S. Park	Senior Staff Fellow	LMC	NCI
	Byung-Joon Song	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

Panels of monoclonal antibodies (MABs) to different forms of cytochrome P-450 have been used to identify the class of P-450 that is responsible for the catalysis of several different drug and carcinogen reactions. These include aryl hydrocarbon hydroxylase (AHH), ethoxycoumarin deethylase (ECD) and ethyl morphine deethylase (EMD) and benzphetamine demethylase (BPD). Several of the MABs inhibit these reactions to different extents in the tissues of different species and strains of rodents. These MABs can thus be used to prepare an atlas of P-450s responsible for various drug and carcinogen reactions in different tissues, strains and species. This approach represents a general method for phenotyping individuals for multi-enzyme systems.

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

Tadahiko Fujino	Expert	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Sang S. Park	Senior Staff Fellow	LMC	NCI
Byung-Joon Song	Visiting Fellow	LMC	NCI

Objectives:

In order to understand the detailed genetics and the role of cytochrome P-450 in carcinogen and drug metabolism, a phenotypic description of the number and quantity of P-450s in various species and tissues is necessary and is examined in this project.

Methods Employed:

Monoclonal antibodies were prepared by the general methods of Kohler and Milstein. Microsomes from different species and tissues, i.e., rat, mouse, hamster, and guinea pig were collected. The genetically defined mouse strains C57BL/6 and DBA/2 were included. Aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase were measured by fluorometric assays.

Major Findings:

1) Monoclonal antibodies (MAbs) to 3-methylcholanthrene (MC)-induced rat liver P-450 inhibit all four enzyme activities, aryl hydrocarbon hydroxylase (AHH) ethoxycoumarin deethylase (ECD) and ethyl morphinedeethylase (EMD) and benzphetamine demethylase (BPD). 2) The MAbs distinguish the contribution of the epitope specific P-450 for each of these reactions in rat liver, lung, and kidney as well as these tissues in DBA mice, C57B1/6 mice, guinea pigs, and hamsters. 3) An epitope specific P-450 atlas has been constructed for the P-450 dependent enzyme activities described above. 4) The pulmonary AHH activities of both C57B1/6 and DBA/2 mice behaved differently from hepatic AHH activities; both were inhibited 80%. 5) Pulmonary 7-ethoxycoumarin deethylase activity was inhibited less than the liver activity in both C57B1/6 and DBA/2 mice. 6) AHH activity in livers of guinea pigs and hamsters were inhibited, but the corresponding ECD activity was unaffected. Preliminary experiments have been done with genetically hybrid mice which indicate MAb sensitive dominance of certain P-450 forms.

Significance to Biomedical Research and the Program of the Institute:

An atlas of cytochromes P-450 classified by MAbs may lead to a better understanding of the multiplicity of the cytochromes P-450, their genetic control, and their relationship to drug and carcinogen metabolism, and to individual differences in rates of drug metabolism and carcinogen sensitivity.

Proposed Course:

Tissue microsomes from different species have been collected. Some crosses of genetically defined mouse strains will be investigated in order to clarify the involvement of genetic factors in the regulation of these systems. MAbs will be used to discriminate between different forms of cytochrome P-450 on the basis of their effects on enzymatic activities. We will make an atlas of the cytochromes P-450 using a library of MAbs to differentiate the different forms.

Publications:

Fujino, T., West, D., Park, S. S., and Gelboin, H. V.: Monoclonal antibody directed phenotyping of cytochrome P-450 dependent aryl hydrocarbon hydroxylase and 7-ethoxycoumarin deethylase in mammalian tissues. J. Biol. Chem. (In Press)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05317-02 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Opal Suppressor tRNA Genes in Human, Chicken, Rabbit and Xenopus Genomes

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

PI:	Dolph Hatfield	Research Biologist	LMC	NCI
Others:	Francine Eden	Expert	LMC	NCI
	Veronica O'Neill	Visiting Fellow	LMC	NCI
	Karen Pratt	NRSA Fellow	LMC	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Protein Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

4.0

## 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.)

Protein phosphorylation is important in many cellular processes, including oncogenic transformation. Opal suppressor tRNAs which have phosphoserine attached to them may insert phosphoserine directly into protein by suppressing termination codons. The structure and function of opal suppressor tRNA genes is being investigated by molecular cloning. The project is divided into three parts: 1) an evolutionary study of the sequence of opal suppressor tRNA genes and their flanking sequences in higher eucaryotes (i.e., human, rabbit, chicken, and Xenopus genomes); 2) *in vitro* transcription of these genes; and 3) site-specific mutagenesis in the promoter and anticodon regions of these genes. Under 1) above, a chicken opal suppressor gene was previously isolated and sequenced. It is encoded in an 87 base pair segment without intervening sequences and has an unusual 5' promoter region. Two human opal suppressor genes, one normal and one pseudogene, have been isolated, subcloned, and sequenced; three rabbit genes have been isolated subcloned, and are in the process of being sequenced; a second chicken gene has been isolated, subcloned, and is being sequenced; and a Xenopus gene has been isolated and is being subcloned for sequencing. Under 2) above, a transcription system has been prepared from Xenopus oocyte nuclei which is capable of transcribing the chicken and human genes. Under 3) above, we are synthesizing oligonucleotides 17 bases in length which correspond to the 5' internal promoter and to the anticodon regions except for having a single or double base change. The oligonucleotides will be used to make site-specific mutations in the gene.

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

Dolph Hatfield	Research Biologist	LMC	NCI
Francine Eden	Expert	LMC	NCI
Veronica O'Neill	Visiting Fellow	LMC	NCI
Karen Pratt	NRSA Fellow	LMC	NCI

Objectives:

The major goal of the project is to understand the structure and function of opal suppressor tRNA genes in higher vertebrates, and the role of these genes in protein phosphorylation.

Specific steps to achieve this goal are:

1) Isolate and characterize opal suppressor tRNA genes from chicken, human, rabbit, and *Xenopus* DNA libraries; 2) sequence the genes and their flanking DNA segments; 3) investigate the structure of the genomic regions that contain these genes with respect to transcription and evolutionary conservation; 4) study the control of transcription using in vitro and in vivo transcription systems; 5) use in vivo transcription systems to study processing and localization of the tRNA product; and 6) make site-specific mutations in the promoter and anticodon regions of tRNA genes in order to understand better their expression and cellular function.

Methods Employed:

The minor seryl-tRNAs are purified by DEAE-cellulose, BD-cellulose, and RPC-5 column chromatography and by two-dimensional polyacrylamide gel electrophoresis. Purified tRNAs are characterized by specific aminoacylation activity, codon recognition in a ribosome binding assay, and RPC-5 chromatography.

Purified tRNAs are labeled for use as probes by dephosphorylation with bacterial alkaline phosphatase and addition of  $^{32}\text{P}$  from gamma- $^{32}\text{P}$ -ATP using T4 polynucleotide kinase.

Genes corresponding to the tRNA probes are identified by hybridization to genomic blots of total DNA digested with restriction endonucleases. Genes are isolated from DNA libraries in bacteriophage lambda by plaque hybridization. The gene-containing segments are subcloned in the plasmid pBR322 for further analysis and for DNA sequencing by the method of Maxam and Gilbert.

DNA segments flanking the tRNA genes are subcloned in pBR322 after construction of the necessary restriction map. Subcloned segments are in turn used as probes against genomic DNA of the same or different species, or are used in in vitro transcription experiments.

In vitro transcription of the opal suppressor tRNA genes subcloned into pBR322 are investigated in *Xenopus* nuclei extracts.

#### Major Findings:

An opal suppressor tRNA purified from chicken liver has been successfully used as a  $^{32}\text{P}$ -labeled probe to isolate the corresponding genes from a library of chicken, human, rabbit, and *Xenopus* DNA cloned in bacteriophage lambda. The cloned chicken, human, and rabbit opal suppressor genes have been localized in the recombinant phage containing them by restriction analysis and subcloned in the plasmid pBR322. After further restriction analysis, a 540 bp chicken segment and a 300 bp human segment of the gene were sequenced by the method of Maxam and Gilbert. The genes are encoded in an 87 bp segment without introns. The CCA terminus of the mature tRNA is not encoded. The genes have a TCA anticodon, demonstrating that the mature tRNA would read the nonsense codon UGA. They contain an unusual internal 5' promoter region which differs from the consensus sequence for eucaryotic tRNAs by the presence of two extra bases. The 3' portion of the internal promoter and the termination signal are both normal. The chicken and human genes have a single base difference and show little homology in their 5' flanking sequences.

A second human gene has been isolated and sequenced. It does not contain a 3' end that would complement the 5' end in the mature tRNA and, therefore, is a pseudogene. Otherwise, it shows a high degree of homology to the normal human gene, differing in only positions 3 and 56. This gene has been inserted between two human Alu family genes in the human genome.

Three genes have been isolated from a rabbit DNA library. Preliminary sequencing of one of the genes suggests that it is a pseudogene. A second gene has been isolated from a chicken DNA library. It has been subcloned into pBR322 and a sequencing strategy is being determined. A 900 bp Bam HI-Ava I fragment of a *Xenopus* opal suppressor gene is being subcloned into pBR322 for sequencing.

Transcription experiments with the chicken gene have indicated that: 1) the gene is active but produces less product than other tRNA genes cloned similarly; 2) three specific transcripts are produced from the gene region; they are 90, 115, and 125 bases in length, respectively. The relationship between these transcripts is presently being determined.

#### Significance to Biomedical Research and the Program of the Institute:

The function of suppressor tRNAs which have phosphoserine attached to them is very unusual and apparently is unique in higher vertebrates. It is important to understand this special means of protein phosphorylation in light of new information relating protein phosphorylation directly to carcinogenesis.

#### Proposed Course:

- 1) Complete the sequencing of rabbit, chicken, and *Xenopus* opal suppressor genes;
- 2) determine where transcription of these tRNAs initiates, how processing proceeds, and where the final tRNA product is localized in the cell (nucleus vs.

cytoplasm); and 3) to make site-specific mutations in the promoter and anticodon regions of opal suppressor genes in order to understand better the expression and function of these genes.

Publications:

Hatfield, D., Dudock, B. and Eden, F.: Characterization and nucleotide sequence of a chicken gene encoding an opal suppressor tRNA and its flanking DNA segments. Proc. Natl. Acad. Sci. USA 80: 4940-4944, 1983.

Wilson, M. J. and Hatfield, D.: A review on "Incorporation of modified amino acids into protein in vivo." Biochim. Biophys. Acta 781: 205-215, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05318-02 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Immunopurification and Characterization of Cytochrome P-450

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

PI: Fred K. Friedman Senior Staff Fellow LMC NCI

Others: Kuo-Chi Cheng Visiting Fellow LMC NCI  
 Richard Robinson Biologist LMC NCI  
 Sang S. Park Senior Staff Fellow LMC NCI  
 Harry V. Gelboin Chief LMC NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Metabolic Control Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

1.5

## OTHER:

0.8

## CHECK APPROPRIATE BOX(ES)

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

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

The mixed-function oxidase system includes cytochromes P-450 that metabolize a variety of drugs and carcinogens. The multiple forms of this enzyme display broad, overlapping substrate specificity. The type and amount of each form varies among species and individuals. The focus of this project is the identification, characterization, and elucidation of structure-function relationships of these isozymes. Monoclonal antibodies (MABs) to specific cytochromes P-450 are an essential tool in these studies. Several cytochromes P-450 have been substantially purified in a one-step immunoabsorption procedure using Sepharose-bound MABs to the major forms of rat liver cytochrome P-450 induced by 3-methylcholanthrene and phenobarbital (MC-P-450, and PB-P-450, respectively). When mixed with solubilized rat liver microsomes, the immunoabsorbent based on MAB 1-7-1 to MC-P-450 binds two polypeptides of MW 56,000 and MW 57,000, while the immunoabsorbent made with MAB 1-31-2 to MC-P-450 binds only the MW 57,000 species. An immunoabsorbent with the MAB 2-66-3 to PB-P-450 adsorbs a species of MW 54,000. These polypeptides are readily desorbed by 0.1 M glycine (pH 3.0). Additional cytochromes P-450 have been purified with MABs 1-7-1 and 1-31-2 from livers of C57B1/6 and DBA/2 mice, guinea pigs and hamsters and from rat lung. Such immunoabsorption experiments based on different MABs reveal epitope relatedness between cytochromes P-450 in different tissues, strains, and species. Cytochromes P-450 isolated by this procedure, although enzymatically inactive, can be further analyzed structurally by peptide mapping and amino acid analysis. Similarities as well as differences in the primary structure of several P-450s were found. In addition we are developing methods for elution of catalytically active cytochrome P-450 from immunoabsorbents.



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

Fred K. Friedman	Senior Staff Fellow	LMC	NCI
Kuo-Chi Cheng	Visiting Fellow	LMC	NCI
Richard Robinson	Biologist	LMC	NCI
Sang S. Park	Senior Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

To identify, purify, and characterize the multiple forms of cytochrome P-450. Monoclonal antibodies (MAbs) are utilized as highly specific reagents for recognition of individual cytochromes P-450.

Methods Employed:

MAbs were prepared to several liver microsomal cytochromes P-450 from rats treated with inducing agents such as 3-methylcholanthrene (MC) and phenobarbital (PB). The MAbs were covalently linked to Sepharose to yield an immunoabsorbent. This resin was combined with microsomes from various tissues and species, and the proteins bound to the column characterized upon elution from the resin. Analytical methods employed include gel electrophoresis, spectral analyses, and measurements of aryl hydrocarbon hydroxylase activity (AHH).

Major Findings:

Spectral and SDS-gel analyses demonstrate that a fraction of the total cytochrome P-450 in microsomes from MC- and PB-treated rats binds tightly to the immunoabsorbents used. Acid conditions (pH 3) are necessary to elute bound P-450. Cytochromes P-450 were purified using Sepharose immunoabsorbents based on the MAbs 1-7-1 and 1-31-2, both to MC-induced P-450; and on MAb 2-66-3 to PB-induced P-450. Sepharose-MAB 1-7-1 binds two species of MW 56,000 and 57,000 from rat MC-microsomes, while Sepharose-MAB 1-31-2 binds only the species of MW 57,000. The latter P-450 therefore contains the epitopes recognized by both MAB 1-7-1 and MAB 1-31-2, while the MW 56,000 species only contains the MAB 1-31-2 specific epitope. Sepharose-MAB 2-66-3 selectively binds an additional P-450 of MW 54,000 from PB microsomes. Additional P-450s were purified with Sepharose bound to MAbs 1-7-1 and 1-31-2 from MC-induced livers of C57B1/6 and DBA/2 mice, hamsters, and guinea pigs. A rat lung P-450 has also been isolated.

The purified P-450s were analyzed for structural homology by peptide mapping studies with SDS-gels and HPLC. The data revealed some similarities as well as differences in the primary structure of several P-450s.

We have modified our immunopurification procedure, which denatures the P-450 during desorption in acid, to obtain an enzymatically active species. When inactive enzyme was added to a Sepharose MAB 1-7-1-P-450 complex, active P-450 desorbed from the immunoabsorbent as measured by AHH activity.

Significance to Biomedical Research and the Program of the Institute:

Purification of individual cytochrome P-450 by a simplified procedure using specific MAbs offers a new approach to studying their multiplicity. Cytochrome P-450 isozymes obtained in the manner from different tissues and species can be further characterized. The methodologies developed can then be applied to human tissues. The relationship of type and amount of cytochrome P-450 to drug and carcinogen metabolism can then be assessed.

Proposed Course:

Purification will proceed from an analytical to a preparative scale. The individual forms of cytochromes P-450 will then be subjected to various physical and chemical structural studies.

Efforts to obtain catalytically active P-450s will be expanded, since characterization of activities is most relevant to our goal of relating isozyme phenotype to metabolic activity. Such detailed characterization should aid in gaining further insight into the role of cytochrome P-450 multiplicity in the metabolism of various drugs and carcinogens. Some emphasis will be placed on isolating P-450s from human tissues.

Publications:

Friedman, F. K., Robinson, R. C., Park, S. S. and Gelboin, H. V.: Monoclonal antibody-directed immunopurification and identification of cytochromes P-450. Biochem. Biophys. Res. Commun. 116: 859-865, 1983.

Friedman, F. K., Park, S. S., Fujino, T., Song, B. J., Robinson, R. C., West, D., Radkowsky, A. K., Miller, H. and Gelboin, H. V.: Phenotyping of cytochrome P-450 dependent drug and carcinogen metabolism with monoclonal antibodies to cytochrome P-450. Toxicol. Pathol. Suppl. (In Press)

Gelboin, H. V., Park, S. S., Fujino, T., Song, B. J., Robinson, R. C. and Friedman, F. K.: Monoclonal antibody directed phenotyping, radioimmunoassay and purification of cytochromes P-450 that metabolize drugs and carcinogens. In Singer, T. P., Mansour, T. E. and Ondarza, R. N. (Eds.): Mechanism of Drug Action. New York Academic Press, 1983, pp. 259-276.

Gelboin, H. V., Fujino, T., Song, B. J., Park, S. S., Cheng, K. C., West, D., Robinson, R., Miller, H. and Friedman, F. K.: Monoclonal antibody-directed phenotyping of cytochromes P-450 by enzyme inhibition, immunopurification and radioimmunoassay. In Omenn, G. and Gelboin, H. V. (Eds.): The Role of Genetic Predisposition in Response to Chemical Exposure, Banbury Report. New York, Cold Spring Harbor Laboratory, 1984, pp. 65-84.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05320-02 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Regulation of Oncogene Expression

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

PI: T. Kakefuda Medical Officer LMC NCI

Others: S. Koizumi Guest Worker LMC NCI  
 H. Imai Visiting Fellow LMC NCI

COOPERATING UNITS (if any) Laboratory of Molecular Microbiology, NIAID, NIH, (Y. Ito) and National Institute of Industrial Health, Tokyo, Japan (M. Kimura)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

(1) Recombinant plasmids for the expression of polyoma virus middle tumor antigen (MT) were constructed in order to obtain a large quantity of MT and to study its properties. To achieve a high level of expression of the MT gene in *E. coli*, a strong promoter, PL, of lambda phage, whose activity is controlled by the temperature sensitive cI gene product was used. Immunoprecipitable proteins of 50K, 53K, and 55K molecular weight were produced and the 53K and 55K proteins were identified as MT by MT specific monoclonal antibodies. They did not react with large T-specific monoclonal antibodies. [<sup>35</sup>S]methionine-labeled 55K protein is indistinguishable in peptide mapping from that of authentic middle T antigen. (2) Based on a hypothesis that metallothionein (MET) or metal-free thionein (apothionein) plays a role in the regulation of the expression of the MET gene, studies are progressing to observe the interaction of apothionein with the control region of the human MET gene. Plasmid DNA containing the human MET gene and apothionein from HeLa cells was isolated. Interactions between apothionein and DNA are studied by DNA affinity column chromatography, DNase protection studies and in vitro transcription experiments. (3) Plasmid vectors containing bovine papilloma virus (BPV), MET promoter-operator regions and mouse beta-interferon gene coding regions are also constructed in such a way that metal-inducible interferon may interact with BPV transforming activity in the cells transfected with the cloned plasmid. These studies will give insight into the molecular and genetic mechanisms of gene expression associated with the oncogenic processes.

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

T. Kakefuda	Medical Officer	LMC	NCI
S. Koizumi	Guest Worker	LMC	NCI
H. Imai	Visiting Fellow	LMC	NCI

Objectives:

1) To observe the molecular and genetic mechanisms of malignant transformation by active expression of transforming genes of viral and cellular origins; 2) to overproduce oncogene products for their characterization; and 3) to establish experimental model systems for the study of the control mechanisms of gene expression and suppression associated with malignant transformation.

Methods Employed:

1) A segment of lambda phage DNA containing cII and O-gene was inserted downstream from the PL promoter. The polyoma virus middle tumor antigen (MT) coding sequence, modified to encode only MT (from Dr. R. Kamen, Genetics Institute, Boston, Massachusetts), was fused to the amino terminal region of the lambda O-gene and then cloned in the pBR322 vector. This plasmid was inserted into a cI857 lysogen of *E. coli* which contains a temperature sensitive mutation in the cI gene. A temperature shift from 32°C to 42°C activated the PL promoter and initiated MT fused protein production.

2) HeLa cells were exposed to Cd<sup>++</sup>, and MET was isolated from cytoplasmic homogenates by passage through Sephadex G-75 and DEAE-Sephadex A-25 chromatographic columns. Cd-binding proteins were detected by atomic absorption analysis, gel electrophoresis, and amino acid analysis.

3) DNA fragments containing promoter-operator regions of the human metallothionein (MET)-II gene and that of bovine papilloma virus (BPV) transforming genes are isolated from the plasmid, pMTII-BPV, (-) (from Dr. M. Karin, University of Southern California). These fragments are used for making a DNA affinity column and for DNase protection studies of apothionein binding.

4) The coding region of the mouse  $\beta$ -interferon gene isolated from the pM $\beta$  3 plasmid (gift from Dr. M. Taniguchi) is inserted downstream of the promoter region of MET gene in pMTII-BPVL(-) DNA. The new plasmid will be transfected into mouse epidermal cells and clones selected by transformation due to BPV functions. Effect of the interferon is assayed by activation of the MET promoter by administration of zinc in the culture.

Major Findings:

When a plasmid consisting of the MT gene with the PL promoter was transfected into *E. coli* lysogenized by cI857, both cII protein and a protein coded by the gene produced by fusion of the MT and lambda O-genes, were produced after shifting the incubation temperature from 32°C to 42°C. Properly constructed plasmids were selected by assaying the production of immunologically reactive proteins using the Western blotting method. Five different positive clones were obtained. In order to obtain a MT fused protein which does not share sequences with large T antigen, the Pst I site, which is located in the coding region of the middle T but in the intron for the large T, was fused with O-gene. This eliminated the entire coding region of the NH<sub>2</sub> terminal side of the large T antigen.

Proteins isolated from these *E. coli* extracts were immunoprecipitable 50, 53, and 55K molecular weight species. The 55K and 53K proteins reacted with MT specific monoclonal antibodies. Monoclonal antibody from clone C4, which recognizes the amino terminal region of all three polyoma T antigens, does not react with the 55K and 53K proteins. This suggests that the recognition site for C4 is within the first 20 amino acid residues of the MT which are missing in our fused protein. The bacterially derived protein does not have MT kinase activity and is not an efficient substrate for MT kinase in spite of the fact that the protein and the authentic protein have essentially the same primary amino acid sequence, differing only in the fused region.

Genetic elements, lying close to the promoter for a human gene for MET, separately mediate the induction of the gene by heavy metal ions, particularly Cd<sup>++</sup>, and glucocorticoid hormones. The element responsible for induction by glucocorticoid hormones is coincident with the DNA-binding site for the glucocorticoid hormone receptor (Karin et al., Nature 305: 513-519, 1984). However, the element responsible for induction by heavy metals is not clearly defined. Experiments are in progress to determine whether apothionein produced excessively may interact (suppression) directly with the control region of the MET gene. Three forms of Cd<sup>++</sup> MET were isolated and purified from HeLa cells by chromatographic separation procedures. Cd<sup>++</sup> was released from thionein by acid and EDTA treatments. DNA fragments containing human MET gene were isolated from the plasmid, pMT11-BPV(-). The potential binding of thionein to specific DNA elements will be studied by DNA affinity chromatography, DNase protection analysis, and in vitro transcription.

Significance to Biomedical Research and the Program of the Institute

Transformation of cells by polyoma virus is the result of the expression of viral early genes. The MT is essential for inducing cell transformation and tumors in animals by polyoma virus. The MT is a phosphoprotein which contains three sites of phosphorylation in vitro, namely, serine, threonine, and tyrosine. In vivo, the substrate for the kinase activity is not necessarily the MT antigen itself. The resulting phosphoproteins could have important roles in cell transformation. The general situation is comparable to that of pp60<sup>Src</sup> and of other retroviral oncogene products that are closely associated with protein kinase activity that transfers the phosphate of ATP to various proteins in vitro and in vivo. It is of particular importance to characterize the structure and function of the MT and its control mechanisms involved in the gene expression. However, isolation

of these proteins without contamination by other cellular and viral proteins is very difficult. Secondary modification and complex formation with other proteins in the virally transformed cells add to the complexity and technical problems.

To overcome these problems, we constructed an expression vector system that is capable of producing MT in large quantities in bacteria. This expression vector system is certainly a logical choice for the synthesis of other cellular and viral proteins related to malignant transformation.

MET gene expression is inducible both by heavy metals and glucocorticoids. The MET induction system might be a useful model for studying how single genes can be regulated by these inducers. Bovine papilloma virus can replicate independently as an episome when introduced into the cells. A recombinant DNA consisting of the MT gene and a portion of BPV also replicates and the MET gene can be expressed after induction by either glucocorticoids or heavy metals. Replacing the MET coding region with other genes such as growth hormone would result in a metal-inducible hybrid gene. Thus the MET promoter system provides a useful tool for the controlled expression of oncogenes or interferon genes and for the evaluation of the activities of the genes introduced. The ability to regulate the expression of an inserted gene by a heavy metal should make it possible to study the oncogenic effects of proteins coded by genes for which functions are unknown.

#### Proposed Course:

Vectors for the bacterial production of oncogene products that have improved efficacy and yields, and that are more closely related to the authentic proteins will be designed.

Plasmid vectors containing BPV, with deletions to prevent expression of its oncogene, and MET promoter-operator regions linked with other oncogenes will be constructed. Another system similar to that described will be constructed such that the transforming sequence of the BPV oncogene will be in a plasmid containing the MET promoter-operator linked to an interferon gene. Using the MET promoter to control the expression of the interferon gene, interferon may be found to interact with the BPV oncogene expression system or with its gene products.

The possibility that apothionein suppresses expression of the MT gene will be studied by DNA binding studies and by studies of apothionein-produced inhibition of in vitro transcription of the MT gene. If positive results are observed, the apothionein suppression system will be applied to the metal-regulated oncogene expression system described above.

#### Publications:

Kakefuda, T.: Mechanistic studies of mutation and cancer by chemical carcinogens. In Yamamoto, M. (Ed.): Recent Advances in Medical Sciences. Tokyo, Japan, Tokyo University Press, 1983, pp. 26-28.

Mizusawa, H., Seidman, M. and Kakefuda, T.: Mutation and modification of DNA molecule by chemical carcinogens. Protein, Nucl. Acid and Enzy. 29: 202-211, 1984.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CE05339-02 LMC

## PERIOD COVERED

October 1, 1983 to September 30, 1984

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

Radioimmunoassay of Cytochromes P-450 Using Monoclonal Antibodies

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

PI:	B. J. Song	Visiting Fellow	LMC	NCI
Others:	S. S. Park	Senior Staff Fellow	LMC	NCI
	F. K. Friedman	Senior Staff Fellow	LMC	NCI
	H. V. Gelboin	Chief	LMC	NCI

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Laboratory of Molecular Carcinogenesis

## SECTION

Metabolic Control Section

## INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

## TOTAL MAN-YEARS:

1.7

## PROFESSIONAL:

1.5

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

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

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

The multiplicity of cytochromes P-450 is being studied with monoclonal antibodies (MABs) to 3-methylcholanthrene (MC)-and phenobarbital (PB)-induced rat liver cytochrome P-450. These MABs directly bind both the corresponding microsomes and purified cytochrome P-450. A semiquantitative, direct radioimmunoassay (RIA) has been developed to measure cytochrome P-450 in the microsomes from various tissues in animals that are untreated, or treated with MC or PB. The amounts of cytochrome P-450 in different tissues and species, including human samples such as placentas and lymphocytes, are also being examined by competitive RIA; individual differences have been observed by this method, which is more reliable than measurements of enzyme activity. RIAs with multiple MABs have also been used to define the epitopic content of cytochromes P-450, with the goal of classifying various tissues with respect to MAB-specific cytochromes. These analyses provide an approach to the study of cytochrome P-450 multiplicity that is complementary to enzymatic and structural studies. Development of rapid, efficient RIA methods will aid in the establishment of a detailed atlas of epitope-defined cytochromes P-450 present in different tissues, species, and strains of laboratory animals as well as humans, and will aid in understanding the diversity of the cytochromes P-450 and their role in individual susceptibility to carcinogenesis.

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

B. J. Song	Visiting Fellow	LMC	NCI
S. S. Park	Senior Staff Fellow	LMC	NCI
F. K. Friedman	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

In order to understand the detailed genetics and role of cytochromes P-450 in carcinogen and drug metabolism, a phenotypic description of their exact number and quantity in various species and tissues is necessary. Monoclonal antibodies (MAbs) to 3-methylcholanthrene (MC)-or phenobarbital (PB)-induced rat liver cytochrome P-450 are used as highly specific probes in the development of radioimmunoassays (RIAs) for different cytochromes P-450.

Methods Employed:

Monoclonal antibodies to rat liver cytochromes P-450 were prepared by general methods. The microsomes were prepared from different tissues from species which were untreated, or treated with either MC or PB. Rat, hamster, guinea pig, and the mouse strains C57B1/6 and DBA/2 were studied along with human placenta and lymphocytes. The cytochrome P-450 content of the microsomes from these tissues were examined by either a direct or competitive RIA using [<sup>35</sup>S] or [<sup>3</sup>H] labeled MAbs. The RIA data were compared with enzymatic activities such as aryl hydrocarbon hydroxylase (AHH) in the absence and presence of monoclonal antibody.

Major Findings:

Monoclonal antibodies are a useful tool in the identification of specific cytochromes P-450 in crude microsome preparations, as evidenced by solid-phase RIA. In rat liver, there is considerable elevation (greater than 50-fold) in the level of the form of cytochrome P-450 specific for MAb 1-7-1 (to MC-induced P-450) in MC-treated rats, relative to the levels in control and PB-treated rats. A similar elevation in the P-450 specific for MAb 2-66-3 (to PB-induced P-450) was observed in PB-treated rats relative to control and MC-induced rats. Similar differences were also observed in livers from C57BL/6 mice, but not in those from hamster, guinea pig, and DBA/2 mice. The species-dependent differences in the amount of MC-induced cytochrome P-450 is consistent with the corresponding data on AHH activity and the data of immunopurification. In MC-induced rats, tissue-dependent differences were also observed; liver has a more than 30-fold higher level of cytochrome P-450 than lung or kidney. Comparable tissue-dependent differences were also observed in measurements of AHH activity. The apparent tissue-dependent difference in the amount of MC-induced cytochrome P-450 was evident with the mouse strain C57B1/6, but not with the relatively nonresponsive DBA/2 strain.



The human placentas and lymphocytes exhibited an MAb 1-7-1 specific epitope in common with rat liver MC-induced microsomes. The cytochromes P-450 responsible for the AHH induced by smoking was observed in human placenta microsomes whereas little was detected in placentas from non-smokers. The antigen appeared to be more stable at 21°C than AHH catalytic activity; thus the RIA is a more reliable and sensitive analytical tool in phenotyping the individual differences in carcinogen susceptibility than enzyme activity measurements.

RIA has been used to define the epitope content of various P-450s. Competitive RIAs have helped define overlapping specificities of MAbs. In addition we have developed a generalized RIA with rat MAb 1-87-1, specific for our mouse MAbs. With this rat MAb we can readily screen tissues for the presence of specific P-450s recognized by our library of mouse MAbs.

#### Significance to Biomedical Research and the Program of the Institute:

Development of RIAs will aid in the construction of an atlas of MAb-defined cytochromes P-450, which will result in a better understanding of cytochrome P-450 multiplicity and genetic control. It will also help to determine the role of particular cytochromes P-450 in drug and carcinogen metabolism. A rapid, efficient RIA system that detects and phenotypes P-450 isozymes in human tissues will aid in assessment of individual differences in cytochrome P-450 content and susceptibility to carcinogenesis.

#### Proposed Course:

Our RIA procedures will be refined to develop a quantitative, sensitive, and reproducible method for detection of cytochromes P-450 that are recognized by specific MAbs. Tissue microsomes from different species will be analyzed. The RIA will also be applied to human tissues such as lymphocytes to examine inter-individual differences. RIA will also be used as a tool for structural analysis of purified cytochromes P-450 with respect to epitope content.

#### Publications:

Gelboin, H. V., Park, S. S., Song, B. J., Fujino, T., Robinson, R., Friedman, F. K.: Monoclonal antibody directed phenotyping, radioimmunoassay, and purification of cytochromes P-450 that metabolize drugs and carcinogen. In Singer, T. P., Mansour, T. E. and Ondarza, P. N. (Eds.): Mechanism of Drug Action. New York, Academic Press, Inc., 1983, pp. 259-275.

Song, B. J., Fujino, T., Park, S. S., Friedman, F. K., and Gelboin, H. V.: Monoclonal antibody-directed radioimmunoassay of specific cytochromes P-450. J. Biol. Chem. 259: 1394-1397, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01CE05415-01 LMC

PERIOD COVERED

October 1, 1983 to September 30, 1984

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

**Structure of Human Actin Genes: Characteristics and Evolutionary Considerations**

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

PI:	T. Kakunaga	Head, Cell Genetics Section	LMC	NCI
Others:	S. Iijima	Visiting Fellow	LMC	NCI
	H. Ueyama	Visiting Fellow	LMC	NCI
	H. Hamada	Guest Researcher	LMC	NCI
	N. Battula	Expert	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:

0.6

PROFESSIONAL:

0.2

OTHER:

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.)

In order to understand the mechanism of carcinogenesis, it is essential to know the molecular mechanisms involved in regulation of gene expression. Human actin genes were chosen to study the structure, organization and expression of genes. A family of human actin genes has been isolated by molecular cloning. Five of these genes (cardiac muscle actin, aorta-type smooth muscle actin, stomach-type smooth muscle actin, pseudo beta-actin and beta-actin), have been characterized by DNA sequencing. Although there are many pseudogenes for cytoplasmic actin (beta- and gamma-actin), the number of functional genes seems to be only one per haploid genome for each actin isoform. Comparison of the primary structure of the actin genes, particularly the location of introns and the presence or absence of a cysteine codon following the initiation codon, led to a hypothesis for the evolutionary pathways of the actin gene families.

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

T. Kakunaga	Section Head	LMC	NCI
S. Iijima	Visiting Fellow	LMC	NCI
H. Ueyama	Visiting Fellow	LMC	NCI
H. Hamada	Guest Researcher	LMC	NCI
N. Battula	Expert	LMC	NCI

Objectives:

The overall goal of this project is to understand the structure and function of the human actin genes and to clarify the regulatory mechanism for expression of actin genes during differentiation and oncogenesis. The immediate objectives are to determine the molecular structure of actin genes and to obtain information on the evolution of the actin gene family by comparing the structure of actin genes between species.

Methods Employed:

Actin is a major protein present in mammalian cells. It constitutes 5-10% of the total protein even in non-muscle cells and a much higher percentage in muscle cells. However, the structure and function of actin genes were not known. From protein analysis, six actin isoforms are known in mammalian cells: four muscle types (skeletal, cardiac, blood-vessel-type smooth muscle, and digestivetract-type smooth muscle) and two non-muscle types (beta and gamma). Each muscle type of actin is functionally involved in muscle contraction and is expressed only in the particular type of muscle cells. On the other hand, non-muscle actins, the so-called cytoplasmic actins, participate in a variety of functions, such as cell motility, mitosis and maintenance of the cytoskeleton and are expressed in many types of cells. Abnormal expression of cytoplasmic actin has been found in neoplastic cells.

Little is known about the arrangement of the actin genes in the mammalian genome. Thus, the structure and organization of human actin genes were investigated to understand the regulation of their differential expression, the evolutionary origin of these related genes, and the involvement of their abnormal expression in carcinogenesis. DNAs were extracted and purified from human cells and inserted into phage DNA. Clones containing human actin genes were screened by colony hybridization with nick-translated probe DNAs such as dictyostelium actin cDNA, the 3'-untranslated region or near the 5'-terminal coding region of the human pseudo beta-actin gene; or synthetic polynucleotides whose base sequences are deduced from the amino acid sequences of the N-terminal region of the human beta-actin gene. The gene structure of cloned genes was determined by making restriction maps and DNA sequencing. Organization of the genes was examined by hybridization using various regions of coding and noncoding sequences of the actin genes as probes. A number of laboratory techniques used in the field of molecular biology were employed, including DNA-DNA and DNA-RNA hybridization, gel electrophoresis, in vitro translation, column chromatography, and autoradiography.

Major Findings:

The pursuit of this project has led to major new findings in five pertinent areas: 1) the first determination of the structure of the cardiac muscle actin gene, stomach-type smooth muscle actin gene, aortic-type smooth muscle actin gene, and beta-actin gene, including restriction map, base sequences and organization; 2) new information on the evolutionary pathway of the actin gene family; 3) discovery of an intronless processed gene; 4) first discovery of potential Z-DNA sequences in nature; and 5) increased information on the unique structure of regulatory genes in the actin gene family.

During the past year our understanding of both the structure and the evolutionary pathway of the actin gene family has been greatly increased. The human aorta-type smooth muscle actin gene contains at least 7 introns at codons 41/42, 84/85, 121/122, 150, 204, 267, and 327/328. All exon-intron junctions follow the GT-AG rule. Like the stomach-type smooth muscle actin gene, the aortic-type smooth muscle actin gene has two unique intron sites, i.e., codons 84/85 and 121/122. The intron site at codon 84/85 has never been found in actin genes other than in these smooth muscle actin genes. The intron site at codon 121/122 has been found in the beta-actin gene and in the sea urchin actin gene, but not in muscle actin genes. The intron sites at codons 150 and 204 are not present in the beta-actin gene, but are present in all muscle actin genes. The intron site at codon 150 was also found in the soybean actin genes, and the one at codon 204 was found in sea urchin actin genes. The other three intron sites are common to all mammalian actin genes examined. On the other hand, the amino acid sequences of actins in lower eukaryotes, such as sea urchin and dictyostelium, resemble vertebrate non-muscle actins more than vertebrate muscle actins. Furthermore, vertebrate non-muscle actin genes and actins from lower eukaryotes such as yeast, slime mold, and soy bean, lack a cysteine codon after an initiation codon, while all vertebrate muscle actin genes have this codon. Based on these structural features of the actin genes, an evolutionary pathway of the vertebrate actin gene family was proposed. The presumptive ancestor actin gene had at least six introns at codons 41, 121, 150, 204, 267, and 327. Through the process in which the intron interrupting at codons 121/122 was lost, skeletal and cardiac muscle actin genes may have evolved from the ancestral actin gene. The smooth muscle actin gene may have been generated from the ancestral gene by obtaining a new intron between codons 84 and 85. On the other hand, the non-muscle actin gene may have been created by loss of the introns at codons 150 and 204. This hypothesis provides for both deletion and insertion as the origin of introns.

Significance to Biomedical Research and the Program of the Institute:

In order to understand the molecular mechanisms of carcinogenesis, it is essential to know how the expression of the genes is regulated. We are the first to isolate and characterize the cardiac muscle actin gene, the aortic-type smooth muscle actin gene and the stomach-type smooth muscle actin gene in any species, and the beta-actin gene in humans. Since the skeletal actin gene has been isolated from chicken and rat by other laboratories, only the gamma-actin gene remains unisolated among the six predicted actin genes in mammalian cells.

Comparison of the primary structure of actin genes led to a hypothesis of the evolutionary pathway of actin genes and the functional relationship between different actins. For example, it is conceivable that, in function, the cardiac muscle actin gene has a greater resemblance to the beta-actin gene than to the muscle actin gene and that the function of the cardiac muscle actin gene is more like that of the skeletal muscle actin gene than that of the aortic-type smooth muscle actin gene. In addition, new information was added to the conceptual hypothesis of the evolutionary pathway of introns. The data obtained by others with immunoglobulin genes and globin genes suggested that introns have been lost during evolution. On the other hand, the previous data on the actin and myosin genes had been against it. We have proposed the hypothesis that both deletion and insertion events have been involved in the evolution of the actin gene family. Our hypothesis was possible because of the strong conservation of coding regions of actin genes through the evolutionary period of the eukaryotic genome. The actin genes which we have isolated will also serve as gene sources and as general or specific probes for studying the expression of actin genes during cell differentiation and carcinogenesis. ' .

#### Proposed Course:

We are in the process of determining the primary structure of the 5' and 3' untranslated regions of the human cardiac muscle actin gene, and the beta-actin gene. Particularly, the structure of the promoter regions of the 5' end will be compared among actin genes of different isoforms and of different species and organisms.

A culture system for studying the differentiation of cardiac muscle cells is being developed by using human embryo heart cells. The conditions for inducing or suppressing differentiation or cell replication will be defined. The expression of actin isoforms during differentiation will be examined at the RNA level by using the 3' or 5' untranslated regions of actin genes which specifically hybridize to the RNA species of each actin isoform. The expression of actin isoforms will also be examined at the protein level, by using two-dimensional gel electrophoresis and by using antibodies specific for each actin isoform. No antiserum specific for each actin isoform has been available. We are planning to raise monoclonal or polyclonal antibodies against each actin isoform by using synthetic peptides equivalent to the amino terminal region of actins, where the amino acid sequences are most variable.

As a future system for studying the regulation of expression of the actin gene, the recombinant plasmids consisting of an ampicillin resistance gene, a replication origin from pBR322, a chloramphenicol acetyltransferase (CAT) gene from IS-2, and combinations of the promoter region poly(A) addition signal sequence from SV40, and the untranslated region of the cardiac muscle or beta-actin gene, will be constructed.

The chromosomal location of each actin isoform will be assigned by *in situ* hybridization and by molecular hybridization with a DNA library derived from specific human chromosomes using DNA fragments specific for each actin isoform. The role of actin genes found in the *fgr* oncogene involved in cell transformation will be investigated. In addition, the possible presence of a part of the actin genes, particularly their untranslated regions, in the oncogenes will be

checked by molecular hybridization. The effects of ligation of a part of the non-muscle actin genes to oncogenes, particularly genes coding for tyrosine-specific protein kinase, will be examined on the basis of their biological activity including transforming activity.

Publications:

Ueyama, H., Hamada, H., Battula, N. and Kakunaga, T.: Structure of a human smooth muscle actin gene (aortic type); It has a unique intron site. Mol. Cell. Biol. (In Press)

ANNUAL REPORT OF  
CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH  
NATIONAL CANCER INSTITUTE

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 carcinogens; inter- and intra-species 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.

Grants and contracts administered by the staff of this Branch support six complementary categories of chemical and physical carcinogenesis research and associated resources: Molecular Carcinogenesis, Carcinogenesis Mechanisms, Biological and Chemical Prevention, Diet and Nutrition, Smoking and Health, and Research Resources.

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 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 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.

Due to a reorganization within the Division of Cancer Etiology during FY 1984, the Diet and Nutrition and Smoking and Health programs have been moved from the Special Programs Branch to this Branch.

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. Grants dealing with epidemiological investigations which focus on the effects of dietary factors in human carcinogenesis will remain in the Extramural Programs Branch (formerly Special Programs Branch) of the Epidemiology and Biostatistics Program.

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. As with the Diet and Nutrition category, those studies in Smoking and Health which are related to epidemiology will remain with the Extramural Programs Branch.

The Research Resources component, supported solely by contract, is principally concerned with the synthesis and distribution of selected chemical carcinogens and certain of their metabolites, with particular reference to polynuclear hydrocarbon carcinogens, their metabolic intermediates, and analogous heterosubstituted compounds, as well as the synthesis and distribution of retinoids including radiolabeled forms.

In the 1983 annual report of the Branch there was a category identified as Special Projects under which were clustered grants from a broad domain of chemical and physical research activities. Some of these represented traditional research program responses to recent RFAs issued by the Branch and other grants included were program project and conference grants. This category of Special Projects has now been completely distributed to the other categories within the Branch and summaries of those ongoing projects will be reported under the relevant topic area for this annual report.

In April of 1983, the Branch introduced a payback system for the Chemical Carcinogen Reference Standard Repository and its associated resource contracts.



The charge billed to the recipient is composed of a unit chemical price, handling cost, and shipping cost. Income from such sales are subtracted from the contractor's monthly invoices to the NCI. Since inception of this payback system the Branch has authorized over \$110,000 in sales to investigators world-wide.

The CPCB co-sponsored a symposium/workshop on December 6-8, 1983, on "Comparison of Mechanisms of Carcinogenesis by Radiation and Chemical Agents." The Low Level Radiation Effects Branch of the Radiation Research Program, Division of Cancer Treatment and the Chemical and Physical Carcinogenesis Branch, Division of Cancer Etiology (DCE), co-sponsored and organized a two-day symposium which was held at the National Bureau of Standards Auditorium in Gaithersburg, Maryland and which attracted nearly 300 participants.

The objective of this symposium was to review the available information on the mechanisms of carcinogenesis by both types of agents and to reveal both the similarities and differences that have been encountered from the initial interaction at the cell and molecular level to the expression of cancer as a tissue disease in various animal models. The challenge to all of the speakers was to go beyond the domain of their own laboratory contributions in order to review the experimental findings in the field at large. In a sense, not only was the state of knowledge reviewed, but the unanswered questions that remain were also examined. Through the process of comparison of the qualitative effects of these agents on animal cells, we may come to a better understanding of the total carcinogenic process.

Immediately following the two day symposium, a one day workshop with approximately 40 participants, primarily the symposium speakers, was convened in order to identify opportunities for new or renewed emphasis and to identify areas where technology or specialized resources are lacking. The Workshop was co-chaired by Drs. I. Bernard Weinstein and Mortimer Elkind.

On May 3-4, 1984, the Biological and Chemical Prevention component of the Branch sponsored a workshop on the "Development of Cancer Chemopreventive Agents" which was chaired by Dr. Lee Wattenberg. This Workshop focused on agents which can protect against free radical species and their mechanisms in chemoprevention. Other discussions covered protease inhibitors as anticarcinogenic agents and the role of inhibitors of the arachidonic acid cascade as anticarcinogenic agents. Recommendations from this workshop have given rise to a Request for Application (RFA) concept which will generate an RFA in FY 1985.

The CPCB also supported or contributed to the support of a number of conference grants (R13 mechanism) dealing with subjects of particular relevance to the Branch. The magnitude of this support is summarized in Table I and the titles of individual conferences can be found after each section in the Branch by looking for grants identified with an R13 prefix.

One RFA for traditional research grant applications was issued this year entitled, "New Natural and Synthetic Inhibitors of Carcinogenesis." This initiative from the Biological and Chemical Prevention component of the Branch attracted 55 applicants. A total of 11 awards will be made from this announcement.

Two Request for Proposals (RFPs) were issued this year for contract-supported initiatives of the Branch. The first was a recompetition of ongoing synthesis efforts in the Research Resources area. The announcement titled, "Synthesis of

Selected Chemical Carcinogens" resulted in two contract awards. The second RFP, "Toxicology and Pharmacology of Anticarcinogenic Agents," is also expected to result in two awards late in FY 1984. The contracts will provide toxicological and pharmacological evaluation of anticarcinogenic compounds. A summary of the number of grants, contracts, and associated funding relative to each of the above categories and to the Chemical and Physical Carcinogenesis Branch, as a whole, follows. Table I focuses on mechanisms of support of extramural research and related activities in the area of Chemical and Physical Carcinogenesis. Table II provides an estimate of grant and contract support, respectively, in each of the six Branch components as described above:

TABLE I  
CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH  
(Extramural Activities - FY 1984 - Estimated)

<u>(Millions)</u>	No. of Contracts/Grants	\$
Research Contracts	15	1.21
Research Grants	397	47.51
Traditional Research Grants (R01) (317 grants; \$34.26 million)		
Conference Grants (R13) (9 Grants; \$0.76 million)		
New Investigator Research Grants (R23) (10 Grants; \$0.54 million)		
Program Project Grants (P01) (10 grants; \$6.12 million)		
Cooperative Agreements (U01) (0 grants; \$0.00 million)		
Small Business Grants (1 grant; \$0.05 million)		
RFAs (50 grants; \$5.78 million)		
Research Resource Contracts	8	1.91
TOTAL	420	50.63

TABLE II  
 CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH  
 (Contracts and Grants Active During FY 1984)

<u>1984</u> <u>GRANTS</u>	<u>FY</u>			
	<u>CONTRACTS</u>			
	<u>No. of Contracts</u>	<u>\$ (Millions)</u>	<u>No. of Grants</u>	<u>\$ (Millions)</u>
Carcinogenesis Mechanisms	0	0	98	11.48
Biological & Chemical Prevention	11	.76	62	7.22
Molecular Carcinogenesis	0	0	196	23.21
Diet and Nutrition	0	0	30	3.20
Smoking and Health	4	.45	11	2.40
Research Resources	8	1.91	0	0
TOTAL	23	3.12	397	47.51

## SUMMARY REPORT

### BIOLOGICAL AND CHEMICAL PREVENTION

The Biological and Chemical Prevention component of the Chemical and Physical Carcinogenesis Branch is responsible for research on agents that can 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 56 grants in this program area with FY84 funding of approximately \$5.91 million and 11 contracts with FY84 funding of approximately \$0.76 million. Three additional support contracts in this program are discussed under Research Resources.

Research grants in the program 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, and pharmacologic disposition. Studies proceed on inhibition of carcinogenesis induced by chemical, physical and 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 repair, binding proteins or receptors for preventive agents, preneoplastic states and cyto-genetic variables.

A new program initiative this year was the RFA "New Natural and Synthetic Inhibitors of Carcinogenesis." This RFA emphasized studies on natural sources of inhibitors of carcinogenesis such as foods consumed by man. The 11 grants resulting from the RFA will provide research programs on isolation, identification and anticarcinogenic efficacy of components of orange peel oil; on the efficacy of crude and purified Bowman-Birk protease inhibitors from soybeans to inhibit colon carcinogenesis; on isolation and antimutagenesis screening for inhibitors from a wide variety of vegetable and vegetable products, and fruits and fruit products; on isolation and identification of inhibitors of carcinogenesis from the coffee bean and various coffee preparations; on the inhibition or decrease in carcinogenesis by endogenous (natural) inhibitors of beta-glucuronidase or dietary inhibitors of this enzyme; on suppression of mammary gland carcinogenesis by vanadium compounds; on isolation, chemical identification and mechanisms of action of potential inhibitors of carcinogenesis obtained from human foods and higher plants selected from the cruciferae, umbelliferae, leguminosae and other sources; and on identification and mechanisms of action of inhibitors of carcinogenesis found in onion and garlic oils.

Contracts in the program support studies on antioxidant inhibition of tumorigenesis in liver, lung, digestive tract and mammary gland; on retinoid inhibition of tumorigenesis in urinary bladder and mammary gland; on synthesis and bioassay of new retinoids for potential future development; on synthesis of large amounts of selected retinoids and studies on their toxicity, and on synthesis of radio-labeled retinoids for metabolic and pharmacologic investigations. Research accomplishments follow on a number of these endeavors.

## Grants Activity Summary

Antioxidants: It is well established in rodents that dietary administration of a variety of structurally diverse hypolipidemic drugs having primarily hypotriglyceridemic properties, as well as certain industrial plasticizers, results in hepatomegaly, the induction of hepatic peroxisome proliferation, elevated peroxisomal enzyme activities of hydrogen peroxide-generating oxidases, catalase, carnitine acetyltransferase and other enzymes involved in the beta-oxidation of long-chain fatty acids, and with chronic treatment, the production of hepatocellular carcinomas. Moreover, peroxisome proliferation and increases in the fatty acid beta-oxidation system have been shown in five other species, including two species of monkey (cat, pigeon, chicken, rhesus and cynomolgus monkeys). These compounds have added interest since they do not appear to have mutagenic activity by eukaryotic or the *Salmonella*/microsome assays, do not produce detectable DNA damage in the lymphocyte <sup>3</sup>H-thymidine incorporation assay nor do they appear to bind to DNA or to cause chromosomal aberrations. However, recent evidence has been obtained for DNA damage during peroxisomal beta-fatty acid oxidation. In these in vitro experiments, purified hepatic peroxisomes, palmitate, and necessary cofactors were coincubated with purified, supercoiled SV40 DNA. Formation of single strand-nicked SV40 DNA was found to be linearly related to the concentration of peroxisomes purified from hypotriglyceridemic drug-treated rat liver with negligible damage seen with control peroxisomes. Further, the extent of DNA damage correlated with the level of H<sub>2</sub>O<sub>2</sub> generated on a chronic basis as a result of increased peroxisomal beta-oxidation of fatty acids.

Recently, the hypothesis was tested that hepatocarcinogenesis induced by peroxisome proliferators is mediated either directly by carcinogenic H<sub>2</sub>O<sub>2</sub> generated by peroxisomal oxidase(s) or indirectly by free radicals produced from H<sub>2</sub>O<sub>2</sub>, and that antioxidants can retard or inhibit such induced neoplasia by scavenging active oxygen species (superoxide radicals, hydrogen peroxide, hydroxyl radicals and ringlet oxygen). In this experiment, the synthetic antioxidants butylated hydroxyanisole (BHA) or ethoxyquin (EQ) were fed for 60 weeks to male Fischer 344 rats concurrently with ciprofibrate, a hypotriglyceridemic drug and one of the most potent peroxisome proliferators. The experimental results showed that EQ markedly inhibited ciprofibrate-induced liver carcinogenesis as seen by a decreased incidence in the percentage of animals with tumor, a decreased number of tumors per animal and a reduced tumor size. A significant decrease in incidence and number of carcinomas per liver larger than 5mm was also found with BHA administration. Since previous studies had shown that these antioxidants neither inhibit ciprofibrate-induced peroxisomal proliferation nor the induction of increased activities of the H<sub>2</sub>O<sub>2</sub>-producing peroxisomal fatty acid beta-oxidation system, the observed inhibition of hepatocarcinogenesis by these antioxidants was deemed to be consonant with this hypothesis (42).

Vitamins and Retinoids: Although retinoids are well known to inhibit or suppress experimental carcinogenesis induced by a variety of carcinogens at several organ sites, little systematic investigation has been directed at elucidating the relationship between retinoid metabolism and chemopreventive activity. Conclusive evidence in even a single instance is lacking demonstrating that a specific chemical form of a retinoid, either natural or synthetic, is associated with chemoprevention of carcinogenesis. The retinoid 13-cis-retinoic acid (13-cis-RA) has inhibited experimental carcinogenesis in the urinary bladder, lung and skin, is equipotent with all-trans-retinoic acid in promoting growth, maintaining differentiation in tracheal organ cultures, and inducing embryonal carcinoma cell differentiation. It is a physiological metabolite of retinol and has been

reported as an *in vivo* metabolite of all-trans-retinoic acid. Little data are available concerning target-tissue metabolites of 13-cis-RA, whether it possesses inherent biological activity or if it must be converted *in vivo* to an active metabolite. Recent studies have shown that the major metabolites of 13-cis-RA in the liver and intestinal mucosa of rats pretreated orally for two weeks with this retinoid are all-trans-RA and the all-trans- and 13-cis-retinoyl glucuronides. These two isomeric conjugates were also the most prominent 13-cis-RA metabolites detected in bladder, prostate, pancreas, kidney, ovary and uterus. Interestingly, these glucuronides were not detected in serum. Nevertheless, the substantial accumulation of these conjugates in target tissues has suggested the possibility that they may serve a biochemical function other than excretory metabolites. The hypothesis raised is that the retinoyl glucuronides could serve as short-term storage forms of all-trans- and 13-cis-RA in those tissues capable of their synthesis via glucuronyl transferase, regenerating the aglycone retinoic acids via beta-glucuronidase activity. In support of this hypothesis are the facts that all-trans-retinoic acid is a significant metabolite of 13-cis-RA in all target-tissues mentioned above, as well as lung, and that UDPGA-dependent glucuronyl transferase activity which synthesizes the isomeric glucuronides has been detected in bladder, kidney and lung microsomes (30).

An important hypothesis regarding retinoid mechanisms of action postulates that retinoid activity is mediated in nonvisual tissues via specific intracellular retinoid binding proteins, cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP). CRBP binds retinol with high specificity and affinity, but does not bind retinal or retinoic acid. CRABP has high affinity for retinoic acid but does not bind retinal or retinol. Both of these intercellular proteins differ as well from the transport protein in blood, retinol binding protein (RBP). The expression of the genes for these two proteins appears to be independently regulated and under developmental control. The levels of the proteins may change also upon neoplastic transformation and vitamin A status. In analogy to steroid hormone mechanisms of action, retinoids are hypothesized to bind to their specific cytosolic binding proteins which act to transport the retinoids to the nucleus where the complexes bind to chromatin, leading to altered expression of the genome. Experimental data exist supporting this hypothesis, although a universal obligatory function for known retinoid binding proteins in retinoid action is still unclear.

Several interesting new developments on retinoid binding proteins have been reported recently. One is the discovery and purification to apparent homogeneity of a new retinol-binding protein from whole neonatal rat pups. The protein is distinct from other retinol-binding proteins by several criteria. These include small but distinct differences in spectra and considerably different immunoreactivity and tissue distribution. It apparently exists in two forms, retinol binding protein (A+B), both of which are single polypeptide chains with molecular weights of about 16,000. Both forms seem to coexist in the tissues examined in equal amounts. Tissue distribution studies showed the presence of the retinol-binding protein in a number of tissues of the neonatal rat. Two tissues, however, intestine and liver, have 100-fold higher levels than other positive tissues. Among the tissues examined only skin had no detectable material by the radioimmunoassay procedure employed. In adult rat tissues high levels of the new binding protein are again found in intestine, particularly the jejunum mucosa. Adult liver, however, had a much lower level than in perinatal liver. Most other adult tissues had non-detectable levels of the new binding protein (less than 4 pmoles per gram of tissue). The high levels in the intestine suggest that the protein may play a role in the absorption process and/or in the esterification of

retinol that occurs prior to its incorporation into chylomicrons. In this regard, the gradient in level of the protein from jejunum to colon of the adult rat is interesting: jejunum levels three-fold greater than ileum and both of these greater than 100-fold the level in colon. Further, as with some other proteins known to be involved in intestinal absorption processes which show increases in their level after birth, the new retinol-binding protein triples in level from day 19 in fetal intestine to that in the newborn rat. Fetal liver levels also appear to increase in the liver of the newborn rat (40).

A large number of systems have been developed for bioassay of various types of retinoid activities. One of the in vitro, cell culture systems employed tests the ability of a retinoid (or retinoid metabolite) to induce terminal differentiation of murine F9 teratocarcinoma cells. In this system some retinoids induce differentiation of F9 embryonal carcinoma cells to non-tumorigenic cells resembling parietal endoderm. This system, like the hamster tracheal organ culture differentiation assay, is extremely sensitive and provides an excellent model system in which to examine the biological activity of retinoid metabolites. Although it is an unresolved issue in human as well as murine cells, a complex between a retinoid and a specific CRABP may be necessary for retinoid promotion of differentiation. Such a specific CRABP has recently been characterized in undifferentiated F9 stem cells. Its molecular weight, isoelectric point and binding affinity for all-trans-RA are similar to other known CRABPs. The binding affinities of all-trans-RA, 13-cis-RA, 5,6-epoxyretinoic acid, and retinol for this F9 CRABP have been determined using a new competitive binding assay for labeled all-trans-RA of greatly increased utility employing high performance size-exclusion chromatography. It was found for these retinoids that a strong correlation existed between binding affinity and differentiation-inducing activity in F9 cells. This system and similar variant embryonal carcinoma cell culture systems provide important assays in which to study the biological activities of retinoids and their metabolites and to probe into fundamental mechanisms of differentiation control (30). In this regard, a specific set of nuclear retinoic acid acceptor sites has been identified and partially characterized in F9 cells. It was found that the complex between retinoic acid and the cellular retinoic acid binding protein mediates specific retinoic acid binding to these nuclear acceptor sites. Moreover, the specific acceptor sites are associated with chromatin and bound retinoic acid can be released by pronase, DNAase I, and micrococcal nuclease digestion (30).

In rheumatoid arthritis, proliferating synovium containing a heterogeneous mixture of synovial cells, polymorphonuclear leukocytes, both T and B cell immunocytes, monocytes/macrophages, and multinucleated giant cells develops into an invasive front which progressively destroys articular cartilage, subchondral bone and tendons. The prime mediators of this tissue destruction are the neutral proteinase collagenase and prostaglandin  $E_2$  ( $PGE_2$ ) which are synthesized and secreted in large quantities by rheumatoid synovial cells. Recently, retinoids have been studied for efficacy in three animal models of arthritis. In two of these cases they have been found effective in reducing severity of disease. Orally administered 13-cis-retinoic acid has suppressed the development of adjuvant arthritis in the rat, and (in a separate study, 9) N-(4-hydroxyphenyl) retinamide has suppressed in a dose-dependent manner the chronic phase of streptococcal cell wall-induced arthritis. However, 13-cis-RA has increased the severity of collagen-induced arthritis. In all three models, synovium from arthritic animals secrete elevated levels of collagenase which retinoids are able to suppress. Up until this time, only corticosteroids have been known to inhibit collagenase production. Suppression by retinoids and by dexamethasone has also

been shown in primary cultures of human rheumatoid synovial cells (which "spontaneously" secrete collagenase and PGE<sub>2</sub>), and in the phorbol ester-stimulated production of collagenase in normal rabbit synovial cells, a standard cell culture system for study of rheumatoid arthritis.

The emerging role of transforming growth factors (TGFs) in neoplastic transformation and their possible essential role in normal cell function have raised the intriguing question as to their possible physiological role in proliferative and invasive but non-malignant diseases such as rheumatoid arthritis. The transforming growth factors constitute two known families of low molecular weight, acid-stable polypeptides distinguishable by their physicochemical and biological properties. They are found in both neoplastic and nonneoplastic tissue. Type alpha TGFs are operationally defined by their capacity to bind to the receptor for epidermal growth factor (EGF) to which they have some sequence homology. They are single-chain polypeptides of molecular weight 5000-7000 and contain three intrachain disulfide bonds. Antibodies to EGF do not cross-react with TGF alpha. EGF itself can substitute for TGF alpha, however, in all known biological responses and has been classified as an alpha TGF. Type beta TGFs do not compete for binding to the EGF receptor, but appear to bind to another unique cell surface receptor, and in their biologically-active form are disulfide-bridged 2-chain peptides of molecular weight 25,000. The outstanding biological characteristic of these two families of TGFs is their synergistic interaction to produce morphological transformation of nonneoplastic indicator cells in monolayer culture, or to confer upon these anchorage-dependent cells the ability to form progressively growing colonies in soft agar. This phenotypic transformation requires the concerted action of the two distinct types of TGF. Type alpha TGFs are mitogenic polypeptides which bind to the EGF receptor and induce anchorage-independent colony formation in the presence of TGF-beta which does not possess mitogenic activity. Neither TGF-alpha nor TGF-beta alone can phenotypically transform cells. Other growth-inducing peptides are also present in normal tissues such as nerve growth factor, fibroblast growth factor and platelet-derived growth factor; however, only the TGFs have been found to stimulate growth of nonneoplastic cells in soft agar.

A recent study has examined the capacity of TGFs to induce proliferation in the normal rabbit synovial fibroblast system. It was found that both a partially purified preparation of TGFs containing both types of TGFs, or a combination of EGF plus purified TGF beta increased 3H-thymidine incorporation, cell number and protein content in confluent monolayers of synovial cells. It was found that retinoic acid added to these cultures before and during exposure to EGF or to the combination of EGF and purified TGF beta blocked the proliferative response induced by these factors. Neither dexamethasone nor indomethacin were capable of inhibiting this proliferative response (9).

### Contracts Activity Summary

Inhibition of Carcinogenesis in Animal Systems: The acquisition of dose-response relationships for the efficacy of chemopreventive agents to inhibit the neoplastic process in various organs, tissues and species is a most important aspect of anti-carcinogenesis research. These relationships along with toxicological and pharmacological information, mechanisms of action and knowledge of the conditions under which inhibition can be demonstrated, provide a basic set of data on which to evaluate a compound's potential for possible human use. Such dose-response studies have been underway on the phenolic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). These compounds are known inhibitors of



carcinogenesis at high levels of administration against many chemical carcinogens, at many organ sites and in several species of animal.

Last year it was reported that BHA inhibits methylazoxymethanol (MAM)-acetate-induced colon carcinogenesis in the CF<sub>1</sub> mouse in a dose-responsive manner when provided in the diet at 300, 1000, 3000, and 6000 mg/kg. In these studies BHA was provided from two weeks before through two weeks after MAM-acetate administration. These results have now been extended in this system to studies in which BHA has been provided from two weeks before carcinogen administration to sacrifice of the mice 40 weeks later. BHA was administered again in NIH-07 diet at 300, 1000, 3000 and 6000 mg/kg, and MAM-acetate was administered at two dose levels. Results show that the incidence of colonic tumors is decreased at the lower dose of carcinogen at all four BHA levels. Not only is inhibition of tumorigenesis found at the lowest level of 300 mg/kg diet, but there is practically no dose-response out to 6000 mg/kg. Thus, the percent of animals with colon tumors decreases from 22% in the control (0 BHA) to 5, 10, 6 and 3% with increasing level of BHA in the diet. The colon tumor multiplicity (tumors per tumor-bearing animal) also decreases in a dose-responsive manner, particularly adenocarcinomas (from 1.0 at 0 and 300 mg/kg to zero at 6000 mg/kg BHA). At the higher carcinogen dose significant inhibition was again found down to 1000 mg/kg BHA in tumor incidence with less pronounced but significant decreases in multiplicity as well. In this model, lung tumors (75% adenocarcinomas) also develop. It is of some interest that at both levels of carcinogenic insult a dose-responsive significant inhibition of these tumors is also seen (63).

A previous report has indicated that a high level of BHT enhances N-2-fluorenyl-acetamide (FAA)-induced hepatic tumorigenesis in the rat. This report has been extended by a recent study employing the same four doses of BHT as indicated above (300 to 6000 ppm) following FAA administration. In this study, a high level of FAA was given in the diet for 8 weeks and then, two weeks later, the BHT-containing diets were provided for an additional 22 weeks. Results of this study showed that BHT feeding after FAA initiation enhanced the frequency of rats with neoplasms only at the highest (6000 ppm) level. This increase (21% to 67%) was due almost entirely to increased neoplastic nodule formation rather than hepatocellular carcinoma which did not increase significantly. At 6000 ppm, BHT also increased the number of gamma-glutamyltranspeptidase (GGT)-positive foci and the area occupied by GGT-positive preneoplastic and neoplastic lesions. Both 316 ppm and 500 ppm phenobarbital (PB) fed under the same conditions as BHT in the post initiation period enhanced the incidence of neoplasms, agreeing with previously reported results. This experiment shows that BHT is a weak enhancer of tumorigenesis under these conditions, compared with PB, and that enhancement is manifest only at known near-toxic doses (67).

Another project on the dose-response relationship for phenolic antioxidants concentrates on the efficacy of 2(3)-BHA and its major isomer 3-BHA in inhibiting pulmonary adenoma formation. Of particular interest has been the effectiveness of these compounds at low levels in the diet, under different experimental conditions, and in combination with other substances. In a recent experiment, the efficacy of 2(3)-BHA was examined in its capacity to inhibit the spontaneous occurrence of pulmonary adenomas at very low levels of the antioxidant, and in a concurrent, parallel experiment, to inhibit benzo(a)pyrene (BaP)-induced adenomas. Two levels of 2(3)-BHA were employed, 100 ppm and 300 ppm in semi-purified diet. The antioxidant was fed for 6 weeks before the first BaP administration and continued until one day after the last administration. Results showed that both levels of 2(3)-BHA inhibited "spontaneously"-occurring pulmonary

adenomas in female ICR/Ha mice when fed from 8 weeks of age until sacrifice at 66 weeks. Both the percent mice with adenoma and the number of adenomas per mouse were reduced. On the other hand, 2(3)-BHA at these levels showed little or no inhibition of BaP-induced pulmonary neoplasia. The results of this study are of some interest in that they suggest that 2(3)-BHA may have two mechanisms of inhibition, one entailing carcinogen detoxification and the other a suppressive mechanism not related to carcinogen metabolism (65).

The capacity of the major isomer of 2(3)-BHA, 3-BHA, to inhibit BaP-induced pulmonary adenomas at low dietary levels has also been studied. The levels in the diet employed were 200 ppm and 500 ppm. These levels in the diet were also studied in combination with cocoa bean powder and theobromine with the objective of enhancing the protective effects of 3-BHA by the simultaneous administration of the second agent. The basis for this rationale was that both cocoa bean powder and theobromine had been found to enhance the induction of glutathione S-transferase (GST) activity by 3-BHA. Theobromine (3,7-dimethylxanthine) is the principal alkaloid in cocoa bean and was found to be an even more effective enhancer of induced GST activity than cocoa bean powder. In addition, 3-BHA fed for 4 or 12 weeks, was known to increase liver GST activity and glutathione (GSH) levels even at 50 and 100 ppm in the diet. GST and GSH are known to be involved in detoxifying pathways of BaP. Mice were fed semipurified diets containing the agents for 6 weeks prior to the first BaP administration through one day after the last dose of carcinogen. Results of this experiment showed that while both 200 and 500 ppm BHA inhibited pulmonary adenoma formation, cocoa powder and theobromine by themselves did not, and combinations of these substances with either level of 3-BHA were less effective than the antioxidant by itself. Thus, although both cocoa powder and theobromine enhance induction of increased GST activity by 3-BHA, this was not translated into an increased protection against BaP-induced pulmonary neoplasia (65). All of these results demonstrate that phenolic antioxidants are capable of blocking or suppressing tumorigenesis even at low levels of administration.

BIOLOGICAL AND CHEMICAL PREVENTION

GRANTS ACTIVE DURING FY84

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. AUERBACH, Arleen D Rockefeller University 5 R01 CA 33948-03	Effects of Anticarcinogens on Fanconi Anemia Chromosomes
2. AWASTHI, Yogesh C University of Texas Medical Branch (Galveston) 5 R01 CA 27967-05	Mechanism of Anticarcinogenic Effect of Antioxidants
3. BAILEY, George S Oregon State University 5 R01 CA 34732-02	Mechanisms of Inhibition of Chemical Carcinogenesis
4. BANERJEE, Mihir R Univ of Nebraska (Lincoln) 5 R01 CA 25304-05	Chemical Carcinogenesis Mammary Gland Organ Culture
5. BENEDICT, William F Children's Hospital of Los Angeles 5 R01 CA 31574-03	Ascorbic Acid Transformation and Oncogenic Progression
6. BENSON, Ann M Johns Hopkins University 5 R01 CA 32479-02	Modulation of Enzyme Profiles by Anticarcinogenic Agents
7. BERNSTEIN, Isadore A Univ of Michigan (Ann Arbor) 5 R01 CA 32470-02	Mechanism for Retinoid Neutralization of Tumor Promotion
8. BERTRAM, John S Roswell Park Memorial Institute 2 R01 CA 25484-04A1	Inhibition of In Vitro Transformation by Retinoids
9. BRINCKERHOFF, Constance E Dartmouth College 5 R01 CA 32476-03	Action of Retinoids on Synovial Cells
10. CHOPRA, Dharam P Southern Research Institute 5 R01 CA 26696-03	Biology of Airway Epithelial Lesions
11. CHUNG, Fung-Lung American Health Foundation 5 R23 CA 32272-02	Screening for Inhibitors of N- Nitrosamine Carcinogenesis

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| 12. | CROCE, Carlo M<br>Wistar Institute of Anatomy<br>and Biology<br>5 R01 CA 32495-02             | Retinoic Acid Induced<br>Differentiation                      |
| 13. | CURPHEY, Thomas J<br>Dartmouth College<br>5 R01 CA 32478-02                                   | Pancreatic Cancer and Retinoids--<br>Model and Mechanism      |
| 14. | DAWSON, Marcia I<br>SRI International<br>5 R01 CA 30512-03                                    | Novel Retinoids for Chemo-<br>prevention of Epithelial Cancer |
| 15. | DAWSON, Marcia I<br>SRI International<br>5 R01 CA 32428-02                                    | Retinoid Tumor Inhibitory<br>Activity-Toxicity Probe          |
| 16. | GRUBBS, Clinton J<br>Southern Research Institute<br>5 R01 CA 30986-02                         | Chemoprevention of Cancer Caused<br>by Anticancer Agents      |
| 17. | HADDOX, Mari K<br>University of Texas Health<br>Science Center (Houston)<br>5 R01 CA 32444-02 | Mechanism of Retinoid Inhibition<br>of Cell Proliferation     |
| 18. | HECHT, Stephen S<br>American Health Foundation<br>5 R01 CA 32519-02                           | Chemoprevention of Nitrosamine<br>Carcinogenesis by BHA       |
| 19. | HILL, Donald L<br>Southern Research Institute<br>5 R01 CA 30604-03                            | Prevention of ENU-Induced Brain<br>Cancer by Retinoids        |
| 20. | HORNSBY, Peter J<br>Univ of California (San Diego)<br>5 R01 CA 32468-03                       | Antioxidant Action in a Model Cell<br>Culture System          |
| 21. | JACOBSON, Herbert I<br>Albany Medical College<br>5 R01 CA 32531-02                            | A Marker for Studying Inhibition<br>of Colon Carcinogenesis   |
| 22. | JOHNSON, Eric F<br>Scripps Clinic and Research<br>Foundation<br>5 R01 CA 34910-02             | Modulation of Carcinogen<br>Activation/Detoxification         |
| 23. | KENSLER, Thomas W<br>Johns Hopkins University<br>1 R01 CA 36380-01                            | Biomimetic Superoxide Dismutases<br>as Antitumor Promoters    |
| 24. | KOEFFLER, H Phillip<br>Univ of California (Los Angeles)<br>5 R01 CA 33936-02                  | Action of Retinoids on Myeloid<br>Leukemia Cells              |

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| 25. | KRINSKY, Norman I<br>Tufts University<br>5 R01 CA 32524-02                                      | Anticarcinogenic Mechanisms of<br>Carotenoid Pigments       |
| 26. | LUDLUM, David B<br>Albany Medical College<br>5 R01 CA 32446-03                                  | Repair of Carcinogenic Lesions in<br>DNA                    |
| 27. | MARNETT, Lawrence J<br>Wayne State University<br>5 R01 CA 32506-02                              | Cancer Chemoprevention and<br>Arachidonate Metabolism       |
| 28. | MATHEWS-ROTH, Micheline M<br>Brigham and Women's Hospital<br>5 R01 CA 23053-06                  | Carotenoids as Antitumor Agents<br>for Skin Tumors          |
| 29. | MAYS, Charles W<br>University of Utah<br>5 R01 CA 28314-04                                      | Reducing Cancer Risk By<br>Radionuclide Chelation           |
| 30. | McCORMICK, Anna M<br>University of Texas Health<br>Science Center (Dallas)<br>5 R01 CA 31676-02 | Metabolism of Chemopreventive<br>Retinoids                  |
| 31. | McCORMICK, David L<br>IIT Research Institute<br>5 R23 CA 30646-03                               | Interactions Among Modifiers of<br>Mammary Carcinogenesis   |
| 32. | McCORMICK, J Justin<br>Michigan State University<br>5 R01 CA 32490-03                           | Inhibition of Carcinogen--<br>Transformation of Human Cells |
| 33. | MEDINA, Daniel<br>Baylor College of Medicine<br>5 R01 CA 11944-12                               | Biology of Mammary Preneoplasias                            |
| 34. | MEDINA, Daniel<br>Baylor College of Medicine<br>5 R01 CA 32473-03                               | Selenium Inhibition of Mouse<br>Mammary Tumorigenesis       |
| 35. | MEHTA, Rajendra G<br>IIT Research Institute<br>5 R01 CA 34664-02                                | Hormone and Retinoid Interaction<br>in Mammary Tissue       |
| 36. | MOORE, Malcolm A<br>Sloan-Kettering Institute for<br>Cancer Research<br>5 R01 CA 32516-02       | Mechanisms of Biological<br>Prevention of Leukemogenesis    |
| 37. | NAPOLI, Joseph L<br>University of Texas Health<br>Science Center<br>5 R01 CA 32474-02           | Determinants of Vitamin A<br>Homeostasis                    |

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| 38. NEWBERNE, PAUL M<br>Massachusetts Institute of Tech<br>1 R01 CA 32520-01A2                      | Anticarcinogenic Effects of<br>Selenium and Vitamin A        |
| 39. NILES, Richard M<br>Boston University<br>5 R01 CA 32543-02                                      | The Effect of Retinoids on Growth<br>and Differentiation     |
| 40. ONG, David E<br>Vanderbilt University<br>5 R01 CA 20850-07                                      | Cancer and Vitamin A   |
| 41. PROUGH, Russell A<br>University of Texas Health<br>Science Center (Dallas)<br>5 R01 CA 32511-03 | Inhibitor Effects on Mono-<br>oxygenase Function             |
| 42. REDDY, Janardan K<br>Northwestern University<br>5 R01 CA 32504-03                               | Antioxidants and Peroxisome<br>Proliferator Carcinogenesis   |
| 43. REINERS, John J, Jr<br>University of Texas<br>System Cancer Center<br>1 R01 CA 34469-01         | Inhibition of Chemical<br>Carcinogenesis by Interferon       |
| 44. ROGERS, Adrienne E<br>Massachusetts Institute of Tech<br>5 R01 CA 32498-03                      | Azaserine Carcinogenesis--<br>Effects of Methionine, Choline |
| 45. RUDDLE, Nancy H<br>Yale University<br>5 R01 CA 32447-03   | Lymphotoxin and Interferon<br>Inhibition of Carcinogenesis   |
| 46. SLAGA, Thomas J<br>Univ of Texas System Can Ctr<br>5 R01 CA 34521-02                            | Inhibition of Tumor Promotion<br>by Antioxidants             |
| 47. STEINBERG, Mark L<br>New York University<br>5 R01 CA 32485-02                                   | Effects of Retinoids on Human<br>Epidermal Keratinocytes     |
| 48. STRAUSS, Bernard S<br>University of Chicago<br>5 R01 CA 32436-03                                | Plasminogen Activator and Error-<br>Prone DNA Synthesis      |
| 49. THOMPSON, Henry J<br>University of New Hampshire<br>5 R01 CA 32465-03                           | Breast Cancer Chemoprevention and<br>Polyamine Biosynthesis  |
| 50. WATANABE, Kyoichi A<br>Sloan-Kettering Institute for<br>Cancer Research<br>5 R01 CA 32535-02    | Chemical Mechanisms of<br>Carcinogenesis Protection          |

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| 51. WATTENBERG, Lee W<br>Univ of Minnesota (Mnpls-St Paul)<br>5 R01 CA 14146-12                  | Inhibition of Carcinogenesis by<br>Phenols and Thiols      |
| 52. WIEBEL, Friedrich J<br>GSF-Research Center Gesellschaft<br>fur Strahlen<br>5 R01 CA 32541-02 | Carcinogen Inactivation by<br>Conjugation with Glutathione |
| 53. WOLF, George D<br>Massachusetts Institute of Tech<br>2 R01 CA 13792-07A1                     | Vitamin A and Glycoproteins of<br>Skin Tumors              |
| 54. WOLF, George D<br>Massachusetts Institute of Tech<br>5 R01 CA 32014-02                       | Mechanism of Retinoid Action on<br>Bladder Cancer          |
| 55. YANG, Chung S<br>University of Medicine and<br>Dentistry of New Jersey<br>5 R01 CA 28298-03  | Effects of BHA on Carcinogen<br>Metabolism                 |

CONTRACTS ACTIVE DURING FY 84

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
56. COHEN, Leonard A American Health Foundation N01-CP-05722	Dose Response Studies on Phenolic Antioxidants (Mammary Gland)
57. DAWSON, Marcia I SRI International N01-CP-05600	Synthesis of New Retinoids for the Chemoprevention of Epithelial Cancer
58. HICKS, Marion R Middlesex Hospital Medical School N01-CP-05602	Chemoprevention of Epithelial Cancer by Retinoids (Bladder)
59. KURTZ, Perry J Battelle Memorial Institute N01-CP-85650	Studies on Toxicology of Retinoids
60. McMURRAY, John E Cornell University N01-CP-05716	Synthesis of New Retinoids for the Chemoprevention of Epithelial Cancer
61. MOON, Richard C IIT Research Institute N01-CP-05718	Chemoprevention of Epithelial Cancer by Retinoids (Mammary Gland)

62. OKAMURA, William H  
Univ of California (Riverside)  
N01-CP-05715  
Synthesis of New Retinoids for the  
Chemoprevention of Epithelial  
Cancer
63. REDDY, Bandaru S  
American Health Foundation  
N01-CP-05721  
Dose Response Studies on Phenolic  
Antioxidants (Intestinal Tract  
Model)
64. SHELLABARGER, Claire J  
Department of Energy/Brookhaven  
National Laboratories  
Y01-CP-00202  
Chemoprevention of Epithelial  
Cancer by Retinoids (Mammary  
Gland)
65. WATTENBERG, Lee W  
University of Minnesota  
N01-CP-05605  
Dose Response Studies on Phenolic  
Antioxidants
66. WELSCH, Clifford W  
Michigan State University  
N01-CP-05717  
Chemoprevention of Epithelial  
Cancer by Retinoids (Mammary  
Gland)
67. WILLIAMS, Gary M  
American Health Foundation  
N01-CP-05723  
Dose Response Studies on Phenolic  
Antioxidants (Liver)



## SUMMARY REPORT

### CARCINOGENESIS MECHANISMS

The Carcinogenesis Mechanisms component of the Branch includes studies relating to the metabolism and mechanisms of action of carcinogens and their metabolites. Studies involving the synthesis of both known and suspect carcinogens or the development of molecular structure-activity relationships are also included. A major component of this portfolio of grants focuses on metabolism, toxicity, and physiological disposition of carcinogens and their ultimate carcinogenic metabolites. Other studies deal with the isolation and/or characterization of carcinogen metabolizing enzymes and structure transforming reactions. Finally, the Mechanisms component also includes a number of studies dealing with endocrine-related biochemistry of cancer and cancerous hosts.

The sole instrument of support for this area is the research grant. In FY 1984 there were three program projects (P01), one young investigator grant (R23), and 115 traditional investigator grants (R01) with a total support funding of \$11.17 million. It should be noted that in Table II of this report only 92 grants are attributed to this Branch component. This lower number represents those which actually received FY 1984 funds and not those in their terminal year or presently operating on a no-cost extension.

#### Grants Activity Summary

The grants in the program are most easily classified by the agent under study. Approximately half are concerned with studies of polycyclic aromatic hydrocarbons, alkylating agents, and aromatic amines. The remainder consists partly of studies involving many carcinogens from more than one of the above-mentioned groups; plus investigations of the mechanisms of action of materials, such as fibers or foreign bodies, and a variety of chemicals that do not fall into the above-mentioned classifications.

Polycyclic Aromatic Hydrocarbon: The oxidation of aromatic hydrocarbons is a subject of intensive investigation because many members of the class of compounds exhibit toxic, mutagenic, or carcinogenic properties. At the present time there are 19 grants in this component that are devoted to various aspects of polycyclic aromatic hydrocarbon (PAH) carcinogenicity. Included are studies on the metabolism of specific PAHs, on mechanisms of action of selected series of PAHs, and of structure-activity relationships.

One project (27) is studying metabolic pathways used by prokaryotic and eukaryotic microorganisms to oxidize benzo(a)pyrene (BaP), benz(a)anthracene (BaA), and 3-methylcholanthrene (3-MC). Available evidence indicates that bacteria use a dioxygenase reaction to initiate oxidation of the aromatic nucleus. The first detectable products are dihydrodiols in which the hydroxyl groups have a cis-relative stereochemistry. In contrast, fungi use a monooxygenase enzyme system to oxidize aromatic hydrocarbons. The initial reaction products are almost certainly arene oxides, which are the precursors of phenols, trans-dihydrodiols, and conjugated derivatives.

Previous studies from this laboratory have shown that a mutant strain of Beijerinckia oxidizes benzo(a)anthracene to cis-dihydrodiols at the 1,2-, 8,9- and 10,11- positions. The dihydrodiols have been separated by high pressure liquid chromatography and have been shown to be formed in a ratio of 73:15:12,

respectively. With collaborators they have synthesized each dihydrodiol in optically pure form from precursors whose configurations were previously known or have been assigned by optical and chemical procedures. These studies have shown that the bacterial metabolites are of very high enantiomeric purity and have (1R,2S)-, (8R,9S)- and (10S, 11R)-absolute configurations. The chemical inter-relationships of absolute configuration have been done in such a manner that the cis 1,2-, 8,9-, and 10,11-dihydrodiols formed by the bacterium are tied directly to structures which have been used to assign the corresponding trans 1,2-, 8,9- and 10,11-dihydrodiols formed from benzo(a)anthracene in mammalian liver.

Experiments with the parent strain of *Beijerinckia* have shown that this organism is capable of degrading the aromatic rings of benzo(a)anthracene. Whole cells oxidized benzo(a)anthracene to two acid products (I and II) which were separated by high pressure liquid chromatography (HPLC).

These investigators made an unusual observation with respect to the initial oxidation of polycyclic hydrocarbons by the *Beijerinckia* species. All previous work with this organism indicates that it utilizes a dioxygenase enzyme system to incorporate both atoms of molecular oxygen into the hydrocarbon substrate to form cis-dihydrodiols. However, when acenaphthene is used as a substrate, the initial product formed is acenaphthenol in which a single hydroxyl group is introduced into the saturated five-membered ring. Further oxidation leads to the formation of acenaphthenone and acenaphthoquinone. All three metabolites were isolated and shown to have identical physical properties to synthetic standard compounds. These observations could provide valuable information on the mechanism of oxygen insertion into polycyclic aromatic hydrocarbons and will be investigated further during the next grant period.

An attempt is being made in one project to study the early critical events in PAH carcinogenesis in mouse skin as a specific target tissue. An initiation-promotion protocol for initiation of skin tumors in mice has been developed for these studies, using inbred mice that have been selectively bred for susceptibility and resistance to two-stage carcinogenesis. The covalent binding of (+) anti-benzo(a)pyrene-7,8-diol-9,10-epoxide (+)-anti-BPDE, the carcinogenic metabolite of benzo(a)pyrene, and its noncarcinogenic (-) enantiomer to macromolecules was investigated in mouse skin in vivo. Levels of the adducts were measured in DNA samples isolated from the epidermis of adult SENCAR mice exposed topically to (+)- and (-)-anti-BPDE for 3, 24 and 72 hrs. The amount of (+)-anti-BPDE bound to epidermal DNA was 3 times higher than that of the (-) enantiomer at all time points studied, with the highest level of adducts observed after 3 hrs. exposure. A similar time course of binding was observed in DNA purified from epidermal basal cells which were isolated from mice treated with the two enantiomers. As with the results for isolated DNA samples from whole epidermis, the investigator also observed a 3:1 ratio of binding with (+)- and (-)-anti-BPDE in basal cell DNA. Interestingly, no significant difference in total binding between the (+) and (-) enantiomers could be detected at any time point in RNA and protein isolated from the basal cells (96).

The formation of individual DNA adducts derived from topically applied (+) or (-)-anti BPDE was monitored at 3, 24 and 72 hrs. using HPLC. The major DNA adduct (64% of total) formed from (+)-anti-BPDE cochromatographed with marker adducts of N<sup>2</sup>-(10-S-(7R,8S,9R-trihydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene)deoxyguanosine, while other minor adducts also were observed. With the (-)-anti BPDE, a greater variety of DNA adducts was formed, with only 20 to 30% of the radioactivity present in HPLC chromatograms corresponding to the N<sup>2</sup>-deoxyguanosine adduct. The

rate of formation and disappearance of individual adducts derived from both isomers of anti-BPDE was similar over the 72-hr. time course. The results suggest that, although differences exist in total binding to DNA between the two enantiomers, they do not appear to be of sufficient magnitude to explain the marked difference in biological activity of (+)- and (-)-anti BPDE in mouse skin.

Several grants support development of new synthetic methods for PAHs and their analogs. In one project, the principal investigator is developing stereo- and regiochemically controlled synthetic methods for bay-region diol-epoxide metabolites of BaA, 7,12-dimethylbenz(a)anthracene (DMBA) and 3-MC plus the putative active metabolites of azuleno(1,2,3-cd)phenalene and cyclopenta(a)-phenanthrene. The chemical properties of the biologically active compounds along with their in vitro and in vivo reactions with nucleic acids are being investigated in conjunction with the bioorganic mode of action of the parent hydrocarbons and their metabolites (52).

Two grants are supporting research where methylated derivatives of PAH are being synthesized for purposes of studying molecular structure-activity relationships. One is studying the mechanistic basis for the enhancing effect in carcinogenicity of a bay-region methyl group and the inhibiting effect of a substituted peri position. Specific methylated isomers of benzo(b)fluoranthene and benzo(j)fluoranthene are being synthesized and bioassayed in order to determine the structural requirements for carcinogenicity of these compounds (41). Another group is evaluating the molecular basis for the carcinogenic differences of the 12 methylbenzo(a)anthracenes (MeBaA). This is being done by assessing the differences in metabolic pathways that may account for their different carcinogenicities and determining their mutagenicity and tumor-initiating ability. These investigators have shown that liver microsomes from 3-MC pretreated male Sprague-Dawley rats metabolized 12-MeBaA predominately to (-)trans-5,6-dihydrodiol with S,S absolute stereochemistry as the predominant enantiomer. Under similar conditions, the major enantiomer formed from BaA is (+)BA-trans-5R,6R-dihydrodiol. According to the authors, this is the first example indicating that the methyl substituent of a PAH can drastically alter the stereo-selective preference of the microsomal drug-metabolizing enzyme systems toward a substrate in the formation of a dihydrodiol metabolite at an unsubstituted aromatic double bond. The same group has reported that in the case of 8-MeBaA, the major enantiomeric trans-dihydrodiol formed at the 3,4- and 5,6-double bonds, respectively, have the same absolute configuration regardless of the rat liver microsomal enzyme system used in the in vitro incubation of 8-MeBaA. However, the results of the study indicate that cytochrome P-448 and cytochrome P-450 interact with different faces of the 8,9-double bond of 8-MeBaA. In these experiments, the rat liver enzyme systems were obtained from rats pretreated with either 3-MC (activates cytochrome P-448) or phenobarbital (activates cytochrome P-450). The 8-MeBaA-trans-8,9-dihydrodiol formed by liver microsomes from 3-MC treated rats or by a reconstituted rat liver enzyme system containing cytochrome P-448 and epoxide hydrolase was enriched with the (-)-enantiomer. The 8-MeBaA trans-8,9-dihydrodiol formed by liver microsomes from either untreated or phenobarbital-treated rats was enriched with the (+)-enantiomer. The authors state that this indicates that a methyl substitution can drastically alter the stereo-selective property of the drug-metabolizing enzyme systems toward a substrate molecule at the methyl-substituted aromatic double bond (116).

In another project, the investigators are synthesizing bay-region diol-epoxides of aza-substituted PAHs, specifically of benz(a)acridine (BaACR) and benz(c)acridine

(BaCR) and testing them for biological activity. The mutagenic activities of BaCR, BcCR and a number of their derivatives, including 12 epoxides and diol epoxides have been examined in *Salmonella typhimurium* strains TA98 and TA100 and in Chinese hamster V79 cells to determine the importance of bay-region activation of aza-PAH. The bay-region diol-epoxides and tetrahydroepoxides of BcCR were found to be from 1 to 4 orders of magnitude more mutagenic to bacterial and mammalian cells than were their non-bay-region counterparts. These results suggest that the bay-region theory of PAH carcinogenicity can be extended to certain aza-PAHs. The most striking structure-activity relationship deduced from the study is that the position of the nitrogen heteroatom in the aromatic ring system has a profound effect in the biological activity of the epoxides. In all mutagenic test systems studied, the bay-region diol-epoxides and tetrahydro-epoxides of BcCR are substantially more mutagenic than are the analogous derivatives of BaCR. Tumorigenicity studies of many of these compounds are in progress, and consistent with the mutagenicity results, the substituted BcCRs are significantly more tumorigenic than the substituted BaCRs (53). Another grant is supporting studies on the metabolic disposition of selected nitrogen containing PAHs in mammalian systems in vivo and in vitro. BcCR is one of the compounds under study (66).

It is known that certain hydrocarbons can potentiate the carcinogenicity of some carcinogenic hydrocarbons and can inhibit cancer induction by others. The effects of these hydrocarbons in the metabolic activation of carcinogenic PAHs and the induction of biological effects in hamster embryo cell cultures is the subject of another grant in this group. A recent report described the effects of benzo(e)-pyrene (BeP) on the metabolism and binding of BaP-7,8-diol in hamster embryo cell cultures. Earlier, this group had shown that simultaneous treatment with BeP increased the diols, decreased the water soluble metabolites, and altered the DNA adducts formed from BaP in early passage Syrian hamster embryo cell cultures. In this study, it was found that as the concentration of BeP increased, the amount of BaP-7,8-diol metabolized decreased. Both oxidation of the BaP-7,8-diol to more polar derivatives and conjugation to glucuronic acid decreased as the concentration of BeP increased. The amount of BaP-7,8-diol bound to DNA decreased as the concentrations of BeP increased. The major DNA adducts formed in all cultures resulted from the binding of the anti- and syn-isomer of BaP-7,8-diol-9,10-epoxide. The ratio of the anti- to syn-diol-epoxide-DNA adducts decreased as the concentration of BeP increased. These results, in the authors' opinion, demonstrate that BeP induces concentration-dependent decreases in the metabolism and DNA binding of BaP-7,8-diol and alters the proportion of the two isomeric diol-epoxides bound to DNA suggesting that the major effects of BeP on BaP metabolism involve alterations in the secondary metabolism of BaP (3).

Alkylating Agents: Thirteen grants fall under this heading, ten of which are concerned exclusively with either nitrosamines or nitrosoarenes. Chemical studies of nitrosamine formation are being performed in depth in work supported by two grants. In one, competitive routes for production of nitrosamines from tertiary amines via nitrosoammonium ion formation are being investigated. The overall goal of this project is to investigate new routes for nitrosamine formation and to find ways of inhibiting these processes (58). These investigators have discovered a new mode of nitrosamine formation which involves what they term ester-mediated nitrosation. The past year has produced a better understanding of the scope of this environmentally significant transformation. Examination of a group of structurally varied amines revealed that nitrosamines are formed from the heating of either a secondary or a tertiary amine with sodium nitrite in the presence of a high boiling ester such as 2-acetoxyethanol in ethylene glycol. All four

secondary and six tertiary amines examined were found to produce nitrosamines in yields ranging from 4% to 80% when equal molar amounts of amine and ester were heated at 120° with one- to ten-fold equivalents of sodium nitrite in ethylene glycol. Secondary amines competitively produced acetamides at a rate slightly greater than nitrosamine formation. Preincubation of a large excess of sodium nitrite and ester led to the rapid formation of nitrosamines in high yield. The reaction of tribenzylamine resulted in the formation of benzaldehyde as well as dibenzyl nitrosamine. N,N-Dimethyltrosamine reacted to give nearly equal molar amounts of dimethylnitrosamine and benzylmethylnitrosamine.

Their preliminary work produced evidence that the nitrosamine yield in many of these reactions proceeds through a maximum. This observation is of interest because it could lead to a method for the destruction of nitrosamines. They have found that yield maximum is not the result of experimental artifact. Dibenzyl nitrosamine was found to undergo facile photochemical decomposition in room light but control of this variable still results in the observation of the maximum.

These reactions are kinetically complex and secondary and tertiary amine nitrosations show different types of time dependence. The results suggest that the nitrosating agent may be a nitrous ester. They have shown that 2-benzyloxyethyl nitrite rapidly nitrosates secondary and tertiary amines under the reaction conditions.

The authors believe that these transformations are good models for the environmental formation of nitrosamines in foods and commercial products. Previous researcher's attention has been focused on acidic nitrosation conditions which are rarely encountered in foods and commercial stuffs. Nitrite is both a common additive and contaminant of many substances containing fat or fat-like materials (high boiling esters such as the model used in this work). Inadvertant heating often occurs during the process of commercial formulation and ester mediated nitrosation could explain nitrosamine formation under these conditions as well as those used in the cooking of nitrite containing meats. Further work will be directed revealing the reaction scope with respect to the ester and elucidation of the reaction mechanism (59).

Several grants support research on metabolism and mechanisms of action of N-nitroso-compounds. One project is investigating the metabolism of various N-nitroso drugs and related compounds in a variety of animals including the pig (61). The mechanism by which two nitrosoureas induce liver tumors in rats is being studied in another project. This observation is unusual since most compounds of this class do not induce liver tumors in the rat (70). Under the mechanisms of action designation, the ability of several N-nitroso compounds to alkylate cellular macromolecules, particularly DNA and RNA, is being studied. In one project, the principal investigator is investigating the mechanism by which certain dialkyl nitrosamines and their beta-oxidized derivatives methylate nucleic acids in rat liver (1). In another project, the ability of a variety of nitrosamines and related hydrazo- compounds to alkylate cellular macromolecules is being studied in both in vivo and in vitro systems (61). The mechanism of action of N-nitrosobis(2-oxo-propanol)amine, a potent pancreatic carcinogen in an in vitro system, is the subject of another grant (73).

A new project is investigating the ability of nitrogen dioxide, an important atmospheric pollutant, to form N-nitroso compounds in vivo. This study developed from experiments showing that N-nitrosomorpholine was produced in mice gavaged with morpholine and exposed to atmospheric nitrogen dioxide (69).

Possible correlations between N-nitroso compounds and colon cancer are under investigation in groups of patients drawn from populations known to be at high or at low risk for development of colon cancer. The experiment involves studying nitrate metabolism in these patients under carefully controlled dietary conditions (57).

Aromatic Amines: Various aspects of the chemistry, metabolism, and mechanisms of action of this class of carcinogens are subjects of 12 grants. The majority of grants in this area are concerned with metabolism. Determining the capacity of rat mammary gland to metabolize N-arylamides and N-arylamidohydroxamic acids is the goal of a very interesting project. Identification of metabolites of these carcinogens excreted in the milk of lactating rats and the consequences of lactational transmittal of these compounds in the suckling young are an important component of this project (62). The role of metabolism in the biliary excretion of drugs is the subject of another grant. In this project, the metabolism of the azo dye, N,N-dimethyl-4-aminoazobenzene is being studied in depth. The function of hepatic glutathione in the metabolism and biliary excretion of this compound is under investigation. The roles of iron, lipid peroxidation, and heme oxygenase are also being considered (54). In studies directed toward determining the genetic susceptibility to xenobiotic toxicity, the capacity of primary cultures of hepatocytes to N-acetylate xenobiotics is being determined. The principal investigator is attempting in these *in vitro* systems to reflect the genetically determined acylator polymorphism. The ultimate goal of this project is to determine at a molecular level the toxicity of xenobiotics that are N-acetylated and to further define the relationship between the risk of toxicity and the acylator phenotype of liver cells (67). 4,4'-Methylene-bis(2-chloroaniline) (MCA) is an industrially used aromatic amine which is carcinogenic for rats, mice, and dogs. It is not known whether MCA is carcinogenic in humans, but it bears a structural resemblance to other aromatic amines which are known to be potent human urinary bladder carcinogens. The objective of this study is to compare the metabolic activation of MCA in rats and humans. The studies on rats, both *in vivo* and *in vitro*, are designed to determine which urinary metabolites are related to activation or detoxification of MCA. The studies on humans consist of examining urine from workers occupationally exposed to MCA to determine whether metabolites are present which correspond to activation or detoxification pathways in rats (72).

Other Agents: This grouping is the largest. It consists of 40 grants supporting research with a variety of agents not in the other categories or with mixtures of agents from more than one category. Four grants in this group are concerned with the physical organic chemistry of carcinogens and their analogs and are supporting studies on mechanisms of action from a purely chemical point of view. In one of the most interesting projects, crystallographic techniques are being applied to determine the three-dimensional structures of biologically active molecules and to determine the possible stereochemistry of interaction of these molecules with other molecules during processes of biological interest. Two areas of study are being pursued. The first is an investigation of the stereochemistry of certain enzyme reactions. The second is an investigation of the structures of series of polycyclic mutagens, carcinogens and their metabolic products, and molecular complexes of such compounds. The latter studies were done in order to derive an accurate three-dimensional description of the interaction of an activated carcinogen with a protein or nucleic acid. (28).

Metabolic studies or mechanisms of action of several carcinogens are the subjects of eight grants. Two groups are involved in a collaborative study on the pharma-

cology of carcinogen activation in intact cells. The major goal of these joint projects is to delineate mechanisms whereby nutritional factors and pharmacological agents influence the metabolic activation and conjugation of precarcinogenic and model drug substances in intact cells using isolated perfused organs as experimental models (50), (106). In another project, the principal investigator has shown that carcinogenic chemicals common in the environment can adversely affect the developing preimplantation embryo without killing it. The effect of exposure to such compounds on implantation rate, birth rate, and the development of birth defects or tumors in live offspring from treated blastocysts following transfer to surrogate mothers is now being studied (45). Another grantee is studying carcinogen activation by cultured mammary cells and has recently shown that intraorgan cell specificity exists. Cells from a 50-55 day-old virgin Sprague-Dawley female rat mammary gland was divided into parenchymal and stromal enriched populations. A mediated mutagenesis assay was used to quantitate the ability of these cells to activate carcinogens. They found that the potent mammary carcinogen, DMBA was activated by both mammary parenchymal and stromal cells, while the non-mammary carcinogen, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), was not activated by either cell type. The weak mammary carcinogen BaP was activated by the stromal cells and not by the parenchymal cells from which mammary carcinomas arise. The authors state that their data suggest that the intraorgan relationship between cell types that activate a carcinogen, and cell types that undergo neoplastic transformation may, in part, explain the organ specificity of a carcinogen (33).

Fibers are the subject of six grants, and foreign body carcinogenesis is the subject of the fifth in this grouping. The foreign body (FB) tumorigenesis project has been the most interesting. In a recent report on chromosomal aberrations in FB tumorigenesis of mice, the authors note that virtually all FB sarcomas of mice possess abnormal chromosome numbers. These observations proved to be remarkably consistent in transplantation experiments and in in vitro cultures. Thus, they suspected an etiological connection. Preneoplastic and neoplastic cells were studied at various stages of progression by means of chromosome banding in an attempt to identify specific chromosome changes relative to the tumorigenic process. A considerable variety of numerical chromosome derangements were observed, leading the authors to note that it appears that many different patterns of genetic disorders and imbalance may lead to initiation. They claim that this hypothesis is consistent with observations which indicated the non-uniformity of initiation events in FB tumorigenesis. The primary alteration of the chromosome number in a preneoplastic parent cell may be explained by faulty mitosis with unequal chromosome distribution to the daughter cells. This may be the result of an inherent karyotype lability which is characteristically seen in murine mesenchymal stem cells of the microvasculature, the cell type of FB sarcoma origin. Other murine cell types, such as fibroblasts, do not share that property to the same extent. In man, the vascular system cells appear karyotypically more stable, and this conforms with the finding that human FB sarcomas are rare. Secondary chromosome aberrations were noted to be unstable and variable during early neoplasia. Only preneoplastic cells of advanced FB-reactive capsules and their homologous tumor cells had structural chromosome aberrations in common. Apparently such aberrations gained stability during advanced stages of preneoplastic progression, possibly by giving affected cells a competitive growth advantage. Thus, they may contribute to tumor promotion and modify the determinants of late tumor characteristics. In another report, the same group states that they have unequivocally shown that FB-induced sarcoma cells of mice produce fibronectin. The presence of fibronectin may be related to the general finding that these sarcomas fail to metastasize hematogenously. In every other way, the tumors must be considered to be malignant: cell doubling time

increases abruptly when autonomous growth commences; growth control seems irreversibly lost; histopathologically, many of the tumors are highly anaplastic; they are transplantable; local invasiveness is always apparent, although initial growth is nodular; metastases per continuity into regional lymph nodes do occur. It follows that these properties of malignancy are not contingent on the absence of fibronectin (6).

The remaining 19 grants cover research on metabolism or mechanisms of action of agents not included in any of the above-mentioned categories. In this group, there are two projects studying the reported mutagenicity and carcinogenicity of malondialdehyde (MDA). They are both using purified malondialdehyde and found, contrary to earlier reports, that the compound is only weakly mutagenic (2), (65). MDA is a product of lipid peroxidation and prostaglandin biosynthesis, and as a result it is widely distributed in mammalian tissue. MDA has been reported to be mutagenic and carcinogenic prompting speculation that it is a mediator of spontaneous carcinogenesis or contributes to the enhancement of carcinogenesis in certain tissues by high levels of dietary fat. Previous work from one laboratory confirmed that MDA is a weak mutagen to *Salmonella typhimurium* but indicated that side products generated during its chemical synthesis are 25-35 times more mutagenic than MDA. They have recently found that highly purified MDA is not a tumor initiator, tumor promoter, or complete carcinogen when applied topically to Sencar mice. This finding implies that the side products (beta-alkoxyacroleins) generated during MDA preparation were responsible for the carcinogenicity reported previously.

The well-established fact that many human breast cancers are estrogen-dependent, invites investigators to study the steps and relationships of estrogen biosynthesis in this disease. One group (21A) found that 16 alpha hydroxyestrone, an unusual and highly active estrogen metabolite, is elevated in breast cancer patients. Recent attention has also been focused on catechol estrogens, since their formation is the principal pathway of metabolism of estradiol and other steroidal estrogens in humans and experimental animals. Using cultures of a heteroploid subclone of mouse fibroblast cells that are particularly sensitive to transformation by carcinogenic polycyclic aromatic hydrocarbons, another group (82) has found that both of the catechol metabolites of estradiol are more effective than estradiol as inducers of the neoplastic transformation of these mouse cells. Using the mouse cells, they have found that the relative frequency of neoplastic transformation induced by an estrogen or an estrogen derivative, parallels its relative rate of metabolism to catechols by the microsomal activating system isolated from these cells. Together, these results support the hypothesis that the formation of catechol estrogens is involved in the mechanism of estrogen-induced transformation in these in vitro model systems. This has a direct application to human breast cancer, since these colleagues have found that a correlation exists between the ratio of the catechols of estradiol formed by microsomes from human breast cancers and the estrogen receptor levels of these tumors. Studies in another laboratory (7) focus on the in vitro transformations of estrogens to catechol estrogens with emphasis on the various enzymes involved. The nature of irreversible binding of estrogens to cellular macromolecules was studied by examining the interactions with DNA in vitro. The results suggest that the "reactive" intermediates of estrogens formed by liver microsomes react with the DNA leading to irreversible binding.

Steroid hormones are thought to act as inducers, promoters, or permissive factors in the carcinogenesis of hormone sensitive tissues in experimental animals and humans. Recent studies in one laboratory (55) indicate that non-hormonal events



may be involved in the tumorigenic activity of both natural and synthetic estrogens in the hamster kidney and that the manner in which estrogens are metabolized by P-450 multisubstrate monooxygenases appears pertinent to the process leading to renal cell transformation. Another group (108) reevaluated the promoting activity of contraceptives using both infant mouse and rats as experimental animals and found that these animal models can be used to explore the promotion of hepatocarcinogenesis and tumor progression by synthetic steroids. Utilizing these models it was found that neither methyltestosterone nor oxy-metholone enhanced diethylnitrosamine (DEN)-induced hepatocarcinogenesis. In contrast, oral contraceptives, mestranol, and norethynodrel appeared to exert weak promotive effects, based on gross nodular lesions of the liver. An 80-100% incidence of multinodular hepatocellular carcinomas was observed by other investigators (55) in castrated male hamsters following synthetic estrogen treatment in the presence of 0.2 - 0.4% alpha-naphthoflourene (ANF) in the diet after 8.5 to 10 months. Induction of these liver tumors was detected as early as 3.5 - 4.0 months in pregnancy. This new estrogen induced liver tumor model could be useful to elucidate the causal relationship which exists between estrogenic hormones and hepatic tumors in the human.

Other investigators (26) have been studying prolactin and its relationship to neoplasia particularly with respect to pituitary hormones and breast cancer. This group is investigating the role of beta-endorphin (B-E) as a possible regulator of anterior pituitary function, with particular emphasis on prolactin and gonadotropins. Preliminary studies indicating variation in B-E levels at various times of the menstrual cycle in rhesus monkeys and between intact and ovariectomized pig-tailed monkeys indicate that B-E is related in some way to gonadotropin and sex-steroid secretion.

How estrogenic hormones regulate prolactin gene expression has been studied by another group (32). Last year they reported that an in vitro nuclear transcription system had been set up and estrogen could be shown to have a marked effect on prolactin gene transcription. They extended this work to show that estrogen effects on prolactin gene transcription are probably biphasic and involve at least two mechanisms. First, it appears that occupied estrogen receptors are essential for and closely correlated with transcription. This response is not effected by inhibitors of protein synthesis suggesting that it is a primary response to the hormone. When so-called "weak estrogens" (estrogens that dissociate from receptors rapidly) are administered, prolactin gene transcription is increased but only transiently. However, after 3 to 4 hours it increases again in a relatively stable manner. This second phase of response is blocked by cycloheximide suggesting it is a secondary response. They have also been able to show that in cell cultures of pituitary cells estrogen also increases at least in the first phase of transcription. However, in these cell cultures the percentage of newly synthesized RNA that is from prolactin gene transcription is small and the kinds of studies that can be carried out are limited.

The growth and differentiation of the mammary gland is regulated by the complex interactions of several polypeptide and steroid hormones. The major goals of another group (86) are to elucidate the mechanisms by which hormones control growth and the expression of differentiated function in the normal mammary gland and how these regulatory mechanisms have deviated in hormone-dependent breast cancer. They placed specific emphasis upon studying prolactin regulation of milk protein gene expression in normal and transformed mammary cells as a unique model system for understanding peptide hormone action or gene expression. During the past year they have had several novel observations. They found that prolactin can

rapidly induce the accumulation of casein in RNA. This was the first example of a peptide hormone which binds to a plasma membrane receptor, inducing the accumulation of a specific mRNA. Hydrocortisone in the absence of prolactin does not induce casein mRNA accumulation, but this steroid hormone markedly potentiates the action of prolactin on casein gene expression. Progesterone, when administered prior to or simultaneously with prolactin, will inhibit the induction of casein mRNA. The effects of prolactin are mediated by an increase in both the rate of synthesis, as well as the stability, of casein mRNA. None of the classical "second messengers" of peptide hormone action, such as cyclic nucleotides, prostaglandins, or polyamines were able to mimic completely the action of prolactin on the induction of casein mRNA. These colleagues have prepared a number of genomic subclones isolated from the beta and gamma-casein genes containing exon and limited intronic sequences. These subclones were used as probes for analysis of the primary transcripts of the casein genes.

The responsiveness of human endometrium to the ovarian hormones makes this tissue particularly well suited for the study of hormone-related biochemical events. The biochemical effects of sex hormones on human endometrium were studied by another group (37). They found that cGMP added to cytosol during the period of labeling with 3-<sup>3</sup>H-estradiol at least doubled the levels of estrogen specific binding. Cyclic AMP, under the same conditions, drastically reduced specific binding levels. Using human endometrium cells, sodium molybdate, ATP, and GTP enhanced estrogen binding on whole cell homogenates, but were ineffective when added to cytosol during labeling. Apparently, the actions of these compounds are exerted on plasma membrane-bound guanylate and adenylate cyclases.

Murine dorsolateral prostate has been shown to undergo estrogen and chemical carcinogen-induced neoplastic transformation. By successful manipulation of the in vitro environment, one group of investigators (77) has established a viable, long-term (22 day) culture of C<sub>3</sub>H mouse prostate and explants of Noble rat dorsolateral prostate for 12 days in culture. With these organ culture systems they will test the concept that sex hormones promote the action of chemical carcinogen by modulating prostatic basal-cell proliferation and epithelial differentiation.

A specific inhibitor of aromatase, which appears to be active in the DMBA rat models, has been devised in another laboratory (21). Tumor proliferation was inhibited during treatment with this aromatase inhibitor, but when treatment was discontinued the tumor started growing again.

Determination of whether there is a relationship between surface charge and the ability of various potentially toxic and carcinogenic particulate metal compounds to be phagocytized by cultured Syrian hamster embryo cells and Chinese hamster ovary cells is the subject of another project in this section (13). Another group is evaluating the possible role of mutagens in rat urinary bladder carcinogenesis induced by the administration of sodium saccharin following freeze ulceration of the bladder. Preliminary results suggested that the carcinogenic process can occur without a mutational event since neither freeze ulceration nor sodium saccharin are mutagenic. A series of experiments have been designed to follow up this interesting observation (10).

Carcinogen metabolism by the adrenal cortex is the subject of another grant. The adrenal cortex is adversely affected by a number of foreign compounds including carbon tetrachloride (CCl<sub>4</sub>) and polychlorinated biphenyls, but the role of adrenal metabolism in the toxicity of such compounds has not been determined. This group

recently reported on studies that were carried out to evaluate the effects of  $CCl_4$  administration on adrenal and hepatic corticosteroid metabolism in rats. Their results indicate that exposure to  $CCl_4$  decreases plasma corticosterone concentration, probably the net result of effects on adrenal corticosterone production and on hepatic steroid metabolism. However, the toxicological significance of these observations are not presently known. This group also reported on a study of the effects of adrenocorticotrophic hormone (ACTH) administration to guinea pigs on the activities of adrenal microsomal monooxygenases. ACTH treatment decreased the rates of adrenal benzphetamine demethylation and BaP hydroxylation but had no effect on the same reactions in hepatic microsomes. Adrenal microsomal steroid hydroxylation reactions were unaffected (21-hydroxylation) or stimulated (17 alpha-hydroxylation) by ACTH. Although ACTH treatment decreased adrenal BaP hydroxylase activity, the relative quantity of the various BaP metabolites as determined by HPLC, did not change. Adrenal microsomal cytochrome P-450 concentrations were decreased by ACTH, but proportionately less than the decreases in adrenal xenobiotic metabolism. The results of these experiments indicate that ACTH selectively decreases the rates of adrenal xenobiotic metabolism, perhaps by producing a selective decline in the concentrations of those cytochrome P-450s involved in the metabolism of foreign compounds (11).

## CARCINOGENESIS MECHANISMS

## GRANTS ACTIVE DURING FY84

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ARCHER, Michael C Ontario Cancer Institute 5 R01 CA 26651-05	Mechanism of Nitrosamine Alkylation of DNA and RNA
2. ASTLE, Lynn University of Utah 5 R01 CA 32628-02	Gastrointestinal Carcinogenicity of Malondialdehyde
3. BAIRD, William M Purdue University, West Lafayette 5 R01 CA 28825-04	Modifiers of Chemical Carcinogenesis in Cell Culture
4. BHARGAVA, Madhu M Yeshiva University 1 R01 CA 32268-01A2	Protein Binding in Hepatic Fate of Chemical Carcinogens
5. BOKKENHEUSER, Victor D St Luke's-Roosevelt Institute for Health Sciences 5 R01 CA 25763-09	Bacteria and Steroid Metabolism
6. BRAND, K Gerhard Univ of Minnesota (Mnpls-St Paul) 5 R01 CA 10712-16	Initiation and Promotion in Foreign Body Tumorigenesis
7. BRUEGGEMEIER, Robert W Ohio State University 5 R01 CA 28578-03	Biotransformations of Estrogens and Cancer
8. BUHLER, Donald R Oregon State University 5 R01 CA 22524-06	Pyrrrolizidine Alkaloid Toxicity, Metabolism and Binding
9. CAVALIERI, Ercole L Univ of Nebraska Medical Center 5 R01 CA 32376-02	Mechanisms of Mammary Carcinogenesis by Hydrocarbons
10. COHEN, Samuel M Univ of Nebraska Medical Center 5 R01 CA 32313-03	Non-Mutational Multistage Urinary Bladder Carcinogenesis
11. COLBY, Howard D West Virginia University 5 R01 CA 22152-05	Adrenal Carcinogen Metabolism
12. CORBETT, Michael D University of Florida 5 R01 CA 32385-03	Hydroxamic Acid Production in Microbial Ecosystems

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| 13.  | COSTA, Max<br>University of Texas Health<br>Sciences Center (Houston)<br>5 R01 CA 29581-03       | Surface Charge and Phagocytosis--<br>Toxic Metal Particulates |
| 14.  | COX, Ray<br>University of Tennessee Center<br>Health Sciences<br>2 R01 CA 15189-10               | Ethionine Carcinogenesis                                      |
| 15.  | CRAIGHEAD, John E<br>University of Vermont and<br>State Agriculture College<br>1 R01 CA 36993-01 | Experimental Asbestos-Induced<br>Mesothelioma                 |
| 16.  | CUCHENS, Marvin A<br>Univ of Mississippi Medical Ctr<br>1 R01 CA 33111-01A1                      | Carcinogenesis of B-Lymphocytes<br>Peyer's Patches            |
| 17.  | DIGIOVANNI, John<br>Univ of Texas System Cancer Ctr<br>1 R01 CA 36979-01                         | Role of DNA-Binding in Skin Tumor<br>Initiation               |
| 18.  | EL-BAYOUMY, Karam E<br>American Health Foundation<br>1 R01 CA 35519-01                           | Nitroaromatics: Carcinogenicity<br>and Metabolism             |
| 18A. | ENSLEIN, Kurt<br>Health Designs Inc<br>1 R43 CA 37494-01   | SAR Estimation of Carcinogenesis<br>Bioassay Results          |
| 19.  | ERNSTER, Lars<br>University of Stockholm<br>5 R01 CA 26261-05                                    | The Metabolism of Polycyclic<br>Hydrocarbons and Cancer       |
| 20.  | FANNING, James Collier<br>Clemson University<br>1 R01 CA 35733-01                                | The Nitrosation of Amines with<br>Iron Nitrates               |
| 21.  | FIELD, Lamar<br>Vanderbilt University<br>5 R01 CA 30321-03                                       | Thiono-Type Compounds and Their<br>Relation to Cancer         |
| 21A. | FISHMAN, Jack<br>Rockefeller University<br>5 P01 CA 22795-06                                     | Specialized Center for Cancer<br>Endocrinology                |
| 22.  | FLOYD, Robert A<br>Oklahoma Medical Research Fdn<br>2 R01 CA 18591-08A1                          | Carcinogen Free Radicals in<br>Arylamine Metabolism           |
| 23.  | FORD, George P<br>Pacific Northwest Research Fdn<br>5 R01 CA 30475-03                            | The Prediction of Nucleoside-<br>Carcinogen Reactivity        |

24. FRANKEL, Fred R  
University of Pennsylvania  
5 R01 CA 17301-08  
Mammary Cancer and the Nuclear  
Estradiol Receptor
25. FRANKLIN, Michael R  
University of Utah  
5 R01 CA 15760-10  
Modification of Procarcinogen  
Enzymatic Activation
26. FRANTZ, Andrew G  
Columbia University  
5 R01 CA 11704-13  
Studies on Prolactin and Other  
Peptides
27. GIBSON, David T  
University of Texas (Austin)  
5 R01 CA 19078-09  
Microbial Degradation of  
Carcinogenic Hydrocarbons
28. GLUSKER, Jenny P  
Institute for Cancer Research  
5 R01 CA 10925-35  
Application of Crystallographic  
Techniques
29. GOLD, Avram  
Univ of North Carolina, Chapel Hill  
5 R01 CA 28622-03  
Activation of Polycyclic  
Environmental Mutagens
30. GOLD, Barry I  
University of Nebraska Med Ctr  
5 R01 CA 24554-05  
Epoxidation in Chloro-Olefin  
Carcinogenesis
31. GOLDMAN, Peter  
Harvard University  
5 R01 CA 34957-02  
Carcinogen Metabolism by Host  
Intestinal Bacteria
32. GORSKI, Jack  
Univ of Wisconsin (Madison)  
5 R01 CA 18110-09  
Prolactin Synthesis in Normal and  
Neoplastic Tissue
33. GOULD, Michael N  
Univ of Wisconsin (Madison)  
5 R01 CA 28954-03  
Carcinogen Activation by Cultured  
Mammary Cells
34. GREENE, Geoffrey L  
University of Chicago  
2 R01 CA 02897-28  
Steroids and Growth
35. GROVER, Philip L  
University of London  
2 R01 CA 21959-07  
Mechanisms of Activation of  
Polycyclic Hydrocarbons
36. GUENTHNER, Thomas M  
University of Illinois (Chicago)  
5 R01 CA 34455-02  
Toxicologic Implications of  
Multiple Epoxide Hydrolases
37. GURPIDE, Erlio  
Mount Sinai School of Medicine  
5 R01 CA 15648-11  
Steroid Dynamics in Human  
Endometrial Cancer

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|-----|--|--|
| 38. | GURTOO, Hira L<br>Roswell Park Memorial Institute<br>5 R01 CA 25362-05       | Genetics of Aflatoxin Metabolism--<br>Role in Carcinogenesis |
| 39. | HARRINGTON, George W<br>Temple University<br>2 R01 CA 18618-09               | Electroanalytical Studies of<br>N-Nitrosamines               |
| 40. | HARVEY, Ronald G<br>University of Chicago<br>1 R01 CA 36097-01               | Mechanism of Carcinogenesis of<br>Polycyclic Hydrocarbons    |
| 41. | HECHT, Stephen S<br>American Health Foundation<br>5 R01 CA 32242-02          | Carcinogenic Methylated PAH:<br>Structural Requirements      |
| 42. | HICKS, Ruth M<br>University of London<br>5 R01 CA 31082-03                   | Carcinogenesis in Human and Rat<br>Bladder Tissues           |
| 43. | HOLLENBERG, Paul F<br>Northwestern University<br>5 R01 CA 16954-08           | Hemoprotein-Catalyzed Oxygenations<br>of Carcinogens         |
| 44. | HYLEMON, Phillip B<br>Virginia Commonwealth University<br>5 R01 CA 17747-10  | Bile Acids and Large Bowel<br>Carcinogenesis                 |
| 45. | IANNACCONE, Philip M<br>Northwestern University<br>5 R01 CA 29675-03         | Effects of Exposure to Carcinogens<br>on Blastocysts         |
| 46. | JEFCOATE, Colin R<br>Univ of Wisconsin (Madison)<br>5 R01 CA 16265-09        | DNA Modification by Polycyclic<br>Hydrocarbons               |
| 47. | JENSEN, David E<br>Temple University<br>2 R01 CA 31503-03                    | Chemical Decomposition of Alkylating<br>Nitroso Compounds    |
| 48. | JUDD, Howard L<br>Univ of California (Los Angeles)<br>2 R01 CA 23093-06A1    | Estrogen and Androgen Studies in<br>Endometrial Cancer       |
| 49. | KAUFFMAN, Frederick C<br>Univ of Maryland (Baltimore)<br>5 R01 CA 20807-07   | Pharmacology of Carcinogen<br>Activation in Intact Cells     |
| 50. | KAUFMAN, David G<br>Univ of North Carolina, Chapel Hill<br>5 R01 CA 32239-03 | Species Comparison of Uterine<br>Carcinogenesis              |
| 51. | KING, Charles M<br>Michigan Cancer Foundation<br>2 R01 CA 23386-07           | Mechanistic Approaches to<br>Carcinogenesis                  |

52. KOREEDA, Masato  
University of Michigan (Ann Arbor)  
5 R01 CA 25185-06  
The Bio-organic Chemistry of Arene Oxides
53. LEHR, Roland E  
University of Oklahoma (Norman)  
5 R01 CA 22985-08  
Diol Epoxide and Other Derivatives of PAH and AZA-PAH
54. LEVINE, Walter G  
Yeshiva University  
5 R01 CA 14231-11  
Role of Metabolism in the Biliary Excretion of Drugs
55. LI, Jonathan J  
Univ of Minnesota (Mnpls-St Paul)  
2 R01 CA 22008-07A1  
Estrogen Carcinogenicity and Hormone Dependent Tumors
56. LIEHR, Joachim G  
Univ of Texas Health Science Center (Houston)  
5 R01 CA 27539-05  
Mechanism of Estrogen-Induced Renal Carcinogenesis
57. LIPKIN, Martin  
Sloan-Kettering Institute for Cancer Research  
5 R01 CA 28805-03  
Nitrate Metabolism in Gastrointestinal Cancer
58. LOEPPKY, Richard N  
University of Missouri (Columbia)  
2 R01 CA 22289-07  
Nitrosamine Fragmentation and Nitrosamine Carcinogenesis
59. LOEPPKY, Richard N  
University of Missouri (Columbia)  
5 R01 CA 26914-05  
Carcinogenesis: Nitrosamine Formation and Inhibition
60. LOTLIKAR, Prabhakar D  
Temple University  
5 R01 CA 31641-03  
Modulation of Mycotoxin Carcinogenesis by Glutathione
61. MAGEE, Peter N  
Temple University  
5 R01 CA 23451-07  
Formation and Metabolism of N-Nitroso Compounds
62. MALEJKA-GIGANTI, Danuta  
Univ of Minnesota (Mnpls-St Paul)  
2 R01 CA 28000-05  
Mammary Carcinogenesis by N-Substituted Aryl Compounds
63. MANDEL, Richard  
Boston University  
5 R01 CA 27324-05  
Additive and Synergistic Effects of Mutagens
64. MARCHOK, Ann C  
Oak Ridge National Laboratory  
1 R01 CA 34137-01A1  
Effects of HCHO and Benzopyrene in A New Tracheal Model



65. MARNETT, Lawrence J  
Wayne State University  
5 R01 CA 22206-06  
Studies on Malondialdehyde
66. McMURTREY, Kenneth D  
Univ of Southern Mississippi  
5 R01 CA 29903-03  
Toxicology of Polynuclear Hetero-  
cyclic Carcinogens
67. McQUEEN, Charlene A  
American Health Foundation  
5 R01 CA 33144-02  
Genetic Susceptibility to Xenobiotic  
Toxicity
68. MILLER, Richard K  
University of Rochester  
5 R01 CA 22335-06  
Transplacental Carcinogenesis
69. MIRVISH, Sidney S  
Univ of Nebraska Medical Center  
1 R01 CA 32192-01A1  
N-Nitroso Compounds Formed In Vivo  
from Nitrogen Dioxide
70. MIRVISH, Sidney S  
Univ of Nebraska Medical Center  
1 R01 CA 35628-01  
Nitrosamine Metabolism in the  
Esophagus
71. MORRISON, Harry A  
Purdue University, West Lafayette  
5 R01 CA 18267-07  
Cutaneous Photobiology and Drug  
Phototoxicity
72. MORTON, Kenneth C  
Michigan Cancer Foundation  
5 R01 CA 32303-02  
Metabolism of Activation of 4,4'-Met  
-Bis(2-Chloroaniline)
73. NAGEL, Donald L  
Univ of Nebraska Medical Center  
5 R01 CA 31016-03  
An In Vitro for Alkylation by  
Pancreas Carcinogens
74. NEWMAN, Melvin S  
Ohio State University  
2 R01 CA 07394-18A2  
Synthesis of Substituted Polycyclic  
Hydrocarbons
75. NICOLAOU, Kyriacos C  
University of Pennsylvania  
1 R01 CA 36196-01  
Synthesis and Biology of Unstable  
Natural Products
76. O'FLAHERTY, Ellen J  
University of Cincinnati  
5 R01 CA 29917-03  
Quantitative Considerations in  
Urethan Carcinogenesis
77. OFNER, Peter  
Tufts University  
5 R01 CA 29513-03  
Androgens in Prostatic and  
Epididymal Culture
78. PAQUETTE, Leo A  
Ohio State University  
5 R01 CA 12115-14  
Unsaturated Polyolefins and Hydro-  
carbon Carcinogenesis

79. PARTHASARATHY, Rengachary  
Roswell Park Memorial Institute  
5 R01 CA 23704-06  
Stereochemistry of Thiol-Disulfide Interchanges
80. PIETTE, Lawrence H  
Univ of Hawaii at Manoa  
5 R01 CA 10977-18  
ESR Studies of Biological Free Radical Mechanisms
81. POUR, Parviz M  
Univ of Nebraska Medical Center  
1 R01 CA 35042-01A1  
Prevention of Nasal Cavity Tumors by Castration
82. PURDY, Robert H  
Southwest Foundation for Biomedical Research  
5 R01 CA 24629-06  
Mutagenic and Carcinogenic Potential of Estrogens
83. REINKE, Lester A  
Univ of Oklahoma Health Science Ctr  
2 R01 CA 30137-04  
Influence of Ethanol on Carcinogen Activation
84. RIGBY, James H  
Wayne State University  
1 R01 CA 36543-01  
Synthesis of Cocarcinogenic Diterpenes
85. ROMAN-FRANCO, Angel A  
University of Puerto Rico  
5 R01 CA 28894-03  
Mechanism of Action of Carcinogenic Fibers
86. ROSEN, Jeffery M  
Baylor College of Medicine  
2 R01 CA 16303-09  
Hormonal Regulation of Breast Cancer
87. SCARPELLI, Dante G  
Northwestern University  
5 R01 CA 34051-02  
Metabolism of Pancreatic Carcinogens: Species Difference
88. SCHAAP, A. Paul  
Wayne State University  
5 R01 CA 15874-09  
Enzymatically Generated Singlet Oxygen in Carcinogenesis
89. SCRIBNER, Norma  
Pacific Northwest Research Fdn  
2 R01 CA 23712-06  
Early and Critical Events in Chemical Carcinogenesis
90. SEGAL, Alvin  
New York University  
2 R01 CA 24124-04A1  
Carcinogenic Acylating Agents and Mode of Action
91. SHIM, Sang C  
Korea Advanced Institute of Science and Technology  
5 R01 CA 21729-06  
Photochemistry of 5,M-Dimethoxy-coumarin

92. SHIMAMURA, Tetsuo  
Rutgers Medical School  
5 R01 CA 30106-02  
Mechanisms of Development of  
Urinary Bladder Cancers
93. SIMPSON, Robert U  
University of Michigan (Ann Arbor)  
1 R23 CA 36507-01  
Actions of 1,25 Dihydroxyvitamin  
D3 on Malignant Cells
94. SINCLAIR, Peter R  
Dartmouth College  
2 R01 CA 25012-06  
Liver Cell Cultures for Study of  
Carcinogen Activation
95. SINHA, Dilip K  
Roswell Park Memorial Institute  
1 R01 CA 36139-01  
Protection Against Mammary Carcino-  
genesis by Pregnancy
96. SLAGA, Thomas J  
Univ of Texas System Cancer Ctr  
1 R01 CA 34962-01  
Polycyclic Hydrocarbon Metabolism  
and Binding in Skin
97. SMITH, Louis C  
Baylor College of Medicine  
5 R01 CA 31513-02  
Cellular Uptake of Carcinogens
98. SOROF, Sam  
Institute for Cancer Research  
5 R01 CA 05945-20  
Macromolecules in Chemical  
Carcinogenesis
99. SPECK, William T  
Case Western Reserve University  
5 R01 CA 23692-06  
Potential Hazards of Phototherapy
100. STOMING, Terrance  
Medical College of Georgia  
7 R01 CA 33586-01  
Metabolism of 3-Methylcholanthrene  
in Liver and Lung
101. STROBEL, Henry W  
Univ of Texas Health Science  
Center (Houston)  
1 R01 CA 37148-01  
Colonic Carcinogenesis/Chemotherapy  
and GI Hormones
102. SULLIVAN, Paul D  
Ohio University (Athens)  
1 R01 CA 34966-01A1  
Structure and Metabolism of  
Substituted Benzo(a)pyrenes
103. SUZUKI, Yasunosuke  
Mount Sinai School of Medicine  
5 R01 CA 29432-03  
Carcinogenic and Fibrogenic  
Effects of Zeolites
104. TANNENBAUM, Steven R  
Massachusetts Institute of Tech  
5 P01 CA 26731-05  
Endogenous Nitrite Carcino-  
genesis in Man

105. THURMAN, Ronald G  
Univ of North Carolina, Chapel Hill  
5 R01 CA 23080-07  
Pharmacology of Carcinogen  
Activation in Intact Cells
106. TOTH, Bela  
Univ of Nebraska Medical Ctr  
1 R01 CA 31611--2  
Carinogenesis and Chemistry of  
Cultivated Mushrooms
107. VESSELINOVITCH, Stan D  
University of Chicago  
5 R01 CA 25522-06  
Role of Sex Hormones in  
Hepatocarcinogenesis
108. VOLLHARDT, K Peter  
Univ of California (Berkeley)  
5 R01 CA 20713-07  
Activated Mutagenic and Aromatic  
Hydrocarbons
109. WEBER, George  
Indiana University  
5 P01 CA 13526-11  
Correlated Study of Metabolic  
Regulation in Neoplasia
110. WHALEN, Dale L  
University of Maryland  
2 R01 CA 26086-04A1  
Kinetics of Nucleic Acid-  
Catalyzed Epoxide Hydrolyses
111. WHITLOCK, James P, Jr  
Stanford University  
5 R01 CA 32786-02  
Carcinogen-Metabolizing Enzymes:  
Action in Variant Cells
112. WOTIZ, Herbert H  
Boston University  
2 P01 CA 28856-04  
The Role of Hormones and Binding  
Proteins in Cancer
113. YANG, Chung S  
Univ of Medicine and Dentistry  
of New Jersey  
5 R01 CA 16788-09  
Monoxygenase: Properties and  
Carcinogen Activation
114. YANG, Chung S  
Univ of Medicine and Dentistry  
of New Jersey  
1 R01 CA 37037-01  
Enzymology and Mechanisms of  
Nitrosamine Metabolism
115. YANG, Shen K  
US Uniformed Services University  
of Health Sciences  
5 R01 CA 29133-02  
Metabolic Activations of Mono-  
methylbenz(a)anthracenes

## SUMMARY REPORT

### DIET AND NUTRITION

The Diet and Nutrition component within the Chemical and Physical Carcinogenesis Branch contains 32 grants with funding during FY84 of \$2.77 million. The component supports laboratory investigations searching for etiologic factors related to diet, nutrition, and cancer. 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, the Program promotes studies which focus on specific dietary factors (i.e., nutrients, or micro-nutrients, 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.

One recent study, supported by the program, entailed the measurement of fatty acid profiles in the mammary parenchymal cell and mammary fat pad cell as well as whole gland homogenates in rats. All rats were placed on either a low fat (LF) (2% linoleic acid) diet or a high polyunsaturated fat (HPF) (20% stripped corn oil) diet at weaning (21 days of age) and sampled from this zero time at weekly intervals up to 11 weeks of age. Neutral lipids and phospholipids were separated, methylated, and tested using gas-liquid chromatography.

Within 2 to 3 weeks on the diet, the various fatty acid patterns had manifested themselves and tended to maintain these levels throughout the remainder of the sampling time. In general, animals consuming the LF diet showed almost three-fold increases in oleic acid (18:1) in both the neutral and phospholipid (PL) fractions. Animals on the HPF diet showed almost a two-fold increase in linoleic acid (18:2) in all three fractions being measured, but more so in the neutral lipid fraction than in the PL fraction. Oleic acid levels in the HPF-fed animals remained relatively constant. Linoleic acid levels markedly decreased in animals consuming the LF diet and in both the neutral and PL fractions. Other shorter chain fatty acids 12:0, 14:0, 16:0, 16:1, 18:0 tended to show only slight changes, if any, as a result of feeding the two dietary fat extremes. From these data one can conclude that it is likely the polyunsaturated fatty acid 18:2 (linoleic acid), by being excessively high in the HPF-fed animals, may be labeled as the factor in enhancing mammary gland tumorigenesis. What the mechanism of this action is remains to be elucidated. The primary conclusion that can be drawn at this time is that the different levels of linoleic acid in the diet has a definite role to play in the resultant tissue levels.

Measurements of levels of epoxide hydratase (EH) and aryl hydrocarbon hydroxylase (AHH) were conducted on whole mammary gland microsomes prepared from 50 day-old female Sprague-Dawley rats which had consumed the LF, HPF or a high saturated fat (HSF) (18% coconut oil + 2% linoleic acid) from 21 days of age, both with and without the addition of 0.3% butylated hydroxytoluene (BHT).

Routinely microsomes from animals consuming the HSF diet, with or without BHT, showed significantly higher EH activity than the LF (43% lower) or HPF-fed (30% lower) animals. The inclusion of BHT in the diets resulted in a four-fold

increase in EH activity in both the LF and HPF fed animals and a seven-fold increase in the HSF animals. AHH levels were found to be higher in the BHT supplemented animals and in all three dietary groups. Levels in the non-supplemented diets were greatest in the HPF followed by the HFS and lowest in the LF-fed animals, although the differences were not statistically different.

In an effort to pinpoint whether or not the antioxidants BHT and propyl gallate (PRG) were indeed inhibiting DMBA-induced mammary carcinogenesis through some influence on the metabolism of the carcinogen, the two antioxidants were tested using the direct-acting carcinogen (not requiring metabolic activation to be carcinogenic) nitrosomethyl urea (NMU). Only the HPF diet was utilized in these studies since this diet causes the greatest enhancement of tumorigenesis. Neither antioxidant, each included in the diet at 0.3%, showed any protection against NMU mammary tumorigenesis. This is very good evidence that these two antioxidant compounds are acting in some way to alter the metabolism of pre-carcinogens such as DMBA, however, further studies are needed to discern the exact mechanism of their influence.

It has been shown previously that a calorie restricted diet dramatically decreased the incidence of spontaneous tumors not only in C<sub>3</sub>H strain mice but in other strains of mice with a high incidence of murine mammary tumor virus (MuMTV) induced mammary cancer. Recently, it has been shown that the effect of calorie restriction can be overcome by increasing the fat content of the diet.

The degree of calorie restriction necessary to produce the inhibition of tumor development of C<sub>3</sub>H mice is not debilitating and does not inhibit the morphological development of the mammary gland. An examination of the influence of total calorie restriction on the estrous cycle of mice revealed no change in the sex cycle. Moreover, a decrease in the calorie intake did not affect the amount of serum growth hormone (GH) or thyroid stimulating hormone (TSH), but did significantly reduce the level of circulating prolactin. In calorie restricted mice, it was shown that, while the production of both type A and type B particles was reduced, the synthesis of type B particles was lowered to a much greater extent and the production of anti-MuMTV antibody was greatly reduced.

Recent studies have been completed on the effect of high dietary corn oil 5 DMBA administration on plasma prolactin, estrogen and progesterone in female Sprague-Dawley rats. Rats bearing in-dwelling atrial cannulae were bled serially at all four stages of the estrous cycle and at several times in the proestrus cycle. They had been fed control diet (5% corn oil) or a diet containing 24% corn oil that enhances mammary tumorigenesis and given 2.5 mg DMBA or the sesame oil vehicle only 4 weeks or 12 weeks earlier. There was no effect of either high dietary corn oil or DMBA treatment on plasma content of prolactin, estrogen or progesterone at any time during the estrous cycle in either age group. This result is in direct contradiction to results reported in decapitated or anesthetized (stressed) rats and shows that basal hormonal levels are not changed by high corn oil diets and that corn oil therefore acts by some other mechanism to enhance mammary tumorigenesis. Another experiment was conducted on the timing of enhancement of DMBA mammary tumorigenesis by a high lard diet. Rats were fed a control diet (4% lard, 1% corn oil) throughout the experiment or a high lard diet (23% lard, 1% corn oil) (1) before DMBA treatment only, or (2) before DMBA treatment for 3 weeks, or (3) 6 weeks after DMBA treatment or (4) throughout the experiment. Tumorigenesis was increased in all four groups fed the high lard diet. There were no significant differences between the four groups, but there was a trend toward shorter tumor latency with longer periods of feeding the high

lard diet. Therefore, it appears that lard exerts its major effect before or at initiation, although it continues to exert some effect after DMBA administration.

Previous studies have demonstrated that the degree of dietary lipid saturation influences the expression of ultraviolet (UV)-induced carcinogenesis. A study has been completed on the influence of dietary lipid level upon UV-carcinogenesis and its modification by antioxidants. Seven hundred twenty female, SKH-HR-1 hairless mice, divided into 16 groups of 45 animals each were employed in the study. The animals received defined diets whose lipid source consisted of 4%, 2%, and 0.75% corn oil. Half of the animals on the respective diets received an antioxidant supplement (2% w/w) consisting of 1.2% ascorbic acid, 0.5% butylated hydroxytoluene, 0.2% tocopherol, and 0.1% reduced glutathione. Eight groups, representing each of the preceding treatments, served as non-irradiated controls, while the remainder received daily, suberythemic UV-radiation until 70 J/cm<sup>2</sup> had been delivered. Cumulative distribution curves, median tumor times, and tumor multiplicity were determined for each irradiated group. A direct relationship of the cumulative tumor distribution to dietary lipid level was found with 4% lipid level demonstrating the shortest latent period and 0.75% exhibiting the greatest latent period. The tumor distribution plots showed a rather marked influence by antioxidants at the 4% lipid level, diminished at 2%, and was completely negated at the 0.75% lipid level. Median tumor times were 18.4, 19.9, and 21.0 weeks for 4%, 2% and 0.75% lipid levels, respectively and 20.6, 21.1, and 20.3 for their respective antioxidant supplemented groups. With regard to tumor multiplicity, the animals exhibited 1.89, 1.30, and 0.61 tumors/animal for 4%, 2% and 0.75% lipid levels respectively. Again, only at the two highest lipid levels did significant reductions in tumor multiplicity, due to antioxidants, occur. When lipid peroxidation of epidermal homogenates was examined using thiobarbituric acid (TBA) and peroxide values (PV), TBA values increased upon incubation to 1.99, 1.72, and 0.87 nMol/mg protein for 4%, 2% and 0.75% dietary lipid levels, respectively. TBA values for the antioxidant supplemented animals remained relatively constant at 0.45 nMol per mg at all dietary lipid levels. PVs were in good agreement with the observed TBA values. These data suggest (1) that dietary lipid level has a direct effect upon the carcinogenic response, both in regard to tumor latency and multiplicity; (2) antioxidants produce an inhibitory effect almost exactly equal to the degree of exacerbation of carcinogenesis evoked by increasing lipid levels; (3) from examination of both cumulative tumor distribution plots and multiplicity data, it appears that antioxidants produce their inhibitory effects early on in the carcinogenic process and that lipids are able to overwhelm such inhibition, possibly by enhancing promotion events in the carcinogenic continuum; and (4) these data indirectly implicate peroxidative reactions in UV-carcinogenesis and show that antioxidants inhibit such events.

A study has been completed to determine whether vitamin E deficiency can lead to an enhancement in mammary carcinogenesis, and secondly, whether excess vitamin E can abrogate the augmentation due to selenium deficiency. The study demonstrated that a low vitamin E intake (7.5 mg/kg of diet) had minimal effect on mammary carcinoma development in rats fed a 5% corn oil diet, but resulted in a marked enhancement in tumor incidence and yield in those rats fed a 25% corn oil ration. Control animals in this experiment received an adequate supply of vitamin E (30 mg/kg). Thus, the effect of vitamin E deficiency on mammary carcinogenesis was accentuated in rats maintained on a high polyunsaturated fat diet, an observation similar to that of selenium deficiency. The second part of the study was to determine whether supranutritional supplementation of vitamin E (1000 mg/kg) could block the enhancement in mammary tumorigenesis due to selenium deprivation. Results of this experiment indicated that vitamin E excess failed to overcome the

augmented tumor yield in selenium-deficient rats, suggesting that the effect of selenium deficiency is probably more specific and may not be related solely to the extent of oxidant stress. In summary, a deficiency of endogenous antioxidant, in general, may predispose the host to a higher risk of cancer, especially when it is coupled to a high polyunsaturated fat intake.

Work continues on the role of protease inhibitors as anticarcinogens. It has been demonstrated that when soybean diets containing two principal protease inhibitors, Kunitz (KI) and Bowman-Birk (BB), were fed to mice and to Sprague-Dawley rats, tumor promotion and x-ray-induced breast cancer were blocked. Recently, studies have been completed on the distribution of BB inhibitors by feeding I<sup>131</sup>-labeled BB inhibitor to mice and rats. BB inhibitor distributed largely into fecal content combined with pancreatic enzymes, trypsin and chymotrypsin. Some active free inhibitors were observed in the colon. Complex inhibitors could be dissociated by acid into free inhibitors. Only insignificant quantities of the protease inhibitor were found in the skin or breasts of experimental animals. A possible explanation for the anticarcinogenic effect of feeding protease inhibitors is that they indirectly prevent protein digestion reducing the availability of amino acids formed from protein digestion. These excess nutrients may be necessary for the cancer cells' development, while contributing little to the host.

#### DIET AND NUTRITION PROGRAM

#### GRANTS ACTIVE DURING FY84

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ABRAHAM, Samuel Children's Hospital Medical Center 5 R01 CA 29767-02	Effect of Dietary Fat on Mammary Neoplasia
2. ASANO, Tomoaki University of Notre Dame 5 R01 CA 28276-02	Experimental Carcinogenesis by Dietary Nitrite
3. AYLSWORTH, Charles F Michigan State University 1 R23 CA 36364-01	Dietary Fat - Cell Communication and Breast Tumorigenesis
4. BLACK, Homer S Baylor College of Medicine 2 R01 CA 20907-03A1	Effects of Dietary Factors on UVL-Carcinogenesis
5. BURKE, James P Pennsylvania College of Podiatric Medicine 5 R01 CA 32256-02	Relationship of Zinc to Cellular Membrane Composition
6. CAMPBELL, T Colin Cornell University 1 CA 34205-01A1	Dietary Protein and Chemical Carcinogenesis



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| 7.  | GRUBBS, Clinton J<br>Southern Research Institute<br>1 R01 CA 33808-01A2            | Effect of Alcohol on Chemically<br>Induced Cancers       |
| 8.  | HAMILTON, Stanley R<br>Johns Hopkins University<br>2 R01 CA 29714-03               | Role of Beer and Ethanol in<br>Experimental Colon Cancer |
| 9.  | HEINIGER, Hans-Jorg<br>Jackson Laboratory<br>5 R01 CA 19305-06                     | Cholesterol in Normal and<br>Malignant Lymphocytes       |
| 10. | IP, Clement C<br>Roswell Park Memorial Institute<br>5 R01 CA 27706-03              | Selenium Supplement and Dietary<br>Fat in Breast Cancer  |
| 11. | JANGHORBANI, Morteza<br>Massachusetts Institute of Technology<br>5 R01 CA 27917-03 | Dietary Bioavailability of<br>Selenium in Man            |
| 12. | KING, M Margaret<br>Oklahoma Medical Research Foundation<br>8 R01 CA 34143-05      | Dietary Fat and Mammary<br>Carcinogenesis                |
| 13. | MIRVISH, Sidney<br>University of Nebraska Medical Center<br>5 R01 CA 30593-02      | Significance of Nitrosoarea<br>Formation from Creatinine |
| 14. | NEWBERNE, Paul<br>Massachusetts Institute of Technology<br>2 R01 CA 26917-04A1     | Dietary Fat in Colon<br>Carcinogenesis                   |
| 15. | PARIZA, Michael W<br>University of Wisconsin<br>5 R01 CA 29618-02                  | Structure and Origin of<br>Mutagens in Fried Beef        |
| 16. | PAULING, Linus C<br>Linus Pauling Institute of Science<br>5 R01 CA 26541-02        | Diet and Breast Cancer in Mice                           |
| 17. | PAWLOWSKI, Norman E<br>Oregon State University<br>5 R01 CA 25766-04                | Mechanisms for Biological<br>Activity of Cyclopropenes   |
| 18. | REDDY, Bandaru<br>American Health Foundation<br>1 R01 CA 36892-01                  | Macro and Micronutrient<br>Interaction in Colon Cancer   |
| 19. | ROEBUCK, Bill D<br>Dartmouth College<br>5 R01 CA 26594-03                          | Modulation of Pancreatic<br>Carcinogenesis by Diet       |

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| 20. | ROGERS, Adrienne E<br>Massachusetts Institute of Technology<br>3 R01 CA 25538-03S1       | Dietary Fat, Prolactin and<br>Mammary Cancer               |
| 21. | ROSS, Morris H<br>Institute for Cancer Research<br>5 R01 CA 16442-08                     | Regulation of Tumor<br>Susceptibility                      |
| 22. | RUDOLPH, Frederick B<br>Rice University<br>5 R01 CA 14030-10                             | Regulation of Metabolism by<br>Purine Interconversions     |
| 23. | SARKAR, Nurul H<br>Sloan-Kettering Institute<br>for Cancer Research<br>5 R01 CA 25679-03 | Effect of Diet on Murine Mammary<br>Tumorigenesis          |
| 24. | SCANLAN, Richard A<br>Oregon State University<br>5 R01 CA 25002-12                       | Nitrosamines in Foods                                      |
| 25. | SELIVONCHICK, Daniel P<br>Oregon State University<br>5 R01 CA 30087-02                   | Membrane Protein Composition:<br>Cyclopropanoid Fatty Acid |
| 26. | SHILS, Maurice E<br>New York Academy of Medicine<br>5 R01 CA 32241-02                    | NY/NJ Regional Center for<br>Clinical Nutrition Education  |
| 27. | SHINOZUKA, Hisashi<br>University of Pittsburgh<br>5 R01 CA 26556-03                      | Diet Modification and Promotion<br>of Liver Carcinogenesis |
| 28. | THANASSI, John<br>University of Vermont<br>1 R01 CA 35878-01                             | Vitamine B-6 Metabolism in<br>Hepatores                    |
| 29. | THOMPSON, Henry J<br>University of New Hampshire<br>5 R01 CA 28109-03                    | Nutrition and Mammary Carcino-<br>genesis                  |
| 30. | TROLL, Walter<br>New York University Medical Center<br>5 R01 CA 16060-11                 | Inhibition of Tumor Promotion<br>by Protease Inhibitors    |
| 31. | VISEK, Willard J<br>University of Illinois<br>5 R01 CA 28629-03                          | Hormones, Dietary Fat and Mammary<br>Carcinogenesis        |
| 32. | WEINDRUCH, Richard H.<br>University of California (Los Angeles)<br>2 R01 CA 26164-04A1   | Dietary Restriction Cancer and<br>Immune Functions         |

33. WEISBURGER, John H  
American Health Foundation  
5 PO1 CA 29602-02

Nutritional Carcinogenesis

## SUMMARY REPORT

### MOLECULAR CARCINOGENESIS

The Molecular Carcinogenesis program area includes 228 grants with FY84 funding of approximately \$19.73 million. There are no contracts in this program area. The grants consist of 214 R01 (Research Project) grants, eight R23 (Young Investigator) grants, five P01 (Program Project) grants and one R43 (SBIR) grant. Research in the program area focuses on the characterization of carcinogen-macromolecule interactions (17 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 (41 grants); the genetics and mechanisms of cell transformation (7 grants); the development of carcinogenicity/mutagenicity testing procedures (12 grants); the mechanisms of carcinogen-induced mutagenesis and genetic damage (10 grants); the identification and properties of tumor promoters and mechanisms of tumor promotion (39 grants); interspecies comparisons in carcinogenesis (17 grants); the genetics and regulation of enzymes associated with carcinogenesis induced by chemical and physical carcinogens (8 grants); development of analytical methodology for detecting chemical carcinogenesis in body fluids and environmental samples (4 grants); and the role of DNA repair in carcinogenesis (32 grants). Expanded descriptions of individual subject areas, along with examples of research accomplishments, are provided below.

#### Grants Activity Summary

Carcinogen-Macromolecule Interactions: The projects in this subject area focus on studies on the identification, quantitation and characterization of carcinogen-nucleic acid adducts. The interest in the identification and characterization of DNA adducts stems from the role alterations in DNA play in the initiation of carcinogenesis. Most of the carcinogens used in these studies 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 radioisotope techniques. The levels and persistence of specific DNA adducts are often related to the organ specificity of the carcinogen and 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<sub>1</sub>, 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. In addition, computer analysis of possible carcinogen-DNA adduct conformations has allowed the building of molecular models for the most likely conformations. 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 the metal carcinogens is the focus of several studies. 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.

Chromium (VI) has been identified as a human carcinogen from epidemiological studies. Chromium (VI) compounds have been shown to be tumorigenic in animals and mutagenic in various bacterial and mammalian cell systems. Although chromium (III), which is produced upon reduction of chromium (VI), is the form ultimately bound to cell DNA and protein, it produces no direct mutagenic and carcinogenic effects. An uptake-reduction model has been used to explain the carcinogenicity of chromium (VI) which can enter the cell using the sulfate transport system. Chromium (III), which forms octahedral complexes, is assumed to be impermeable to the cell membrane. The reduction of chromium (VI) to its active form has been shown to be associated with the cytochrome P-450 electron transport system. The binding of chromium to DNA and protein may occur during the production of intermediate, labile oxidation states and ultimately produce chromium (III) complexes bound to cellular macromolecules. In studies conducted by Tsapakos, et al. DNA interstrand cross-links, strand breaks, and DNA-protein cross-links were observed in kidney, liver and lung nuclei of rats exposed to sodium dichromate. DNA interstrand cross-links were repaired in all three organs by 40 hrs. after exposure. DNA-protein cross-links, however, were repaired in liver within 30 hrs. but not in lung or kidney. These results suggest that the lung and kidney may be more sensitive than liver to chromium-induced DNA damage, an observation which correlates with the reported toxicity and carcinogenicity data for chromium (VI) in both animals and humans. In further studies native and denatured calf thymus DNA and homopolyribonucleotides were incubated in vitro with rat liver microsomes, NADPH and chromium (VI) or chromium (III). The binding of chromium (III) to native or denatured DNA was shown to be relatively small and independent of the microsomal reducing system. For both native and denatured DNA incubated with chromium (VI) the amount of protein bound to DNA increased with the amount of chromium bound to DNA. The results with the homopolyribonucleotides indicated that the activated chromium (VI) binds preferentially to guanosine residues and results in DNA-protein cross-links (217).

The major modified nucleoside formed after reaction of the carcinogen N-acetoxy-2-acetylaminofluorene (N-acetoxy-AAF) with DNA in vitro and in vivo is the result of AAF binding at the C-8 position of guanosine. The reaction at the C-8 position of deoxyguanosine residues in native DNA has been shown to result in large conformational changes in the B-form of DNA and has been described in a base displacement model. The carcinogen is inserted into the DNA helix perpendicular to the long axis, and the modified guanine residue is displaced and is in the syn rather than the normal anti conformation. The modified regions show evidence of denaturation. A similar model, called the insertion-denaturation model has been proposed by others. A different conformation has been observed when the alternating purine-pyrimidine copolymer, poly(dG-dC).poly(dG-dC) is modified. This copolymer has been shown to be able to undergo a conformational change to a left-handed behind structure termed Z-DNA. In the Z-DNA form the deoxyguanosine is in the syn conformation while the deoxycytidine is in the anti conformation. The modification of poly(dG-dC).poly(dG-dC) or poly(dG-m<sup>5</sup>dC).poly(dG-m<sup>5</sup>dC) by AAF has been shown to induce the Z conformation, the methylated polymer requiring a much lower degree of modification (10%) than for the nonmethylated polymer (greater than 20%). Base pairing of the modified polymer remains intact as demonstrated by its resistance to digestion with S<sub>1</sub> nuclease and lack of reactivity to anticytidine

antibodies. From equilibrium binding studies using various polymers and increasing levels of N-acetoxy-AAF, it was shown that the order of reactivity follows the ease of formation of the Z conformation. The C5 methylation of cytosine residues in CpG sequences, which stabilizes Z-DNA under physiological condition, has been implicated in the inhibition of transcription of some eukaryotic genes. Since AAF can also stabilize the Z-DNA conformation under physiological conditions, it is hypothesized that this carcinogen can also inhibit gene expression indirectly by causing a conformational switch in a region which, under normal conditions, would be transcribed. The feasibility of such a proposal is currently under investigation (212).

A major advance in the effort to determine the biological relevance of the left-handed Z-DNA helix came when it was found that Z-DNA, unlike B-DNA, is immunogenic. In the laboratory of Alexander Rich (171) the first antibodies were elicited by immunization with poly(dG-dC).poly(dG-dC) in which the 8-position of guanine and the 5-position of cytosine were brominated. In this study rabbits were immunized with a complex of methylated bovine serum albumin and AAF-modified poly(dG-dC).poly(dG-dC). Several populations of antibodies specific for Z-DNA determinants were isolated, purified and analyzed. It was found that the AAF-modified polymer shaped common Z-DNA determinants with poly(dG-dC).poly(dG-dC) in 3.0M NaCl, poly(dG-m<sup>5</sup>dC).poly(dG-m<sup>5</sup>dC) in 1.5M NaCl and brominated poly(dG-dC).poly(dG-dC) in 0.2M, 1.5M and 3.0M NaCl. Determinants present on the AAF-modified polymer but not on the other Z-DNAs were recognized by another distinct population of antibodies. The greater part of this population appeared to recognize Z-DNA-associated conformational characteristics that were unique to the AAF-modified polymer. The results of these studies are consistent with the interpretation that a continuum of Z-DNA determinants exists which might be capable of functioning as recognition signals for regulatory DNA-binding proteins (212).

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 which are presumed to result from a substantial reprogramming of the cellular genome during neoplastic transformation. Not all of this reprogramming is thought to be due to direct alterations of the DNA genome. It has been hypothesized that non-DNA factors, so called "epigenetic" effects, play a role in the eventual appearance of neoplastically transformed 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 also is involved in the control of cell differentiation. Thus, a greater understanding of the effects of 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 postrep-

lication 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, is being studied by using compounds such as 5-azacytidine which are known to affect the transfer of methyl groups to DNA.

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.

Studies in the laboratory of Dr. Hnilica (91) have focused on the nature and role of chromosome nonhistone protein-DNA complexes in cell differentiation and hepatocarcinogenesis. Three Novikoff ascites hepatoma antigens p39, p49 and p56 (molecular weights, 39,000, 49,000 and 56,000, respectively), have been characterized in chromatin preparations. Subsequent studies have established that the p39 and p49 antigens are specific for rat carcinoma cells and that they are cytokeratins. The cytokeratins are characterized as the most heterogeneous class of polypeptides that comprise the cytostructural intermediate filaments. The proteins exhibit overall patterns of cell-type specificity, allowing classification of epithelial and carcinoma cells on the basis of cytokeratin expression. One goal of the laboratory has been to clarify the relationship of these antigens to the nucleus and chromosomal proteins. Attempts to assign a role for keratin filaments in nuclear morphology and function have been complicated by their extreme insolubility and their tenacious ability to remain with purified nuclei. In this investigation a protein-DNA cross-linker, cis-dichlorodiammineplatinum and gamma irradiation have been employed with intact, viable Novikoff hepatoma cells to assess the in vivo interaction of p39 with DNA. Following the treatments, cells were solubilized in sodium dodecyl sulfate-containing buffer and the DNA was pelleted by high speed centrifugation. Using immunotransfer analysis, p39 cytokeratin was found in the DNA pellet of the cross-linked samples but not in the controls. The results obtained indicate that a part of the p39 antigen, perhaps the filament ends, is situated with or within cross-linking distance or bound to the DNA in intact cells (91).

Approximately 3 to 6% of cytosine residues in the DNA of all vertebrate is modified to 5-methylcytosine, which is predominantly found in the dinucleotide sequence 5'-CpG. Substantial evidence has accumulated over the past several years that the methylation of cytosine residues in vertebrate DNA is implicated in the control of gene expression. The interrelationship between the effect of enzymatic DNA methylation on gene expression and neoplastic transformation is not clear as contradictory results on levels of DNA methylation and methylase activity in tumors have been reported. In one study designed to investigate the relationship between DNA methylation and cancer, DNA methylation was measured in five established human tumor cell lines and in freshly explanted pediatric tumors which included rhabdomyosarcomas, Wilm's tumor, medulloblastoma, neuroblastomas and retinoblastomas. The results showed that discrepancies existed between values obtained from fresh tumors and their corresponding cell lines and that tumors of

the same histological type had similar 5-methylcytosine values. It was concluded that tumor cell lines may be inadequate models for studies on the relationship between DNA methylation and cancer. The results also suggested that no generalized trend in DNA methylation levels will be found in fresh tumor explants or in tumor cell lines (106).

In another study the methylation of the alpha-fetoprotein (AFP) gene in different rat transplantable hepatocellular carcinomas in which the gene is either actively expressed or repressed was investigated. The extent of methylation of Hpa II (CCGG)-Msp I (CmCGG) and Hha I (GCGC) sites in DNA isolated from normal rat livers and from the transplantable hepatocellular carcinomas THC 7777 and THC 252 was compared. The results showed that the overall level of methylation of the internal cytosine in CCGG sequences was lower in the THC DNAs than in the normal liver DNAs. The AFP gene, which is repressed in normal adult liver and in the nonproductive THC 252 and is highly active in the productive THC 7777, contains certain CCGG sites which are not methylated in the THC 7777 but methylated in the THC 252. Also, GCGC sites in the AFP gene were found to be less methylated in both hepatoma DNAs than in normal liver DNA (9).

Most chemical carcinogens bind covalently to DNA and these reactions are considered to be critical in the initiation of carcinogenesis. The carcinogen N-2-acetylaminofluorene (AAF) primarily binds to the C-8 position of deoxyguanosine in DNA following metabolic activation to esters of N-hydroxy-AAF (N-acetoxy-AAF). The modification of DNA by N-acetoxy-AAF creates a locally denatured region recognizable by repair enzymes. There is also evidence that AAF modification impairs the DNA template functions in replication and transcription processes. In one study the ultimate chemical carcinogen N-acetoxy-AAF was shown to inhibit the enzymatic methylation of newly replicated DNA in cultured mouse P815 cells in a dose-dependent manner. Following removal of the carcinogen, a significant de novo methylation of newly replicated DNA takes place, the level of methylation being higher than in control cultures. The aberrant methylation pattern persists in the absence of carcinogen for at least six cell cycles. Cell cloning experiments showed the isolation of clones belonging to either of two classes, one with hypermethylated DNA and one with hypomethylated DNA. The existence of cells containing aberrantly methylated DNA of these two different types indicated to the investigators that both the inhibition of maintenance methylation and aberrant de novo methylation of DNA occurred during the N-acetoxy-AAF treatment. These results, in addition to other work, suggests that the induction of aberrant methylation patterns may be related to the initial step in chemical carcinogenesis (212).

Two other groups have shown that a diverse range of carcinogens inhibit the transfer of methyl groups from S-adenosylmethionine to hemimethylated DNA or to a synthetic DNA polymer, poly(dC-dG) catalyzed by purified mouse spleen or rat liver DNA methyltransferase. The carcinogens used included methylating agents, N-acetoxy-AAF and benzo(a)pyrene diol epoxide. Methylating carcinogens were shown to directly inactivate the enzyme as well as to inhibit its activity by forming DNA adducts. The inhibitory effects of AAF appeared to depend on the recognition of an altered dG base configuration by DNA methyltransferase which is responsible for the altered binding and methylation kinetics that is observed (124,106).

One hypothesis of how methylating carcinogens can exert its biological effect is by the alkylation of deoxyribonucleoside triphosphates (dNTPs) in the cellular pool. The incorporation of modified nucleotides into DNA could then result in mutagenesis and carcinogenesis. The hypothesis is being tested in the laboratory



of Dr. Topal (201). DNA sequencing methodology has been employed to study the behavior of modified dNTPs during DNA replication and in particular the incorporation properties of  $O^6$ -methyl-dGTP during DNA replication in vitro by the "Klenow" fragment of *E. coli* DNA polymerase I and phage T4 DNA polymerase. The results show that  $O^6$ -methyl-G:T base pairs arising during DNA synthesis could be a factor contributing to the mutagenicity of  $O^6$ -methyl-guanosine lesions in DNA. The results showed further that incorporation at non-pyrimidine-rich DNA sequences in place of dATP arrests DNA synthesis. This phenomenon of DNA arrest may produce cellular abnormalities. For example, phenomena seen with other compounds that arrest DNA synthesis include chromosome abnormalities, mutation, neoplastic transformation, and cell death of cells in culture. It is thought that  $O^6$ -methyl-dGTP incorporation may have similar consequences (201).

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 multistep 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 animal and cell culture, are being used. Of the animal model systems, the 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 endpoint 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. There are other interesting model systems which are being established and analyzed by one or more laboratories. For example, a model of renal carcinogenesis in the rat is being established in which adenocarcinomas or mesenchymal tumors are selectively induced following a single dose of dimethylnitrosamine. Cell cultures representative of the renal tumor types are being established in order to correlate in vivo phenomena in renal carcinogenesis with events occurring in vitro.

Another 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.

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. Properties of normal and carcinogen-treated human respiratory

epithelium can also be studied by using the denuded rat trachea implants in nude mice. These types of studies are being initiated 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 being handled primarily by the Organ Systems Program of NCI, although some of these model systems are being used in projects supported by this 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 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 has been and will continue to be vigorously supported by NCI.

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. 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 and 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 and aldehyde dehydrogenase isozymes are being evaluated. 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.

The laboratory of Dr. E. Farber at the University of Toronto has provided results which have had a seminal influence in establishing working models and hypotheses for aspects of epithelial carcinogenesis. The liver model system of carcinogenesis may serve as a paradigm for other epithelial tissues. It is now being considered that there may be at least two patterns of development of liver cancer in rats with several chemical carcinogens which may be designated the chronic enzyme induction model and the resistant hepatocyte model. The chronic enzyme induction model utilizes a brief exposure to one of several carcinogens to induce initiation, followed by a relatively long exposure to one of several different promoters, such as phenobarbital, PCBs, etc. All such promoters have been shown to be effective enzyme inducers in the liver. The resistant hepatocyte model utilizes a brief exposure to an initiating dose of a carcinogen to induce resistant liver cells followed by the selection of resistant hepatocytes to form focal proliferations or hyperplastic nodules. The selective proliferation of resistant hepatocytes is accomplished by a short exposure to another carcinogen, such as 2-acetylaminofluorene (AAF), which inhibits the proliferation of a majority of hepatocytes, coupled with a stimulus for liver proliferation. A study was designed to obtain more insight into the biochemical basis for the resistance

in the new population of hepatocytes in hyperplastic nodules induced by diethylnitrosamine and selected by dietary AAF plus partial hepatectomy. The nodule cells exhibited a very low uptake of AAF relative to surrounding and normal liver and low levels of cytochromes P-450 and aryl hydrocarbon hydroxylase activity. Elevated levels of glutathione and high levels of gamma-glutamyltransferase, microsomal epoxide hydrolase, soluble glutathione-S-transferase and soluble UDP-glucuronyltransferase activity are also found in nodules. This metabolic pattern appears to maximize the resistance of the nodules to xenobiotics generally and may therefore account for the resistant behavior of nodule hepatocytes to the inhibition of cell proliferation and the cytotoxicity by AAF and other carcinogens (52).

In another study, hepatic hyperplastic nodules were studied as an experimental prototype of oral contraceptive-related hepatic tumors due to the histological similarities of the human hepatic adenoma and the AAF-induced hepatic hyperplastic nodules. The formation of benign hepatic adenomas and focal nodular hyperplasia have been linked to the use of oral contraceptives. Since an association between oral contraceptives and hepatocellular carcinoma is suspected but has not been established, the following studies were undertaken. The demonstrated presence of cytoplasmic estrogen receptors in hyperplastic nodules produced by AAF raised the possibility that these tumors may be hormone dependent. To test this possibility, the effects of estrogen and tamoxifen (an estrogen antagonist) alone and in combination on the progression and malignant transformation of liver hyperplastic nodules was studied. The treatment of rats with AAF-induced hyperplastic nodules for 8 months with 17 beta-estradiol and tamoxifen resulted in the regression of nodular involvement and no evidence of malignant transformation. Nodular proliferation was shown to decrease after 2 and 4 months treatment with 17 beta-estradiol and after 8 months with tamoxifen. The evidence of hepatocellular carcinoma after 8 months of treatment was significantly less after treatment with estrogen (40%) or tamoxifen (42.9%) when compared to AAF-treated controls (87.5%). The results of this study suggest that the malignant transformation of receptor-positive liver hyperplastic nodules is hormone dependent. The identification of specific hormonally controlled steps in this process may have important implications in preventing malignant transformation of liver and other tumor types (52).

Studies on the elucidation and characterization of mechanisms involved in the expression of a series of tumor-specific aldehyde dehydrogenase (ALDH) isozymes are being conducted in the laboratory of R. Lindahl (131). The ALDH phenotype in aromatic amine-induced rat hepatomas is qualitatively and quantitatively different from that of normal liver. The tumor-specific ALDH phenotype is characterized by increased total ALDH activity due to the appearance of several cytosolic enzymes not detectable in normal liver. The tumor isozymes preferentially oxidize aromatic aldehyde substrates using NADP as coenzyme and have physical and immunochemical properties distinct from normal liver ALDH. It has also been shown that several distinct ALDHs, in addition to the basal normal liver isozymes, can be induced by various xenobiotics such as phenobarbital and TCDD. These are also physically and functionally distinct from the tumor-specific ALDHs. In order to assess the generality of this ALDH phenotype the ability of another family of carcinogens, the nitrosamines represented by diethylnitrosamine, and an additional tumor induction protocol to induce this change was evaluated. Of the 21 tumors found in diethylnitrosamine, phenobarbital-treated rats, 16 of them were shown to possess the tumor-specific ALDH phenotype. Only one of 17 phenobarbital control and one of 16 basal-diet control animals had marginally elevated NAD-dependent ALDH activity. This ALDH activity was distinguishable from normal liver ALDHs and

from tumor-specific ALDHs by a number of properties. The results of these studies extend the induction of the tumor-specific phenotype to another family of carcinogens, the nitrosamines, and confirm that the phenotypic change is due to an initiator-induced, stable genetic change that is expressed relatively late in hepatocarcinogenesis. The promotion-associated ALDH phenotype which was observed represents a genetically independent change in ALDH activity. This change in phenotype, requiring both initiator and promoter for its expression, may be useful in studying the interactions between initiators and promoters (131).

The studies of changes in ALDH activity during hepatocarcinogenesis, to date, have been limited by the fact that it has not been possible to identify which liver cell populations are responsible for the changes observed. An effective, reliable histochemical procedure for detecting the various hepatic ALDH isozymes in situ would identify the population or subpopulation of cells expressing the tumor- and promotion-associated ALDH phenotypes and would also establish the time of earliest appearance of the tumor ALDH phenotype. This could prove useful as a marker for certain specific events in the progression through neoplastic transformation. For this reason a simple, rapid, and specific procedure for the histochemical localization of rat liver ALDH has been developed. The procedure involves the use of either propionaldehyde-NAD to detect normal liver ALDH or benzaldehyde-NADP to detect tumor ALDH. A characteristic staining pattern is observed in normal liver with ALDH activity being strongly centrilobular with only slight periportal activity. During hepatocarcinogenesis, ALDH staining patterns in grossly normal liver ranged from normal-appearing to patterns of distinct, focal hepatocyte staining. ALDH-positive foci were found in both in normal regions of tumor-bearing livers and prior to the appearance of gross neoplasms. Neoplastic nodules and carcinomas possessed a wide variety of ALDH staining patterns between and within lesions. Changes in ALDH could be identified histochemically at a time in hepatocarcinogenesis when other analytical methods did not detect significant changes. Also, the use of histochemistry demonstrated a considerable heterogeneity in the expression of tumor ALDH (131).

An apparently unique calcium binding protein, which has been given the name oncomodulin, has been found in several rat Morris hepatomas. This protein has been purified to homogeneity and a specific antiserum has been raised against it. It has been characterized as an acidic (isoelectric point of 3.9) protein of molecular weight 11,500 containing two calcium binding sites. It has an amino acid composition devoid of tryptophan and with a high phenylalanine to tyrosine ratio. Its amino acid sequence has been determined to be homologous to the parvalbumins, but unlike the parvalbumins, has some modulator activity analogous to calmodulin. A radioimmunoassay for rat oncomodulin has been developed. This antisera cross-reacts with extracts from several human solid tumor tissues. This human immunoreactive protein was purified and characterized as a calcium-binding protein with a molecular weight (12,000) and amino acid composition similar to the rat protein. The inability to find oncomodulin in any normal adult rodent tissue and the present finding of the inability to detect an oncomodulin-like protein in normal adult human tissues suggests that this protein has a strong potential as a specific tumor marker (139).

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 the identification of 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 supports

the hypothesis that somatic mutations are involved in the process leading to neoplasia. Somatic cell hybridization techniques have been used to study the types of mutations leading to the transformation of BHK cells by chemical carcinogens. The chemically induced transformants examined show the characteristics of a single-step, recessive mutation. Temperature-sensitive transformants which result primarily from base change mutations rather than frameshift mutations have also been isolated. Similar types of studies are being conducted to determine the number of complementation groups into which the various transformants isolated fall. This will allow a determination of the number of functional alterations which are needed to lead to the expression of transformation. In another study, the role of H-2 haplotypes in influencing the relative resistance (or relative susceptibility) to local tumorigenesis induced by 3-methylcholanthrene is being determined. It has been determined that the H-2<sup>K</sup> haplotype confers a high degree of susceptibility of local tumorigenesis by 3-methylcholanthrene and that this effect is a recessive trait. Further studies to understand the mechanism of the observed effect are in progress.

Newer studies on the role of specific genes and gene products in chemically induced cell transformation have been initiated. In one study of this type the hypothesis to be tested is that the transcriptional activation of genes, which are progenitors of sarcoma virus genes, is required before chemical mutagens can initiate transformation in cultured rat cells. It is also proposed that initiation involves the production of mutations in at least one copy of the sarcoma virus genes. In these studies the techniques of somatic cell hybridization and two-dimensional gel electrophoresis are being used to provide information concerning the nature of the genetic lesion and altered gene expression resulting from the chemically induced transformation of cells. In support of the above hypothesis, there has been a veritable explosion of publications demonstrating the isolation and characterization of genes responsible for the transformation of cells to malignancy. Newly developed recombinant DNA, gene cloning and DNA sequencing techniques have been employed in this research. To date, several different transforming genes have been isolated from different human tumor cells and their homology to various viral oncogenes has been established. Recently, it was shown that there is increased transcription of the cellular homologue of the transforming gene of Harvey sarcoma virus (c-Ha-ras gene) during the early stages of liver regeneration. Increased transcription of the c-Ha-ras gene was also observed during chemically induced hepatocarcinogenesis. Further studies are underway to extend these findings. In another study the expression of the Ha-ras oncogene during the course of bladder carcinogenesis using the N-4-(5-nitro-2-furyl)-2-thiazolyl)formamide/Fischer rat and the butyl-4-hydroxybutyl-nitrosamine/C3H mouse bladder carcinogenesis models will be evaluated. The studies will involve the quantitation of the mRNA and gene to the sequencing of the gene, localization of the gene within cells and also its localization on the human chromosome. The goal of other newly funded studies is to examine oncogene activation during tumor progression of nasal tumors in rats induced by the inhalation of direct-acting carcinogens or during the development of thymic lymphomas induced spontaneously in nude mice or by the chemical carcinogen N-methylnitrosourea in young mice.

Another possible mechanism of cell transformation by chemicals could involve the induction of DNA sequence rearrangements, 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 studies. 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 10T 1/2 cell line and in a human diploid cell line transformed by chemicals and radiation.

Another major 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 C3H 10T 1/2 cells, G<sub>1</sub> 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 initiation effect of carcinogenic chemicals.

Development of Carcinogenicity Test Systems and Mechanisms of Mutagenesis: The development of carcinogenicity test systems subject area includes projects in which epithelial and fibroblast cell culture systems, specially constructed bacterial strains, erythroid cells, and a <sup>32</sup>P-labeling test 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 generation of DNA damage. The differential sensitivity of cells to low or high calcium concentrations in the medium is a selective property in two of the test systems. 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. Work is in progress to isolate and characterize cloned epidermal cell lines for use in a test system, to identify and resolve the sources of variability in this system and to identify additional markers of transformation of epidermal cells.

In another laboratory epidermal cells from skin tumor sensitive (SENCAR) mice are being used to develop a reliable and quantitative in vitro transformation system. In this system the epidermal cells are grown on a mouse fibroblast feeder layer in medium containing standard calcium levels. Under these conditions the epidermal cells can grow, be subcultured and terminally differentiate (stratify, form keratin, and cornify) in a manner analogous to normal skin. It was felt that this should allow studies of epidermal carcinogenesis and differentiation to be conducted under more normal conditions. The SENCAR mouse skin tumorigenesis system has been used extensively for the testing of chemical compounds for their carcinogenic activity using an initiation-promotion protocol. Investigations on the critical biochemical events in initiation and promotion have been conducted using this animal model. The development of an in vitro transformation system

should facilitate investigations into mechanistic questions involving initiation and promotion as well as allowing the detection of carcinogens and promoters.

A system to investigate factors associated with susceptibility to mammary cancer and to develop methods to identify environmental chemicals that may induce mammary cancer is being developed in the laboratory of Dr. Gould (69). The goals include the further characterization of cultured populations of mammary cells through the development and use of antibodies to specific proteins such as keratins, the development of a direct clonal specific locus mutagenesis assay for rat primary cells, the further investigation of the cell-mediated mutagenesis assay with respect to the differential ability of epithelial and stromal cells to activate carcinogens, and a comparison of the in vivo and in vitro systems in order to assess the validity of the in vitro model. In a related project the development of a human mammary gland in vitro system will be pursued.

The bacterial mutagenesis systems currently in use have been much less successful in predicting the carcinogenicity of metal compounds than they have for predicting the activity of organic compounds. In one project a bacterial short-term test system with a genetic endpoint of broad specificity for detecting carcinogenic metal compounds is to be developed. The investigator has developed a microtitre assay using a number of *E. coli* B strains which allow the analysis of toxicity, lambda prophage induction and mutagenicity of metal compounds simultaneously. The system will be developed further to increase its sensitivity and to broaden the genetic endpoints detectable. These studies will be extended to the Chinese hamster V79 cell system, which have the known capability to phagocytize insoluble metal compounds. This will allow the study of particulate metal compounds which do not enter bacteria.

The basis for development of a different bacterial test system for carcinogens comes from a theoretical paper published by Dr. Martin Pall (Proc. Natl. Acad. Sci. USA 78: 2465-2468, 1981) in which he describes a mechanism by which carcinogenesis might occur. The mechanism proposed is that initiation involves a mutation which produces a tandem duplication of certain genes (a proto-oncogene is suggested) and that promotion involves the further amplification of the same genes by unequal crossing over and sister chromatid exchange. When sufficient gene product is produced, the cell will then become transformed. In the proposed work certain predictions of the theory will be tested. The main prediction to be tested using derivatives of the bacterium, *Salmonella typhimurium*, will be that carcinogenic chemicals can cause tandem duplications more efficiently than noncarcinogenic chemicals can. The results obtained to date have confirmed this prediction in that tandem duplications of the histidine operon were induced by a number of direct-acting chemical carcinogens but not by toxic agents. Some problems with the current procedure for selecting for tandem duplications have been noted. Further work to make the system more specific and to investigate the mechanism of tandem duplication induction is in progress. It is felt that the work can lead to the understanding of some molecular events in carcinogenesis and to a potentially valuable carcinogen test system.

The proposition that gene rearrangements or gene amplification may be more significant in carcinogenesis than classical point mutations is the rationale for a project to construct a mammalian cell line derived from Chinese hamster V79 cells, which will have deletion mutations in the cell's thymidine kinase (TK) genes and a single copy of the herpes simplex virus TK gene. With this system the principal investigator is expected to be able to determine whether a given carcinogen or tumor promoter produces TK+ to TK- mutations and if so, whether

these involve base pair substitution, frameshift mutations, or gene rearrangements or gene amplification. Gene amplification or gene rearrangements will be determined by using restriction endonuclease and Southern blot analysis.

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, two of them are focusing on measuring the level of base substitution mutations produced in erythrocytes. Monoclonal antibodies are being produced which can recognize mutant hemoglobin and spectrin molecules. These will be labeled by conjugation with fluorescein. Erythrocytes are labeled with the fluorescently conjugated antibodies specific for the variant proteins. The mutant cells are then enumerated using high-speed fluorescence-activated cell sorting and automated scanning microscopy. The method has the capability of detecting one abnormal cell in  $10^7$  cells and thus the frequency of background and mutagen/carcinogen-induced somatic mutations can be determined. In another laboratory the goal of the proposed work is to develop a quantitative in vitro assay for measuring the increase in frequency of thioguanine resistant T lymphocytes in humans exposed to environmental mutagens. Because practical methods to measure frequency of mutants 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. The proposed series of experiments, if successful, may ultimately allow the evaluation of mutagen dose in cases of human exposure. In a similar system using normal human T-cells and T-cell leukemia/lymphoma, the proposed studies will determine the efficacy and feasibility of combining the electrophoretic multiple locus mutation system and T-cell cloning procedures to analyze the role and impact of somatic cell mutagenesis in vivo and in vitro.

In addition to the development of mutagenicity test systems are projects which seek to understand how mutations and DNA or chromosome damage are generated by carcinogenic chemicals. Specifically synthesized oligonucleotides of defined base sequence are being used to examine the molecular mechanism of frameshift mutagenesis. The base sequence specificities of the interactions of frameshift mutagens with oligonucleotides are being studied and correlated to their mutagenic activity in Ames testor strains having known base composition in the frameshift site. Studies are also being supported for the study of the mechanism and genetic control of frameshift mutagenesis in yeast. The recently sequenced His4 gene system with existing (+1 G/C) and new (-1) frameshifts are to be used to construct testor strains. DNA sequencing and recombinant DNA technology have been used in these studies. The types of studies to be undertaken include an assessment of the effect of transcription on mutation frequency, the effect of having an origin of replication in close proximity, an analysis of gene duplication, and a study of the potential role of the nuclear envelope in mutagenesis.

Mutagenesis by aflatoxin B<sub>1</sub> is being investigated at the molecular level in a prokaryotic experimental system involving *E. coli*, phage  $\phi$ XI74 and the plasmid pBR322. It has been demonstrated that the activated form of aflatoxin B<sub>1</sub> causes the covalent modification primarily of guanine residues, leading to alkali-labile sites in DNA. DNA sequence analysis was used to identify alkali-labile sites induced by aflatoxin B<sub>1</sub> and to determine the frequency of alkali-labile aflatoxin



B<sub>1</sub> modifications at particular sites on a DNA fragment of known sequence. The influence of flanking nucleotide sequences on aflatoxin B<sub>1</sub> modification in a number of DNA fragments of known sequence was investigated. The results show that, in general, all guanine residues in single-strand DNA are equally subject to aflatoxin B<sub>1</sub> modification and that the DNA sequence does play a role in the site of binding of aflatoxin B<sub>1</sub>. The determination of what specific types of mutations are produced in bacteria in response to DNA modification by aflatoxin B<sub>1</sub>, which produces bulky DNA adducts, and N-methyl-N'-nitro-N-nitrosoguanidine and methyl methanesulfonate, which transfer simple alkyl groups to DNA, is the focus of one new study. It is expected that significant new information on the mechanisms of mutation by carcinogens will be forthcoming from these studies.

DNA damage also leads to aberrations at the higher organizational level of the chromosome. Projects are being supported whose goals are to understand the molecular mechanisms which lead to the formation of chromosome aberrations and to investigate the biological significance of the sister chromatid exchange assay. Standard cytogenetic techniques as well as cell fusion techniques to form prematurely condensed chromosomes, DNA elution techniques, and techniques required to get drugs and enzymes into cells are being utilized to study chromosome damage by various chemical agents. A combination of classical somatic cell genetic and recombinant DNA technologies are being used to study the effects of chromosome rearrangements on gene expression and mutation or deletion. Also, a cell line that is heterozygous for four different linked genetic or cytogenetic markers will be utilized to assay mitotic recombination, gene inactivation, chromosomal rearrangements, deletions, or chromosome segregation events leading to the expression of APRT-recessive mutant phenotypes in mutagen-treated or untreated cell cultures. This system will be used to determine whether extramutational events are induced by known tumor initiators or promoters.

In two other studies methods for the analysis of mutations induced in human cells at the DNA sequence level are being developed. The approaches used depend on the use of a recombinant DNA shuttle vector composed of the simian virus (SV40) early region, sequences derived from the bacterial plasmid pBR322, and the herpes simplex virus thymidine kinase gene. A shuttle vector containing, in addition, the *E. coli lac I* gene has also been used. The studies seek to determine the types of DNA sequence changes induced by chemical carcinogens, to characterize host processes that determine the frequency or types of mutations induced by chemical carcinogens and to identify the primary determinants of sequence specificity in mammalian mutagenesis.

Investigations on the binding of chemicals to DNA are considered to be important not only for the identification of potential carcinogens but also for the qualitative and quantitative analysis of temporal relationships between such binding and the appearance of preneoplastic and neoplastic lesions in treated tissues. A new <sup>32</sup>P-postlabeling method for the analysis of a variety of carcinogen-DNA adducts has been developed in the laboratories of Drs. K. Randerath (169) and R.C. Gupta (77). The method was developed to allow the detection of unlabeled carcinogen-DNA adducts, since many of potential test compounds would not be available in radiolabeled form. The method has been further developed and applied to the analysis of the DNA binding of 28 different chemicals following exposure of mouse or rat tissues *in vivo*. The 28 compounds included 7 arylamines and derivatives, 3 azo compounds, 2 nitroaromatics, 12 polycyclic aromatic hydrocarbons, and 4 methylating agents. After treatment with the individual carcinogens, DNA was isolated from mouse skin, mouse liver and rat liver and digested with micrococcal nuclease and spleen exonuclease to deoxyribonucleoside

3'-monophosphates. These were converted to 5'-<sup>32</sup>P-labeled deoxyribonucleoside 3', 5'-bisphosphates by the transfer of (<sup>32</sup>P)phosphate from (gamma-<sup>32</sup>P)ATP using T4 polynucleotide kinase. The nucleotides were resolved by polyethyleneimine-cellulose anion-exchange thin-layer chromatography and detected by autoradiography. The removal of normal nucleotides prior to the resolution of adduct nucleotides was required to determine low levels of aromatic carcinogen binding to DNA. To accomplish this, an alternative procedure was devised using LKC18 reverse-phase thin layer chromatography, which offered advantages for detecting quantitatively minor adducts. The limits of detection for the described procedures were about one aromatic DNA adduct in 10<sup>8</sup> normal nucleotides and about one adduct in 6 x 10<sup>9</sup> nucleotides for methylated adducts. The results show that a great number of carcinogen-DNA adducts can be <sup>32</sup>P-labeled by T4 polynucleotide kinase. Because covalent DNA adduct formation in vivo appears to be an essential property of the majority of chemical carcinogens, the <sup>32</sup>P-postlabeling method for analyzing carcinogen-DNA adducts in mammalian tissues is being proposed as a useful test for the screening of chemicals for their potential carcinogenicity (77, 169).

A goal of the laboratory of M. Calos (31) is to develop methodologies for the delineation of the nature of spontaneous and induced mutation in mammalian cells at the DNA sequence level. Because of the inherent complexity of higher eukaryotic cells, this type of information is difficult to obtain. In contrast, a large body of information, particularly for the lac I gene of E. coli, pertaining to both spontaneous and induced mutation has been obtained in prokaryotic systems. For these studies recombinant DNA techniques have been used to link the lac I gene to a plasmid vector, which also contains the SV40 origin of replication and the chicken thymidine kinase (TK) gene, that can replicate and be selected in both bacterial and mammalian cells. The lac I gene can thus be introduced into mammalian cells for mutagenesis and returned to E. coli for rapid detection and analysis of mutations. As constructed, the plasmid vector can be integrated into the chromosomes of a TK-mammalian cell line, mutagenized, and rescued by fusion to cells permissive for SV40 replication or it can be directly introduced into permissive cells where it can be mutagenized during its replication. Initially, the studies have been focused on the latter approach by introducing the plasmid into COS 7 simian cells by DEAE-dextran transfection. Mutants in lac-I were observed at frequencies of one to several percent during the passage of the plasmid in the mammalian cell in the absence of any mutagenic treatment. This is compared to a spontaneous mutation rate in E. coli of less than 10<sup>-5</sup>. The lesions detected include a large number of base substitutions, in addition to deletions, duplications, and more complex rearrangements such as the insertion of host genome sequences into the plasmid. These results suggested the possibility that the majority of the mutations were initiated by intracellular pathways of degradation and completed when the damaged DNA was processed by error prone repair systems in the nucleus. Attempts are being made to extend these experiments to cells of several different species. If it is shown that the mutagenic effect of DNA transfection is widespread, then caution must be exercised in the interpretation of experiments involving DNA transfer. The high mutation frequency observed implies that DNA introduced by transfection may acquire alterations by the time it is established in the nucleus (31).

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 compound in these studies. They have been shown to exert their effects by

binding to specific receptors on the cell surface membrane. 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 and the mimicry of the transformed phenotype by normal cells and the enhancement of transformation by chemicals and oncogenic viruses. Two of the studies are focused on the perturbation of ion fluxes by the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Since the action of TPA may be mediated by the phosphorylation of proteins and lipids, seven 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 five of the studies the effect of TPA on cell differentiation is being characterized. 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 arachidonic acid cascade by TPA and other first and second stage promoters, is the focus of two of the studies. The activation of the 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 at least five studies.

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 Grant 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 non-phorbol tumor promoters and/or cocarcinogens; and (5) interdisciplinary studies involving epidemiologists and experimentalists to test hypotheses concerning tumor promotion generated by either.

In FY82 12 grants were funded from applications submitted in response to this RFA; ten were approved for three years of funding and two for four 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 is 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 is being 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 is being studied. Using a heterotopically transplanted rat bladder system one laboratory is investigating the promoting effect of urine components on bladder carcino-

genesis induced by N-methyl-N-nitrosourea and N-butyl-N-(3-carboxypropyl)-nitrosamine. The hypothesis that asbestos and selected non-asbestos minerals act as tumor promoters in carcinogenesis of the respiratory tract is being studied using a hamster trachea model. The determination of whether EBV-related oncogenic mechanisms in in vitro virus-cell interaction models involves promotion is 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, is being conducted using the 7,12-dimethylbenz(a)anthracene skin tumor model system. Two in vitro model systems for testing for tumor promoters are being developed. One model system uses hepatocytes or liver cells from carcinogen-treated rats which are then promoted in culture using selected compounds. The other model system uses various rodent and human cells to test the hypothesis that the induction of mutations at the 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 is being studied. Finally, one of the studies involves a biochemical epidemiology project in which sex hormone levels in breast and prostatic cancer will be studied.

In many of the studies funded from the RFA, only preliminary data are currently available. In some of the studies interesting results obtained will be described in greater detail below. It is also evident that this RFA may have stimulated more studies on nonphorbol tumor promoters of relevance to humans. Some recently funded projects seek to study the activity of compounds such as n-alkanes, cyclosporin A, endogenous hepatic growth modulators, hormones and dietary L-tryptophan as tumor promoters. In addition, epithelial cell and organ culture systems from human endometrium and human prostate are being developed to study the process of tumor promotion by a variety of agents such as hormones and TPA.

Tumor promoters have been shown to modulate the action of epidermal growth factor (EGF) by reducing EGF binding and internalization and by potentiating the mitogenic activity of EGF in quiescent cells. EGF has been shown to stimulate the tyrosine phosphorylation of its receptors via a receptor-associated kinase activity. The potential regulatory role of tyrosine kinase activity, which is suggested by studies showing its association with the action of other growth factors and its apparent requirement for the oncogenic transformation of cells by a number of retroviruses, led to an examination of whether the effects of various tumor promoters on the action of EGF involved changes in receptor phosphorylation. In these studies three chemically distinct classes of tumor promoters were shown to block tyrosine-specific phosphorylation of the EGF receptor in hormonally stimulated human epidermal carcinoma A431 cells. The promoters tested included phorbol esters, indole alkaloids (teleocidin and lyngbyatoxin A), and polyacetates (aplysiatoxin and debromoaplysiatoxin). Non-tumor-promoting analogs did not have any effect on the levels of receptor phosphorylation. The results obtained from specific EGF binding studies demonstrated a correlation between the modulation of EGF receptor binding and phosphorylation of tyrosine by tumor promoters. The data suggests a possible role for protein kinase C, the putative cellular receptor for these tumor promoters, in the regulation of EGF-stimulated tyrosine kinase activity (174).

Diethylstilbestrol (DES) administration has been causally linked to the development of vaginal adenocarcinoma in women exposed to the drug in utero. Both male

and female children of DES-treated mothers have been reported to have other non-cancerous genital tract abnormalities. The mechanism by which DES produces genital lesions and adenocarcinomas is unclear. DES has been shown not to be mutagenic, although it can transform cells in culture. Recently, DES has been shown to act as a promoter in the C3H 10T 1/2 cell transformation system, mimicking the prototype promoting agent TPA in causing the emergence of transformed foci following a subthreshold dose of 3-methylcholanthrene. The promotion of altered phenotypes by TPA in human endometrial stromal cells which were initiated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was previously demonstrated in the laboratory of D.G. Kaufman (112). Using the same cells pretreated with MNNG, chronic treatment with DES was shown to increase the expression of phenotypic alterations. The indicators of preneoplastic changes evaluated (abnormal morphology, enhanced gamma-glutamyltranspeptidase expression and colony formation under restrictive conditions) were all more pronounced after combined treatment with MNNG and DES compared to carcinogen or DES alone. The results showed that the DES effect was not due simply to growth enhancement or an alteration in the toxicity of MNNG by DES. A shift in the isozyme pattern of lactate dehydrogenase was detected which would be indicative of a hormonal response. The results of this and other studies suggest that the effects of DES as a tumor promoter or cocarcinogen in human cells may be mediated through processes such as superoxide production or interruption of the mitotic spindle apparatus, although this may be coupled with hormonal effects (112).

It is well established that consumption of alcoholic beverages has a multiplicative effect on the risk of epithelial cancer of the oral cavity, oropharynx, larynx, and esophagus among tobacco smokers. The mechanism underlying the enhancing effect of ethanol is still unknown. In one study to address a possible mechanism, the effect of ethanol on the carcinogenic potency and organo-specificity in F344 rats of one of the most abundant tobacco-specific N-nitrosamines, N-nitrosornicotine (NNN) was investigated. In order to mimic the human orodigestive tract exposure, both ethanol and NNN were given in a liquid diet. The results showed that nasal mucosa were the main target tissue of NNN when it was given by sc injection and that ethanol consumption had no effect on the distribution and incidence of nasal cavity tumors. The nasal mucosa and esophagus were the major target tissues when NNN was given in the liquid diet. The incidence of nasal cavity tumors was enhanced and esophageal tumor incidence was decreased by ethanol consumption. The effects of ethanol on the alpha-carbon-hydroxylating enzyme system which activates NNN were studied. The results of those experiments showed that the increased susceptibility of the rat nasal mucosa to the carcinogenic effects of NNN added to an ethanol-containing diet could be due, in part, to an induction of activating enzymes by ethanol. However, since chronic ethanol consumption had no apparent effect on the incidence of nasal cavity tumors in rats given NNN by sc injection, it was concluded that factors other than enzyme induction are probably important in determining the effects of ethanol on NNN carcinogenicity (85).

Asbestos has been shown to act as a cocarcinogen in the respiratory tract and exhibits properties of classical tumor promoters. These include the ability to stimulate proliferation of cells and to inhibit normal cellular differentiation. Most experimental evidence obtained to date indicates that physical parameters such as the length and diameter of fibers are important in influencing the pathogenicity of asbestos. The intrapleural inoculation of materials composed of fibers which are long and thin results in the experimental induction of mesotheliomas in rats, whereas short, thick fibers and particles are less tumorigenic. The fibrous morphology of asbestos was hypothesized to also be important

in the induction of cell proliferation and squamous metaplasia. To test this hypothesis, several different fibrous materials and their nonfibrous analogs were examined for their ability to induce these alterations in organ cultures of hamster trachea. Chrysotile and crocidolite asbestos, attapulgite, a naturally occurring fibrous clay material, and two forms of fiberglass were examined. The results demonstrated that exposure to both crocidolite asbestos and fiberglass resulted in significant increases in squamous metaplasia over a range of dosages. Attapulgite and both "long-" and "short-" fiber preparations of chrysotile asbestos gave similar but less marked effects. Metaplasia was not produced by nonfibrous analogs of each material (riebeckite, antigorite and glass particles). Thus, these studies confirm the hypothesis that asbestos, and fibrous materials in general, appear to stimulate squamous metaplasia because of their fibrous geometry. Since squamous metaplasia in the human respiratory tract is topographically associated with squamous cell carcinoma, its stimulation could lead to respiratory tract cancer through a number of possible mechanisms (155).

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 fill a perceived need 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 in the response to chemical carcinogens between experimental animals and humans. 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 FY81, 16 grants were funded from applications submitted in response to this RFA; 15 were approved for three years and one for a five 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 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.

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 the cells transformed by chemical carcinogens. In addition, many more investigators utilize more than one species or strain of animal in their proposed studies.

It has become clear from a number of studies that hydrocarbon metabolism and DNA adduct formation can differ in different tissues and species, in microsomes compared to whole cells, and under different conditions of induction and inhibition of such enzymes as aryl hydrocarbon hydroxylase and epoxide hydrolase. Thus, it is not surprising that the use of various different activation systems can result in quantitative differences in the biological activity of a given carcinogen. Some studies have indicated that intact cells, which have a full complement of oxidative and conjugative enzymes, may be more appropriate as activators in short-term tests than are subcellular fractions and that activation by human cells may be warranted if the primary interest is to determine the potential genotoxic risk of a chemical to humans. The use of aneuploid tumor cell lines for studying hydrocarbon metabolism in human cells is considered to have the advantage of being established cell lines from which large amounts of reproducible material can be obtained and they frequently have a high hydrocarbon metabolizing capacity. The present study is part of an overall effort in the laboratory of L. Diamond (44) to establish the validity of using a human hepatoma cell line, HepG2, as an exogenous activation system for polycyclic aromatic hydrocarbons (PAHs) in short-term assays. HepG2 cells were compared with low-passage hamster embryo (HE) cells for their ability to metabolize and activate 7,12-dimethylbenz(a)anthracene (DMBA) to mutagenic and DNA-binding metabolites under similar conditions of cell-mediated mutagenesis. The results showed that the HE cells were much more effective than were HepG2 cells in activating DMBA to metabolites mutagenic for Chinese hamster V79 cells, the mutation induction to 6-thioguanine resistance being about ten times greater at 0.1 ug/ml DMBA with HE cells than with HepG2 cells as activators. The maximum value of DMBA binding to DNA was about 15-fold higher in HE cells versus HepG2 cells (180.7 pmoles/mg DNA vs. 12.1 pmoles/mg DNA), although the pattern of DNA adducts formed was nearly identical. The studies also show that there is a good correlation between the level of DNA adduct formation in the activating cells and the induced mutation frequency in the target cells. The results obtained indicated that the marked difference in the ability of HepG2 and HE cells to activate DMBA in cell-mediated mutation assays is not due to a lower metabolizing capacity of HepG2 cells for DMBA. Instead, it is concluded that there are significant differences in the metabolic pathways used by the two cell types which lead to the marked reduction of DNA-binding metabolites in one cell type (HepG2) compared to the other (HE). Additional evidence is accumulating that HepG2 cells are an excellent intact human cell activation system for PAHs and other mutagens/carcinogens in short-term assays. The data, more importantly, demonstrate the need to use more than one activation system before drawing conclusions about the relative biological activity of individual chemical compounds.

In recent studies the laboratory of G. Michalopoulos (148) has described a technique for the isolation of intact, viable, human hepatocytes which maintain relatively high levels of cytochrome P-450 in primary culture and respond with unscheduled DNA synthesis after the addition of carcinogenic chemicals. Human hepatocytes have been combined with human fibroblasts to constitute a system composed entirely of normal human cells that can be used to investigate the mutagenicity of chemicals that require metabolic activation. Mutations at the hypoxanthine-guanine phosphoribosyltransferase locus in human fibroblasts were shown to result following the addition of diethylnitrosamine to this system. A comparative analysis of diethylnitrosamine-induced unscheduled DNA synthesis and hepatocyte-mediated mutagenesis indicated that there was a great degree of similarity between the human and the previously studied rat hepatocytes in their response to diethylnitrosamine in vitro.

It has been previously established that there are interindividual variations in the metabolism of foreign compounds such as therapeutic drugs by humans. This variation appears to have a strong genetic component, although the environment can modify metabolizing capacity and there is also intraindividual tissue variation in metabolizing capacity. The variation in metabolism that leads to varying effects of drugs might also be expected to be associated with differences in the susceptibility of different individuals to the effects of chemical carcinogens. While variations in metabolism are often attributed to qualitative and quantitative differences in the isozyme composition of cytochrome P-450 and other enzymes which metabolize xenobiotics, little biochemical evidence has been available up to now to support this hypothesis and to probe its molecular basis. Multiple forms of cytochrome P-450 have been shown to exist in experimental animals, but the demonstration of similar enzyme polymorphisms in humans has lagged behind experimental animal models due to technical considerations. The laboratory of F.P. Guengerich (76) has now successfully purified to homogeneity six different cytochrome P-450 isozymes from human liver and has characterized them for some of their electrophoretic, spectral, catalytic and immunochemical properties. Differences were noted among all six cytochrome P-450s for some or all of the parameters determined. The data obtained provide a strong biochemical basis for the view that distinct isozymes of cytochrome P-450 exist in humans and that these isozymes differ in catalytic activity toward drugs and carcinogens (76).

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 six 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 can also be examined. In two of the projects, mutants or variant cells, altered in their ability to induce the carcinogen metabolizing enzymes, are being isolated and characterized. Using the inbred hamster model the genetic variation in arylhydroxamic acid acyltransferase, sulfotransferase, N-acetyltransferase, and N-deacetylase enzymes which are involved in the metabolism of aromatic amines is being studied. The characterization, localization and regulation of the enzyme arylsulfotransferase IV is the focus of one other study.

Aryl hydrocarbon hydroxylase (AHH), a cytochrome P-450 containing enzyme complex, and epoxide hydrolase are the enzymes which are involved in the activation of polycyclic aromatic hydrocarbons (PAHs) to their ultimate carcinogenic derivatives. Certain P-450 and non-cytochrome P-450 dependent activities can be induced in cells by PAHs and compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The induction of these various enzymes has been shown to be mediated by a cytosolic receptor protein (the Ah receptor) which binds PAHs and then translocates to the nucleus, where it is believed to enhance transcription of the relevant genes. Both dominant and recessive AHH-deficient mutants of the mouse hepatoma line, Hepa-1 have been isolated in the laboratory of Oliver Hankinson (81). Using somatic cell genetic techniques, the recessive mutants have been shown to belong to three complementation groups, the group B mutants possessing greatly reduced amounts of Ah receptor and the group C mutants being characterized as unable to translocate the PAH-receptor complex into the nucleus. The primary defects in the group A recessive mutants and in the dominant mutants have not yet been identified. The purpose of the present study was to determine whether the



various mutations affected only cytochrome P-450 catalyzed activities, or whether they also affected other enzymes associated with PAH metabolism. In addition to AHH, another cytochrome P-450-dependent activity, ethoxyresorufin-O-deethylase was measured. It was found that all mutants lacked this activity as well as AHH. However, all mutants retained normal activities of NADPH-cytochrome P-450 reductase, epoxide hydrolase and ornithine decarboxylase. Also, it was shown that the mutant cells retained two liver-specific functions possessed by the parental line, albumin and transferrin secretion. From these studies it was concluded that the phenotypic alterations in the mutants were restricted to the cytochrome P-450 activities measured (81).

The development of a selection procedure for isolating cells possessing the AHH enzyme system could provide further insight into the mechanism of induction of AHH. A single-step selection procedure has been developed by the laboratory of Oliver Hankinson (81) making use of the observation by Miller and Whitlock (*J. Biol. Chem.* 256: 2433-2437, 1981) that the mouse hepatoma cell line used in these experiments, Hepalclc7 cells, rapidly eliminate PAHs and that certain PAHs can be rendered highly cytotoxic when illuminated with near-UV light. Twenty PAHs were screened for phototoxicity and toxicity in the absence of light in Hepalclc7 cells which have high inducible AHH activity and in AHH-deficient mutants derived from this line. It was found that the PAH, benzo(g,h,i) perylene, had the greatest phototoxicity in cells lacking AHH but was not toxic to cells possessing AHH either in the presence or absence of near-UV light. It was also shown that cells possessing AHH can be selected from a majority population of cells lacking this enzyme activity by this procedure. The developed selection procedure has a number of important applications. Experiments are planned to isolate revertants of the AHH-deficient mutants, to isolate regulatory constitutive mutants and to potentially detect and isolate clones of the AHH-deficient mutants which have regained AHH activity through transfection with wild-type DNA. Also, the technique may be useful in experiments aimed at the identification of human chromosomes involved in the regulation of AHH, in which the mouse/human hybrid mapping strategy will also be used (81).

In other studies using the same Hepalclc7 mouse hepatoma cells, the laboratory of James Whitlock (218) has used a cDNA probe which is specific for a PAH-inducible form of cytochrome P-450, P<sub>1</sub>-450, to analyze the accumulation of enzyme-specific mRNA in wild-type Hepalclc7 cells and in variant cells defective in AHH induction. The experiments show that in wild-type cells, TCDD induces a 25- to 50-fold increase in the steady state concentration of cytochrome P<sub>1</sub>-450-specific mRNA which migrates in agarose gels as a single species of about 2900 nucleotides in length. The RNA induction precedes AHH induction by 2-4 hrs and can occur in cells in which protein synthesis is inhibited by 95 to 97%. The results demonstrated that variant cells with decreased TCDD receptors have decreased basal and induced levels of P<sub>1</sub>-450 mRNA and variant cells with a defect in nuclear localization of the inducer-receptor complex have virtually no basal or inducible levels of P<sub>1</sub>-450 mRNA. Thus, it was concluded from these and other experiments that the expression of the cytochrome P<sub>1</sub>-450 gene is under transcriptional control and that the amount of the inducer-receptor complex in the nucleus regulates the synthesis of enzyme-specific mRNA (218).

The cytochrome P-450s, though functionally related in catalyzing the oxidation of endogenous and foreign compounds, comprise a family of enzymes that differ from one another in primary structures, substrate specificities, antigenic characteristics and spectral properties as well as in their induction response to various xenobiotics. In one study Southern blot analyses of rat, mouse and rabbit genomic

DNA were conducted using a cDNA probe (pP-450b-5) derived from a major phenobarbital-inducible cytochrome P-450 mRNA species in Sprague-Dawley rats. The Southern blot analyses suggested that multiple sequences homologous to the cDNA probe are present in rat and mouse genomes. The cDNA probe detected six polymorphic DNA fragments when hybridized to DNA from C57BL/6J and DBA/2J mice restricted with endonucleases Eco RI, Bam HI, and Pvu II. This gene family appears to be distinct from the gene sequences encoding pregnenolone-16 alpha-carbonitrile and 3-methylcholanthrene-inducible cytochrome P-450s. Using the B<sup>6</sup>D recombinant inbred strains of mice, five of the DNA polymorphic sequences detected were mapped to the Coh (coumarin hydroxylase) locus on chromosome 7 of the mouse. It is concluded that the region of the Coh locus on chromosome 7 may be the site of a cluster of cytochrome P-450 genes (149).

Development of Analytical Methodology for Detecting Chemical Carcinogens in Body Fluids and Environmental Samples: Technological or methodological breakthroughs which enable a great increase in resolution and/or a significant reduction in the technical labor intensity required to obtain information are generally associated with a new advent in our understanding at the mechanistic level. A small number of grants are supported in this Branch which have taken an approach based on improvement of our analytical technology in chemical carcinogenesis.

Studies of DNA alkylation in vivo challenge analytical chemistry by simultaneously requiring both qualitative and quantitative analyses at high sensitivity on closely related compounds, some of which are involatile and thermally unstable and all of which occur in particularly complex matrices. Fortunately, recent progress in liquid chromatography and mass spectrometry offers capabilities to match these requirements.

The primary objective of one interesting project (32) is to develop a highly sensitive liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) system for the analysis of methyldeoxyribonucleosides formed in carcinogen-treated DNA. This system will allow the detection and quantitation of methylated nucleosides generated from nonradioisotopically labeled carcinogens at levels comparable to those which induce biological effects in vivo.

The task of separating of isomeric alkylated nucleosides utilizes high pressure liquid chromatography. Satisfactory separations of the various methylated compounds in reasonable times can be achieved by a procedure which uses an ion-paired reverse-phase column. The technique of tandem mass spectrometry (MS/MS) also provides separation capabilities as well as specific, sensitive detection and very rapid sample throughput. In the MS/MS mode of analysis, one analyzer serves as the separator and the second as the means of sample identification. MS/MS complements LC in distinguishing separated isomeric compounds; a combination of the two methods should allow optimization of separation, specificity and speed of analysis.

The goal set by another group (57) is to find a way around the current technological impasse on the analyses of N-nitrosamides. Nonvolatile N-nitroso compounds, especially the N-nitrosamides, have been implicated as possible causative agents in gastric and other cancers. N-nitrosamides are also known to be rapidly formed from their precursors under a wide variety of conditions. At present, it is not known whether these highly carcinogenic compounds are present in human body fluids or whether they are contained in foodstuffs, cosmetics, drugs, or pesticides. This paucity of knowledge is due entirely to the lack of

viable analytical chemistry procedures for the analysis and detection of these compounds at the parts-per-billion (ppb) sensitivity level.

The key technique is based on the Thermal Energy Analyzer (TEA), an instrument developed by the applicant for the detection of N-nitrosamines at the ppb level. This detector thermally cleaves nitrosamines at the N-NO bond to yield NO which is detected by chemiluminescence following its reaction with ozone. The instrument cannot be directly applied to nitrosamides, however, since these compounds do not cleave at the N-NO bond, but undergo irreversible thermal rearrangement to give diazo esters. By increasing the pressure in the catalytic pyrolyzer and by use of a new nickel-manganese-chromium alloy as a catalyst, the applicant has been able to achieve almost quantitative thermal degradation of nitrosamides to NO. As this technology is optimized, the focus will also be applied to the establishment and qualification of analytical procedures for the N-nitrosamides so that they can be rapidly screened and quantitated in selected matrices, including human body fluids at the ppb concentration level.

Role of DNA Repair in Carcinogenesis: Though not yet rigorously proven, it is highly likely that mutagens effect neoplastic transformation by causing damage to the nucleotide bases of DNA in the genome of living cells. For example, studies on the human hereditary disease, xeroderma pigmentosum (XP), have shown an increased susceptibility of these cells in culture to mutagenesis and a markedly increased incidence of skin cancers in patients exposed to sunlight. The recent demonstrations that the T24 human bladder carcinoma oncogene differs from the naturally occurring gene by the replacement of a single nucleotide provides further evidence suggesting a role of DNA damage and mutation in somatic cells in the pathogenesis of at least some forms of human cancer, though the significance of this particular observation is not yet definitive.

It has been known for many years that a variety of organisms, including bacteriophages, bacteria, and lower and higher eukaryotes are capable of repairing certain forms of base damage to their DNA. The most extensively studied form of DNA damage is that following exposure of living cells or DNA in vitro to ultraviolet irradiation (UV) at wavelengths of about 260nm. Under these conditions the principal and probably the biologically most significant photoproduct in DNA is the pyrimidine dimer. The repair of DNA-containing pyrimidine dimers and other bulky forms of base damage caused by agents such as photoactivated psoralen, mitomycin C, 4 nitro-quinoline-1-oxide etc., occurs by a process called nucleotide excision repair.

Nucleotide excision repair involves a series of enzyme catalyzed events by which damaged bases are excised as part of an oligonucleotide and the resulting gaps repaired by DNA synthesis and phosphodiester bond rejoining. Very specific biochemical events during excision of DNA are the recognition of bulky base damage in DNA and the incision (nicking) of the deoxyribose-phosphate backbone at or near sites of such damage. The substrate specificity of the putative endonuclease (damage-specific DNA incising activity) that recognizes such damage is broad and includes pyrimidine dimers, as well as base damage produced by the various chemicals mentioned above.

A damage-specific DNA incising activity has not been isolated from any eukaryotic source, and several studies indicate that the biochemistry of the incision of damaged DNA in higher organisms is even more complex than in E. coli. For example, nine genetic complementation groups have been defined in the human disease XP, suggesting that as many as nine different genes may be required for

nucleotide excision repair in normal human cells. More detailed studies on these complementation groups indicate that at least five of them are severely, if not completely, defective in the incision of UV-irradiated DNA *in vivo*. Cells from the other four complementation groups appear to be leaky in this respect; i.e., they carry out residual incision of DNA, suggesting that in human cells some genes are absolutely required for excision repair (presumably genes coding for the damage-specific DNA incising activity), while others have a more secondary role. The latter may code for accessory proteins such as DNA helicases or single strand binding proteins which possibly increase the efficiency and/or fidelity of excision repair *in vivo*. Indeed, in *E. coli*, mutants defective in DNA helicase II (coded by the *uvrD* gene) or in single strand binding protein, are abnormally sensitive to DNA damaging agents such as UV radiation.

The purification of damage-specific DNA incising activity from extracts of human diploid fibroblasts in culture requires inordinately high concentrations of protein in incubation mixtures and as a consequence the activity is very labile to fractionation.

The lower eukaryote, *Saccharomyces cerevisiae* (a yeast), is not subject to these limitations. This organism has been extensively characterized genetically, and with respect to DNA repair, over twenty distinct loci have been identified, mutations which confer abnormal sensitivity to killing by UV or ionizing radiation. Most of these are designated as RAD loci. Nine genes (RAD1, RAD2, RAD3, RAD4, RAD7, RAD10, RAD14, RAD16, and MMS19) are required for, or involved in, the excision repair of bulky base damage (e.g., pyrimidine dimers) *in vivo*. Studies carried out in two laboratories (62, 168), with grant support, have shown that mutants in the RAD1, RAD2, RAD3, RAD4, and RAD10 genes are totally defective in the incision of DNA at pyrimidine dimers during post-UV incubation of cells *in vivo*. Thus, at least five genes are required for DNA incision during excision repair in yeast--a remarkable biochemical complexity that bears a striking analogy to that apparently operative in human cells. Mutants defective in the other genetic loci mentioned above are deficient rather than defective in DNA incision and may either be leaky mutants, or as suggested above with respect to the XP complementation groups, may reflect the action of genes which although not required for DNA incision, nonetheless participate in this process in living cells. Thus in general the genetics of excision repair in human and in yeast cells bear remarkable similarities and in fact may be identical.

Work is proceeding on the isolation of individual plasmids containing the five cloned Rad genes from a yeast genomic library. The genes will then be subcloned and the 5' noncoding regions of the subcloned genes will be tailored so as to place the coding regions under the control of a regulative yeast promoter, so that transcription results in over-expression of the genes. Isolation of the highly purified Rad proteins will enable study of the *in vitro* biochemistry of the incision events associated with excision repair of DNA in eukaryotic cells.

Excision repair and postreplication repair are two important mechanisms for the survival of cells exposed to radiations or mutagenic chemicals. Postreplication repair, a process studied most extensively in cells exposed to ultraviolet light, takes place when DNA containing damaged bases undergoes replication. The newly synthesized daughter strands are formed with postreplication gaps opposite pyrimidine dimers or other damaged bases in the template strands. During subsequent repair processes, the postreplication gaps cause sister strand exchanges and the gaps are filled with the correct base sequences (94). Postreplication repair in bacteria depends on the recombination gene *recA*<sup>+</sup>. Strains

carrying  $recA^-$  mutants are very sensitive to ultraviolet light. Double mutants carrying both  $recA^-$  and  $uvrA^-$  or  $uvrB^-$  mutations which block excision repair are many times more UV sensitive than either  $recA^-$  or  $uvrA^-$  single mutants.

Human skin fibroblasts carrying the trait xeroderma pigmentosum show defects in DNA repair synthesis and nine complementation groups have been recognized. In addition the so-called xeroderma variants appear to be normal for repair synthesis but are defective in postreplication repair, and little is known of the nature of the defect or even the mechanism of postreplication repair in human cells.

Postreplication repair in E. coli is a complex process which is thought to depend upon sister chromatid exchanges between newly replicated duplexes. The finding that most or all postreplication gaps cause sister chromatid exchanges, led to the conclusion that DNA containing such gaps is a good substrate for imitating genetic recombination. As with other forms of genetic recombination, sister chromatid exchanges involve the cutting and joining of DNA strands and it is with this cutting by recombination enzymes, referred to as cutting in trans (CIT), that one group (94) of investigators has been concerned. By using a series of mutants of E. coli K12 the investigators are working to clone the gene responsible for the nicking of cross-linked DNA (CIT). The gene product is to be isolated and purified and used to study the mechanism of recombination.

Other groups (20 and 72) are studying postreplication repair in mammalian cells in response to a potent carcinogenic metabolite of benzo(a)pyrene. The diol epoxides of benzo(a)pyrene are being studied in Chinese hamster ovary cells (CHO) (20) and in human IMR-90 and hamster V79 cells (72) to determine the effect on replication pattern and rate of DNA replication when the carcinogen is applied before and during the S phase of the cell cycle. They are looking for the effect on utilization of small replication intermediates into larger regions and the production of gaps or discontinuities in replicated daughter strands. These should help to indicate the presence of single strand regions in the template DNA or signal the presence of blocked replication forks.

DNA glycosylases constitute a ubiquitous class of DNA repair enzymes that catalyze the hydrolysis of the N-glycosyl bonds linking nitrogenous bases to the deoxy-ribose-phosphate backbone of DNA, resulting in the excision of damaged bases as free bases. This DNA repair mode is thus sometimes referred to as base excision repair to distinguish it from the nucleotide excision repair mode described earlier. The apurinic/aprimidinic sites in the genome generated by the action of DNA glycosylases are generally repaired by excision/resynthesis, following the incision of DNA containing such sites by another class of repair enzymes called apurinic/aprimidinic (AP) endonucleases. Several groups have focused attention on the purification and characterization of specific glycosylases responsible for removal of various modified bases from DNA. One group (35) is capitalizing on previous experience gained from the purification of a glycosylase from E. coli which recognizes DNA that is altered by aflatoxin B<sub>1</sub>. This enzyme, FABy-DNA glycosylase, is also present in rat liver and is now being purified and characterized. In a similar vein, another group (67) is working on an overall study of the mechanisms of enzymes involved in repair of DNA and chromatin components after treatment with alkylating agents. Of particular interest is a glycosylase which removes 3-methyladenine which is being studied in both E. coli and rat liver cells.

Another laboratory (48) is also attempting purification of the endonuclease specific for photoalkylated DNA from human placenta and from Micrococcus luteus

which is more stable and which may provide information about suitable purification procedures, optimum assay conditions, and appropriate substrates. The lack of knowledge of the enzymes that initiate human DNA excision repair is not surprising because many of them combine low activity and high lability, making definitive purifications difficult. The problem, however, is a vital one in that we must gain knowledge of the types of lesions which are recognized by human cells and repaired as contrasted to those which are unrecognized, remain uncorrected, and whose persistence may lead to carcinogenesis.

DNA repair in eukaryotic cells must be ultimately understood in the context of the structure of chromatin rather than free DNA. By applying the new tools of DNA technology, huge advances in our understanding of the programmed structure of the eukaryotic gene have been accomplished in a relatively short time. To complement this explosion of information, it is important to ascertain how these recently appreciated properties of DNA are packaged within domains of polynucleosomes and what mechanisms are operative in "housekeeping" and repair of chromatin in its highly condensed conformation. One rapidly emerging area of interest is focused on the importance of poly(ADP-ribose) polymerase in chromatin structure and repair (192, 102, 103). Poly (ADP-ribose) polymerase is a chromatin associated enzyme which catalyzes the successive covalent transfer of the ADP-ribose moiety of NAD to histone acceptors to generate poly (ADP-ribose) on these proteins. A significant feature of this reaction is the fact that the purified polymerase possesses a strict requirement for DNA for activity; more importantly, the ability of DNA to support poly (ADP-ribose) synthesis is completely dependent upon the number and type of single breaks it contains.

It has been long recognized that NAD is a major metabolite of the animal cell nucleus. The enzymes involved in both the synthesis and breakdown of NAD are associated with chromatin within the nucleus. The rate of NAD biosynthesis in HeLa nuclei is extremely high (on the order of  $10^5$  molecules/sec/cell), yet approximately 95% of this replaces the NAD that is catabolized in the nucleus for poly (ADP-ribose) synthesis, presumably for repair replication, and only 5% maintains the cytoplasmic NAD pool size during growth. Normal cells showed an increase in poly (ADP-ribose) synthesis in response to both alkylating agents and UV irradiation, whereas XP lines only show a response to the former agents.

It is particularly noteworthy that highly specific inhibitors of the polymerase (i.e., aminobenzamide, nicotinamide) prevent the *in vivo* NAD depressions induced by DNA strand breakers; furthermore, investigators (192) have shown that they increase the cytotoxicity of the strand breaking agent. The latter point emphasizes the potential practical importance of poly ADP-ribosylation when related to cancer chemotherapy. The next obvious consideration must be how the resultant alteration of nuclear proteins, caused by increased poly ADP-ribosylation, aids in the complicated series of reactions leading to cellular recovery from strand breakage (i.e. supercoil stabilization, ligation reaction, etc.). The operating hypothesis is that the poly(ADP-ribose) system functions for stabilization of the higher order structure and supercoiling of chromatin, as well as its local unwinding to achieve accessibility in chromatin to repair and replicative enzymes.

Two supported groups (192 and 103) have developed antibodies to poly(ADP-ribose) and have characterized immuno-affinity columns that are proving successful in the assessment of DNA strand breaks induced in biological systems by UV-irradiation and various chemical carcinogens. The antibody columns have also enabled the fractionation of chromatin domains (ca. 10% of the total) which are undergoing

poly ADP-ribosylation. These ADP-ribosylated nucleosomes contain significant numbers of internal DNA strand breaks compared to unbound chromatin.

MOLECULAR CARCINOGENESIS  
GRANTS ACTIVE DURING FY84

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ACS, George Mount Sinai School of Medicine 5 R01 CA 16890-09	Studies on Chemotherapeutic Deoxyribonucleosides
2. ADAIR, Gerald M Univ of Texas System Cancer Ctr 5 R01 CA 28711-03	Expression of Genetic Variation in Cultured Cells
3. ALBERT, Roy E New York University 5 P01 CA 26724-04	Inhalation Carcinogenesis of Environmental Agents
4. ALBERTINI, Richard J University of Vermont and State Agriculture College 5 R01 CA 30688-03	Direct Mutagenicity Testing in Man
5. ASHENDEL, Curtis L Purdue University West Lafayette 1 R01 CA 36262-01	Interactions of Tumor Promoters with Receptors
6. AVADHANI, Narayan G University of Pennsylvania 5 R01 CA 22762-06	Cellular and Molecular Targets of Chemical Carcinogenesis
7. BAIRD, William M Purdue University West Lafayette 5 P01 CA 30234-03	Molecular Mechanisms of Carcinogen-DNA Interactions
8. BAKER, Donald G University of Virginia (Charlottesville) 2 R01 CA 25890-03	Influence of Hyperthermia on X-ray Carcinogenesis
9. BECKER, Frederick F Univ of Texas System Cancer Ctr 2 R01 CA 20657-08	Phenotypic Analysis of Chemical Carcinogenesis
10. BECKER, Frederick F Univ of Texas System Cancer Ctr 5 R01 CA 20659-07	Analysis of Cellular Events in Chemical Carcinogenesis
11. BECKER, Frederick F Univ of Texas System Cancer Ctr 2 R01 CA 28263-04	Chromosomal Proteins During Chemical Carcinogenesis



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| 12. | BENFIELD, John R<br>City of Hope National Med Ctr<br>5 R01 CA 28045-03                  | Esophageal and Pancreatic<br>Carcinogenesis                 |
| 13. | BENFIELD, John R<br>City of Hope National Med Ctr<br>5 R01 CA 29373-03                  | Model of Bronchogenic Lung Cancer                           |
| 14. | BERNHARD, William A<br>University of Rochester<br>5 R01 CA 32546-09                     | Effects of Ionizing Radiation<br>on Nucleic Acids           |
| 15. | BERRY, David L<br>U.S. Agricultural Research Serv<br>5 R01 CA 28968-02                  | Mode of Action of Phorbol Esters<br>in Epidermal Cells      |
| 16. | BIRT, Diane F<br>Univ of Nebraska Medical Center<br>1 R01 CA 33368-01                   | Urinary Bladder Cancer Promotion<br>by Dietary L-Tryptophan |
| 17. | BLOOM, Stephen E<br>Cornell University (Ithaca)<br>5 R01 CA 28953-03                    | Chick Embryos for Detecting<br>Environmental Mutagens       |
| 18. | BLUMER, Jeffrey L<br>Case Western Reserve University<br>5 R23 CA 30067-03               | Lymphocyte Carcinogen Metabolism<br>in Acute Leukemia       |
| 19. | BOUCK, Noel P<br>Northwestern University<br>5 R01 CA 27306-06                           | Genetic Analysis of Malignant<br>Transformation             |
| 20. | BOWDEN, George T<br>University of Arizona<br>5 R01 CA 26972-05                          | Postreplication Repair in Cultured<br>Mammalian Cells       |
| 21. | BOX, Harold C<br>Roswell Park Memorial Institute<br>2 R01 CA 29425-04                   | Molecular Studies of<br>Carcinogenesis and Mutagenesis      |
| 22. | BRANSCOMB, Elbert W<br>Univ of California (Berkeley)<br>5 R01 CA 30613-03               | Somatic Point Mutation Monitoring                           |
| 23. | BRANSCOMB, Elbert W<br>Univ of California (Berkeley)<br>5 R01 CA 31714-02               | Monitoring In Vivo Somatic<br>Mutations In Animals and Man  |
| 24. | BRASITUS, Thomas A<br>Michael Reese Hospital<br>and Medical Center<br>1 R01 CA 36745-01 | Colonic Epithelial Cell Plasma<br>Membranes                 |

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| 25. | BRESNICK, Edward<br>Univ of Nebraska Medical Center<br>1 R01 CA 35994-01A1    | Epoxide Hydrolase in Hyperplastic<br>and Neoplastic Livers     |
| 26. | BRESNICK, Edward<br>Univ of Nebraska Medical Center<br>5 R01 36105-02         | Comparison of Hamster Trachea<br>& Human Bronchus              |
| 27. | BRESNICK, Edward<br>Univ of Nebraska Medical Center<br>5 R01 CA 36106-02      | Polycyclic Hydrocarbon Metabolism<br>and Carcinogenesis        |
| 28. | BRESNICK, Edward<br>Univ of Nebraska Medical Center<br>5 R01 CA 36679-02      | DNA Repair After Polycyclic<br>Hydrocarbon Administration      |
| 29. | BROYDE, Suse B<br>New York University<br>2 R01 CA 28038-04                    | Carcinogen - DNA Adducts: Linkage<br>Site and Conformation     |
| 30. | BUTEL, Janet S<br>Baylor College of Medicine<br>5 R01 CA 33369-02             | Tumor Promotion and Murine Mammary<br>Cancer                   |
| 31. | CALOS, Michele P<br>Stanford University<br>5 R01 CA 33056-02                  | Mutation in Human Cells at the DNA<br>Sequence Level           |
| 32. | CHANG, Ching-Jer<br>Purdue University West Lafayette<br>1 R01 CA 35904-01     | Chemical Carcinogens and DNA<br>Interactions in Tissue Culture |
| 33. | CHEN, Fu-Ming<br>Tennessee State University<br>5 R01 CA 29817-03              | Binding of Benzo(a)pyrene<br>Metabolites to DNA                |
| 34. | CHEN, Lan B<br>Dana-Farber Cancer Institute<br>5 R01 CA 29793-03              | Chemical Carcinogenesis of<br>Epithelial Cells                 |
| 35. | CHETSANGA, Christopher J<br>Univ of Michigan (Ann Arbor)<br>5 R01 CA 33025-02 | Excision Repair of Alkylated DNA                               |
| 36. | CHRISTMAN, Judith K<br>Mount Sinai School of Medicine<br>5 R01 CA 25985-05    | Response of Phagocytic Leukocytes<br>to Tumor Promoters        |
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Cell-Cycle Related DNA Repair  
Mechanisms
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Replication in Human Fibroblasts
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in Mammalian Cells
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Dentistry of New Jersey  
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Carcinogenesis
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and Biology  
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Differentiation
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Optoacoustic Detection of  
Carcinogens
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Mechanism of Mouse Skin Tumor  
Promotion by Chrysarobin
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Induced Mutations
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Mouse and Human Cells

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Carcinogenesis
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Repair
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Mediated Mutagenesis Assay
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therapy Leukemogenesis
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Membranes
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Mutagenesis, Repair
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Xeroderma Pigmentosum
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Mutations in Humans
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Mutagenesis
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DNA Helices
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Crosslinks
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Repair in Human Cells

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Unexpressed Mammalian Genes
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in Rats
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Malignant Melanoma

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Cells by Carcinogens
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Epoxides with DNA
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of Human Hepatocytes
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Carcinogenesis
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Protein Kinases
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Carcinogenesis
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Mammalian Cell Extracts
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genesis in Fish
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in Bronchogenic Carcinoma

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Spontaneous Tumors
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and Biology  
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Promotion
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and Other Agents
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Carcinogenesis
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Carcinogen Screening
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Pancreas Carcinogenesis
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Invasion Mechanisms
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Carcinogenesis
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Cancer Model
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Psoralen + 360 nm Irradiation

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Cell Markers
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Structure
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Dimer DNA Damage
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in Colon and Esophagus
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Genes in Human Cells
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Somatic Mutation
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Populations
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Epidermal Cells
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Carcinogenesis
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Carcinogens and Chromatin  
Structure and Function
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Carcinogenic Damage to DNA
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of Preneoplastic Markers
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Cellular Processes
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Genes in Carcinogenesis
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Massachusetts Institute of Tech  
5 R01 CA 33297-02  
Gene Amplification and Tumor  
Promotion
205. VERMA, Ajit K  
Univ of Wisconsin (Madison)  
5 R01 CA 35368-02  
Ca<sup>2+</sup>-Dependent Processes Involved  
in Phorbol Ester Tumor Promotion
206. VOLSKY, David J  
Univ of Nebraska Medical Ctr  
5 R01 CA 33386-02  
Epstein-Barr Virus and Tumor  
Promotion
207. WALBORG, Earl F, Jr.  
Univ of Texas System Cancer Ctr  
5 R01 CA 27377-05  
Membrane Glycoproteins During  
Hepatocarcinogenesis
208. WALDSTEIN, Evelyn A  
Tel Aviv University  
1 R01 CA 35895-01  
Regulation of Induced O6-Methyl-  
Guanine Repair in Cells

209. WALKER, Graham C  
Massachusetts Institute of Tech  
5 R01 CA 21615-08  
Mutagenesis and Repair of DNA
210. WEBBER, Mukta M  
University of Colorado Health  
Sciences Center  
5 R01 CA 28279-03  
Human Prostatic Growth Regulation  
and Carcinogenesis
211. WEBBER, Mukta M  
University of Colorado Health  
Sciences Center  
1 R01 CA 33169-01A1  
Intrinsic & Extrinsic Tumor  
Promoters in Prostate Cancer
212. WEINSTEIN, I Bernard  
Columbia University  
5 P01 CA 21111-08  
Molecular Events in Chemical  
Carcinogenesis
213. WEINSTEIN, I Bernard  
Columbia University  
5 R01 CA 26056-05  
Cellular and Biochemical Effects  
of Tumor Promoters
214. WEISBURGER, John H  
American Health Foundation  
5 R01 CA 30658-03  
Strain Differences in  
Carcinogenesis
215. WENDER, Paul A  
Stanford University  
2 R01 CA 31841-04  
Synthetic Studies on Tumor  
Promoters and Inhibitors
216. WENNER, Charles, E  
Roswell Park Memorial Institute  
5 R01 CA 13784-11  
The Effect of Cocarcinogens  
on Cellular Membranes
217. WETTERHAHN, Karen E  
Dartmouth College  
5 R01 CA 34869-02  
Interaction of Chromate with  
Mitochondria
218. WHITLOCK, James P, Jr  
Stanford University  
5 R01 CA 32786-02  
Carcinogen-Metabolizing Enzymes:  
Action in Variant Cells
219. WILLIAMS, Jerry R  
George Washington University  
5 R01 CA 31015-03  
Mechanisms of Procarcinogenic  
Metabolism in Rat and Mammals
220. WILLIAMS, Jerry R  
George Washington University  
5 R01 CA 33482-02  
Cellular Hypermutability in  
Cancer Promotion
221. WINKLE, Stephen A  
Rutgers State University  
5 R01 CA 34762-02  
Cooperative, Selective Carcinogen,  
Drug Binding to DNA



222. WITSCHI, Hanspeter R  
Oak Ridge National Laboratory  
5 R01 CA 33795-02  
Enhancement of Lung Tumor  
Formation: Cell Kinetics
223. WITZ, Gisela  
Rutgers Medical School  
5 R01 CA 33270-02  
Free Radicals in Tumor Promotion
224. YAGER, James D Jr  
Dartmouth College  
1 R01 CA 36701-01  
Role of Gonadal Steroids in  
Hepatocarcinogenesis
225. YAGER, James D Jr  
Dartmouth College  
5 R01 CA 36713-02  
DNA Sequence Changes During  
Hepatocarcinogenesis
226. YANG, Nien-Chu C  
University of Chicago  
5 R01 CA 10220-14  
Chemistry of Biologically Active  
Oxiranes
227. YU, Fu-Li  
Rockford School of Medicine  
5 R01 CA 30093-03  
Aflatoxin B1 and Nucleolar RNA  
Synthesis
228. ZURLO, Joanne  
Dartmouth College  
1 R23 CA 36782-01  
Inducible DNA Repair in Pancreatic  
Carcinogenesis

SUMMARY REPORT  
SMOKING AND HEALTH

The Smoking and Health component within the Chemical and Physical Carcinogenesis Branch includes 14 grants with FY84 funding of \$1.96 million and four contracts with FY84 funding of \$0.45 million. It continues to support research directed toward understanding and mitigating the deleterious effects of smoking on health. Significant past efforts have included development of practical techniques for making and testing less hazardous cigarettes, epidemiology studies seeking means for identifying groups of individuals at high risk to smoking related diseases, and chemical analyses of major whole smoke components and their subsequent metabolic products. A major finding according to evidence to date, is that low tar, low nicotine cigarette smoke is less harmful to experimental animals than high tar, high nicotine cigarette smoke. These findings are reflected in the current trend to low tar, low nicotine commercial cigarettes by the consumer. Current program emphasis is focused on the toxicological and pharmacological aspects of the problem, with emphasis on nicotine and nicotine metabolites.

Grants Activity Summary:

Nicotine gives rise to relatively high concentrations of tobacco-specific N-nitrosamines (TSNA) during tobacco processing (less than or equal to 100 ppm) and during smoking (less than or equal to 2.5 µg/cigarette). It has been shown by analyzing saliva of 40 young people that TSNA are also formed during snuff-dipping, a habit becoming increasingly popular among young people. Chewing of betel quid with tobacco leads not only to the formation of TSNA, but also to at least three nitrosamines deriving from the alkaloid arecoline. Of these, 4-methylnitro-samino)propionitrile (MNPN) is a powerful carcinogen in rats which induces nasal and esophageal tumors. The pyrosynthesis of TSNA during smoking is greatly enhanced by the addition of tobacco leaf ribs with high nitrate content to the cigarette blend (U.S. commercial cigarettes contain about 20% of tobacco ribs).

Experimental studies in rats exposed to concentrations of 1,3 and 9 mmol/kg of the tobacco-specific N-nitrososornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) revealed high rates of nasal tumors. In addition, NNK causes lung tumors at all three dose levels, and at the high dose, it causes hepatomas, whereas NNN at the higher two doses elicits esophageal tumors and nasal tumors.

The nitrate content of tobacco influences not only the smoke yields of nitrosamines but also the yield of carcinogenic aromatic amines, which may contribute to bladder cancer in cigarette smokers.

Contrary to previous assumptions, cotinine and nicotine-N'-oxide, the major urinary metabolites of nicotine, were found to be inactive as tumor promoters or cocarcinogens in the urinary bladder of rats.

The distillates of tobacco blends from low-yield cigarettes contain, in the "flavor fraction," measurable amounts of mutagenic agents, some of which were identified as amino azaarenes.

One study has shown that the urine of cigarette smokers contains higher amounts of the noncarcinogenic N-nitrosoproline (NPRO) than the urine of nonsmokers. Similarly, increased amounts of NPRO was found in the urine of snuff-dippers. These findings suggest that the consumers of tobacco products have the capability for endogenous N-nitrosamine formation including the carcinogenic compounds in this class of substances. Since tobacco smokers and snuff-dippers are exposed to nicotine and nornicotine, it is likely that these amines give rise to carcinogenic NNN by endogenous nitrosation reactions. However, NNN is rapidly metabolized and its urinary metabolites are the same as those of nicotine. In order to document endogenous nitrosation, nornicotine-5'-carboxylic acid was synthesized. It will be administered to rats and the formation of N-nitrosornnicotine-5'-carboxylic acid will be monitored. This compound is presumed to be noncarcinogenic and is expected to be unchanged when excreted in the urine.

Another study is concerned with the metabolic fate of nicotine in rodent and human liver microsomal preparations. An important consideration has been the demonstration of cytochrome P-450-dependent oxidation of nicotine to the reactive iminium intermediate already characterized in rabbit and rat tissues. There is definitive evidence that human liver microsomal preparations are effective in the catalysis of this pathway. Additional mechanistic studies using specifically monodeuterated nicotine analogs (the cis and trans C-5 nicotine-d' analogs) have provided clear evidence that this 2-electron oxidative pathway is mediated by a stereoselective process involving abstraction of the trans C-5 proton (or deuterium). The absence of a significant isotope effect in this reaction suggests that the rate-limiting event involved in this major biotransformation step for nicotine is not the proton abstraction reaction itself, but is likely to be an initial electron abstraction leading to an iminium ion radical which itself may possess alkylating potential. Human liver preparations proved to be more selective in this reaction than rabbit liver preparations, implying a higher degree of enzyme-substrate stereochemical requirements for the human enzyme systems.

A second part of this study has been the development of a reaction scheme that will provide high specific activity tritium-labeled nicotine. Results to date show that 5-bromonicotine can be synthesized in a reaction pathway that leads to the pure (S)-enantiomer (the naturally occurring isomer of nicotine). Conditions for the hydrogenolysis of this nicotine derivative were optimized and using these conditions, the (S)-5-bromonicotine was converted to nicotine-5-d<sub>1</sub> using Pd/C and D<sub>2</sub>. Although a high yield of the deuterated product could be obtained and characterized by chemical ionization and electron ionization mass spectrometry as well as <sup>1</sup>H-NMR analyses, when an attempt was made to perform the same reaction with carrier free T<sub>2</sub>, a complex mixture of products was obtained which proved very difficult to purify. Although the yield of the final product was poor, enough radiochemically pure nicotine T-1, with an estimated specific activity of ca. 10 Ci/mmol was obtained to perform the planned experiments.

Other work on aspects of nicotine has been the characterization of the DNA adducts formed in vivo upon activation of NNN and NNK. NNK was injected IV (0.41 mmol/kg) into F344 rats; DNA from target organs (lung, liver) and a non-target organ (kidney) was extracted, hydrolyzed, and analyzed for methylated guanines. Adducts were assayed by cation exchange HPLC-fluorimetry. Levels of O<sup>6</sup>-methylguanine, a promutagenic lesion, and 7-methylguanine were 3 to 8 times higher in the liver than in the lung. Neither base could be detected in the kidneys. The levels of the two methylated guanines in liver and lung DNA increased between 4 hrs. and 24

hrs. following NNK injection. NNK is rapidly metabolized in F344 rats to 4-(methylnitrosamino)-1-(3-pyridyl)-butan-1-ol (NNA1). The relatively slow methylation of hepatic DNA after NNK injection could be due to a slow release of methylating species from the major circulating metabolite NNA1. This low but sustained level of O<sup>6</sup>-methylguanine induced by NNK could, in part, explain its carcinogenic potency. Additional work is being supported to study the alkylations of DNA by tobacco-specific N-nitrosamines and to develop sensitive methods to measure these adducts in human tissues exposed to tobacco smoke. A sensitive immunosay for O<sup>6</sup>-methyldeoxyguanosine has been developed. Anti-O<sup>6</sup>-methyldeoxyguanosine was raised in two New Zealand white rabbits using O<sup>6</sup>-methyldeoxyguanosine-KLH conjugate as antigen. The specific antibodies were purified by affinity chromatography. A Biotin-Avidin enzyme-linked immunosorbent assay (BA-ELISA) which can measure 2.5 picomoles (50% inhibition) was developed. The limit of detection of the assay was 100 femtomoles corresponding to 15% inhibition of binding of the antibody to the immobilized antigen. The sensitivity of the assay can be further increased by HPLC separation of O<sup>6</sup>-methyldeoxyguanosine from other deoxyribonucleosides. Calf thymus DNA was methylated with <sup>14</sup>C-MNU. The accuracy of the BA-ELISA and HPLC-BA-ELISA methods were compared to radiochromatographic and HPLC-fluorimetric methods. The coefficient of correlations obtained by the four methods were higher than 0.997. F344 rats were injected IV with NNK, and the DNA from various tissues was isolated and hydrolyzed to deoxyribonucleosides. O<sup>6</sup>-Methyldeoxyguanosine was detected in the DNA of nasal mucosa, esophagus, lung and liver (target tissues) but not in the spleen and heart (non-target tissues).

Studies are continuing on carcinogen metabolism in the cigarette smoking baboon. The first task was to obtain baseline information on two groups of baboons: current cigarette smokers and sham-puffing controls. Data have been collected from the two groups to identify differences in 1) production of urinary mutagens; 2) metabolism of benzo(a)pyrene (BaP) by pulmonary alveolar macrophages (PAMs) and bronchial epithelial cells; and 3) mutagenic activation by PAMs cocultivated with V79 cells, target cells which themselves are incapable of metabolic activation of mutagens. In addition, smoking (or sham-puffing) performance was described in terms of dosimetry measures, including 1) number of cigarettes given and puffs made per day; 2) mean smoke and air puff volume and duration; 3) blood carboxyhemoglobin (COHb); and 4) levels of urine cotinine, the major circulating metabolite of nicotine. The principal results obtained from these observations to date are as follows: 1) The mean number of revertants per 100 ml urine produced in *S. typhimurium* tester strain TA1538, by urine concentrates from 10 smokers (18.81 revertants) was significantly greater (P less than or equal to .06) than that by urine concentrates from 10 shams (2.93 revertants). 2) No difference in basal metabolism of BaP was observed between the cigarette smokers and the sham puffers. However, BaP was metabolized to a greater extent by PAMs lavaged from cigarette smokers that were induced with benz(a)anthracene in culture, prior to addition of <sup>3</sup>H-BaP. The metabolism of BaP by bronchial explants has been performed, but data analysis has not been completed. 3) Mutagenic activation of BaP-7,8-dihydrodiol by PAMs from shams and smokers was significantly different (P less than or equal to .02), whereas without added mutagen, no difference was observed between the two groups. This observation supports the BaP metabolism results described in Part 2 above. PAMs lavaged from baboons also were capable of activating the respiratory carcinogens 7H-dibenzo-(c,g) carbazole, NNN, and benzo(a)pyrene in the V79 cocultivation assay. 4) The smokers averaged 250 puffs on 28 cigarettes per day, and mean smoke and air puff volumes were 16 and 116 ml, respectively. This performance resulted in a mean COHb level of 4.05% and 1320 ng cotinine/ml urine in 24 hrs. The behavioral performance of the shams was similar; they averaged 226 puffs on 22 shams per day, with mean "smoke" and "air" puff

volumes of 17 and 102 ml. However, because they were not actually smoking, the shams' mean COHb and cotinine scores were much lower, 0.97% and 50 ng. According to these results baboons are capable of smoking cigarettes in a human-like manner and the pulmonary cells and tissues can activate known respiratory carcinogens, as has been reported previously for human tissues and cells. BaP is metabolized to a greater extent by baboon PAMs compared with human PAMs. The observation that basal levels of activation of BaP by baboon PAMs was not increased by cigarette smoking was unexpected.

### Contracts Activity Summary

Just recently completed was a prospective epidemiologic study designed to determine whether cigarettes with lower tar and nicotine yields are safer to smoke than high-yield cigarettes. In the study, detailed information on current and past use of tobacco products was collected from over 90,000 ambulatory subscribers to a prepaid medical care program. The incidence through 1982 of hospitalized illness in the 83,798 subjects recruited through 1982 was determined using computer-stored records of hospitalizations in Kaiser-Permanente facilities. It was found that the risk of cardiovascular disease was significantly higher in smokers of high yield cigarettes even after control in a Cox regression analysis for other known cardiovascular risk factors. Yield was not significantly associated with the risk of cancer of the trachea, bronchus, and lung; with all smoking related cancers as a group; with chronic obstructive pulmonary disease; or with peptic ulcer disease. Yield was also not significantly associated with any of 15 other categories of disease grouped by ICDA code after control in a Cox regression analysis for age, sex, race, and number of cigarettes smoked per day. On the other hand, smokers of low yield cigarettes (less than 15.0 mg of tar and less than 1.0 mg of nicotine) had higher rates of hospitalization for a number of diseases compared with never-users of any form of tobacco. Mortality through 1980 in the 53,697 subjects recruited through 1980 was ascertained using the National Death Index. The number of deaths in smokers (15) was too small for meaningful analysis. Detailed information on visits to outpatient clinics was collected for a sample comprising 8,821 subjects recruited in 1979 and 1980. There was no difference in the average number of visits for respiratory disease in smokers of low yield compared with high yield cigarettes. Also, there was no difference in the percentage of smokers of low and high yield cigarettes who had at least one visit for a respiratory disease in the year after their complete check-up. Smokers of low yield cigarettes were not less likely than smokers of high yield cigarettes to visit subsequently for a complication of an upper respiratory viral infection. The stability of the smoking habit and of cigarette brand preference was assessed using a follow-up questionnaire mailed to 4,060 subjects, a 10% random sample of the subjects recruited in the first year of the project, and by analysis of information from people who completed more than one questionnaire in the first 3 years of the study. Both data sources showed that over the one or two year period between contacts with the study, the smoking habits of the subjects were stable with 90% of subjects having the same tobacco use at follow-up as at the initial examination. Eighty percent of current, regular cigarette smokers smoked the same brand of cigarette at follow-up as at the initial exam. No association of tar or nicotine yield with the prevalence of peptic ulcer disease was indicated; however, it was demonstrated that tar and nicotine yield were significantly related to the white blood count even after controlling, by multiple linear regression, for other factors known to be associated with white blood count. A separate study was conducted on a retrospective cohort analysis comparing mortality in persistent cigarette smokers with that in those who quit smoking. Mortality in quitters was lower than in those who persisted, even after

control, in a multivariate analysis for differences between the two groups at their baseline examination.

A recently initiated study on cigarette smoke yield of various chemical smoke components and smoker compensation is underway. This study will provide scientific evidence regarding the consequences, if any, of switching to lower tar and nicotine cigarettes. In particular, the study addresses changes in the daily cigarette consumption, in the aggressiveness of puff profiles, in the levels of nicotine and cotinine in blood plasma, and in the carbon monoxide in the expired air and blood. These are the factors that have given rise, during the past several years, to the widespread controversy related to cigarette switching. No convincing evidence of the extent of such changes is now available from the limited studies that have taken place. Millions of smokers today are aware of the health consequences of smoking. Yet, because of their well-established smoking habit, they will not or cannot quit smoking at this time. Their position is similar to that of many alcohol and drug abusers. Without strong evidence one way or the other, specialists who help these smokers could be providing misleading or even harmful guidance. For example, should such a smoker remain on his/her current brand, or is it preferable to switch to a lower tar and nicotine brand, possibly as an intermediate sequential step towards smoking cessation? Scientific evidence on the effects of switching would provide answers for these kinds of questions. Consequently, results from the study will be of significant importance to all researchers in the smoking and health field, to physicians counseling patients who smoke, to public health officials, and, of course, to the general smoking population.

The program maintains resources for chemical support through an Interagency Agreement with the Department of Energy, Oak Ridge National Laboratories, Oak Ridge Tennessee.

Activities carried out under this agreement involve (1) providing quality-assured data on the deliveries of selected chemical constituents by commercial and experimental cigarettes, (2) providing validated methods for the quantitative determination of additional smoke constituents and for the assessment of smoke composition, (3) providing sampling and monitoring services to define exposures accompanying tobacco smoke inhalation exposure experiments, and (4) providing methods and data to establish the relationship of exposures and smoking conditions to the resulting dose of smoke constituents experienced by the smoker.

Work conducted through another Interagency Agreement with the United States Department of Agriculture has just been completed. Several million Low Yield Reference cigarettes have been fabricated, tested, and placed in cold storage for a resource reference. This activity was undertaken after the Surgeon General's Report (1980) indicated that one of the chief research needs is the study of reduced tar and nicotine cigarettes by routine and frequent surveillance of current and new cigarettes for specific chemical constituents and biological activity.

## SMOKING AND HEALTH PROGRAM

## GRANTS ACTIVE DURING FY84

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. BENOWITZ, Neal Univ of California (San Francisco) 1 R01 CA 32389-02	Nicotine and Tar Intake During Cigarette Smoking
2. CASTAGNOLI, Neal Univ of California (San Francisco) 1 R01 CA 35678-01	The Pharmacological Role of Nicotine
3. CASTONGUAY, Andre American Health Foundation 1 R01 CA 32391-01	Tobacco-Specific Nitrosamine: RIA for DANA-Adducts
4. HECHT, Stephen S American Health Foundation 5 R01 CA 21393-06	Metabolism of the Carcinogen Nitrosonornicotine
5. HOFFMANN, Dietrich American Health Foundation 5 P01 CA 29580-02	Experimental Tobacco Carcino- Cigarette
6. HOFFMANN, Dietrich American Health Foundation 1 R01 CA 35607-01	Endogenous Formation of Nicotine Derived Nitrosamines
7. MARSHALL, Milton Southwest Foundation for Biomedical Research 1 R01 CA 33069-02	Carcinogen Metabolism in the Cigarette Smoking Baboon
8. McCOY, George D. Case Western Reserve University 1 R01 CA 32126-01	Role of Ethanol in the Etiology of Head and Neck Cancer
9. POMERLEAU, Ovide F. Univ of Connecticut Health Center 1 R01 CA 38087-01	Cholinergic/B Endophinergic Effects of Tobacco Smoke
10. STITZER, Maxine L. Johns Hopkins University School of Medicine 1 R01 CA 37736-01	Tobacco Yield Changes: Behavioral and Biological Effect

SMOKING AND HEALTH PROGRAM  
CONTRACTS ACTIVE DURING FY 84

<u>Investigator/Institute/Contract Number</u>	<u>Title</u>
11. FRIEDMAN, Gary Kaiser Foundation Research Institute N01 CP 05681	Surveillance of the Health Effects of Tobacco Products
12. GORI, Gio B The Franklin Research Institute N01 CP 31047	Cigarette Smoke Yield and Smoker Compensation
13. GUERIN, Michael Department of Energy Y01 CP 60206	Collection, Separation, and Elucidation of the Components of Cigarette Smoke and Smoke Condensate
14. T'SO, T C Department of Agriculture Y01 CP 20201	Development, Production, and Evaluation of Low Yield Reference Cigarettes



SUMMARY REPORT  
CHEMICAL RESEARCH RESOURCES

The Chemical Research Resources program of the Branch endeavors to make available to the cancer research community those critical resources which are difficult or impossible for most investigators to obtain on their own, but which are necessary for the pursuit of studies on the chemical and physical aspects of carcinogenesis. Eight resource contracts totalling \$1.91 million in FY 84 dollars presently comprise this program. There are no grants included. A major effort of this program has involved the synthesis and distribution of chemical carcinogens, derivatives, and metabolites for use as authentic research standards. Tritium-labeled analogs of vitamin A, known as retinoids, which have shown promise in studies conducted for the Biological and Chemical Prevention program are also synthesized under contract and made available for pharmacologic and metabolic investigations. Also included under the resource category are two initiatives which support the Biological and Chemical Prevention program. These include a contract for the synthesis of kilogram quantities of retinoids for subsequent testing in chemoprevention and toxicity assays and a contract for the tracheal organ culture bioassay of new retinoids developed by the chemoprevention program. The Research Resources program also monitors an instrument loan program, involving NCI-owned thermal energy analyzers, which are placed in laboratories around the world for studies on the environmental occurrence and relevance of N-nitroso compounds such as nitrosamines and nitrosamides.

The Research Resources program currently has six contractors who are involved in the synthesis of compounds, either carcinogen standards or chemopreventive agents. These contractors develop suitable routes for the unequivocal organic synthesis of compounds designated by the NCI project officer. Methods are developed to produce 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.

At the American Health Foundation (2) the current contract objective is to synthesize one gram quantities of key metabolites of benzo(b)fluoranthene (B(b)F), benzo(j)fluoranthene (B(j)F), and benzo(k)fluoranthene (B(k)F) for distribution to the research community through the NCI Chemical Carcinogen Reference Standard Repository. The benzofluoranthenes are among the most prevalent of the carcinogenic environmental polynuclear aromatic hydrocarbons (PAH), but in contrast to other hydrocarbons such as benzo(a)pyrene, relatively little is known about the mechanism by which they cause cancer. It is hoped that, as these standards are made available, research will be stimulated in this area. Synthesis has now been completed on 1-hydroxy(b)fluoranthene and 8,9-dihydroxy-10,11-epoxy-8,9,10,11-tetrahydrobenzo(k)fluoranthene. The 9,10-dihydroxy-11-,12-epoxy-9,10,11,12-tetrahydrobenzo(b)fluoranthene and 6-hydroxybenzo(b)fluoranthene should be completed by the end of FY 1984. That will bring our inventory of benzfluoranthene metabolites to 10.

At the Midwest Research Institute (MRI) (8), in Kansas City, Missouri, there is a large effort invested in the synthesis of NCI-selected, nonlabeled and labeled (<sup>3</sup>H, <sup>14</sup>C) PAH derivatives other than the benzofluoranthenes. The parent compounds

of interest for new synthesis work include benz(a)anthracene, benzo(a)pyrene, benzo(e)pyrene, cyclopenta(c,d)pyrene, chrysene, dibenzanthracene and 3-methylcholanthrene. Derivatives of the following types are prepared as research trends dictate: phenols; quinones; epoxides; dihydrodiols; diolepoxydes; alkyl and hydroxyalkyl substituted parent hydrocarbons; nitro-PAH derivatives; PAH-DNA adducts; and sulfate, glucuronide, and glutathione conjugates. The MRI maintains a Radiochemical Repository for the NCI under this contract. Shipments of isotopically labeled PAH metabolites are prepared and monitored for authorized recipients as directed by the NCI project officer.

During the last year, 35 polynuclear aromatic hydrocarbon derivatives were synthesized, characterized, and shipped to the NCI Chemical Carcinogen Reference Standard Repository or, in the case of the isotopically labeled derivatives, placed in the Radiochemical Repository at MRI. These derivatives have included <sup>14</sup>C- and <sup>3</sup>H-labeled racemic anti- and syn-dihydrodiol epoxides of benzo(a)pyrene (BP); K-region phenols, dihydrodiols, and <sup>3</sup>H-labeled derivatives of indeno(1,2,3-c,d)pyrene; non-K-region A-ring phenols, dihydrodiols, and the epoxide of 7,12-dimethylbenz(a)anthracene; methylpyrene derivatives; multifunctional derivatives of BP (e.g., 9-phenol-4,5-dihydrodiol); and re-synthesis of labeled and unlabeled BP phenols, dihydrodiols and epoxides.

From October 1983 through March 1984 a total of 86 samples of radiolabeled compounds and eight samples of unlabeled compounds were shipped to 38 different investigators after authorization by the project officer and after receipt by MRI of documentation demonstrating that the user (or his institution) possesses a license from the Nuclear Regulatory Commission for handling the isotope and the quantity involved.

The following compounds and compound groups have been selected for synthesis during the next 6 months:

1. Benzo(a)pyrene-1,6- and 3,6-dione: The glucuronides of benzo(a)pyrene-diones are major metabolites of benzo(a)pyrene (BP) metabolism and have been considered as detoxification metabolites of BP. However, the diones and their semiquinones are potential alkylating agents. In order to further studies on their transport, secondary and ternary metabolism, and activity, the tritium-labeled quinones and their conjugates will be prepared.
2. Quinone-methides: Recently, there is evidence that some ternary metabolites such as triol-epoxides and phenol epoxides are involved in the metabolic activation of PAHs. Investigators have proposed that the phenolic OH-group present in such epoxides can rearrange to quinone-methides. The quinone-methides are highly reactive compounds with a strong alkylating capacity. Only epoxides that also possess a phenolic OH-group in a certain position will form quinone methides. Examples of phenol-epoxides which can form quinone-methides are 9-hydroxybenzo(a)pyrene-4,5-oxide and the triol-epoxides 9-hydroxy-trans-1,2-dihydro-1,2-dihydroxy-chrysene-3,4-oxide and 2-hydroxy-trans-9,10-dihydro-9,10-dihydroxybenzo(a)pyrene-7,8-oxide.

The 9-hydroxybenzo(a)pyrene-4,5-oxide is very unstable and therefore cannot be isolated. However, a number of precursor and rearrangement products of the 9-OH-BP-4,5-oxide have been prepared.

During the coming year, the synthesis of another candidate quinone-methide, trans-1,2-dihydrochrysene-1,2,9-triol, will be undertaken.

3. Perylene, benzo(g,h,i)perylene, and coronene: All three of these PAHs are widely distributed in the environment and are listed on the EPA Priority Pollutant list. All are also mutagenic; however, due to limited testing, the carcinogenicity data are inconclusive. In order to stimulate further studies on the carcinogenicity of these compounds, a few of the more promising metabolites will be prepared.
4. Nitro PAH derivatives of selected PAHs of wide environmental distribution with special emphasis on PAHs that appear on the EPA priority pollutant list.

Companion contract efforts at MRI (1) and at SRI International (5) provide for the re-synthesis of PAH derivatives in order to maintain the inventory at the Repository. Once an unequivocal route has been developed and tested several times by the previously mentioned contractors, then contractors at MRI and SRI International provide the future re-syntheses in order to maintain a continuing supply. Each contractor has specific parent PAH compounds for which responsibility is assigned for the preparation of derivatives. A second objective for these contractors is the syntheses of compounds from other chemical classes that are needed in the Repository: nitrosamines, aromatic amines, additional parent polynuclear aromatic hydrocarbons, aflatoxins, steroid derivatives, and physiologically active natural products, to name a few. These two contracts were re-competed during the last fiscal year and in each case the incumbent contractor won the new award.

All nonlabeled compounds prepared by the four previously mentioned contractors are forwarded to the Chemical Carcinogen Reference Standard Repository operated for the NCI by IIT Research Institute (4). Other items of inventory are derived from surplus, re-analyzed chemicals that are tested by the National Toxicology Program (within the National Institute of Environmental Health Sciences) and other chemicals which are purchased commercially and re-analyzed. Most commercial purchases are made as a result of a need to obtain a given chemical for in vitro testing. The Repository participates in a program for the Coordinator for Environmental Carcinogenesis, Office of the Director, Division of Cancer Etiology, in which selected chemicals are submitted as blind-coded samples for in vitro testing and subsequent evaluation as candidates for in vivo testing. During the past year, 681 shipments were made to the research community at large. These shipments contained custom packaged samples usually with 5 to 100 milligrams of material. Samples were furnished with analytical documentation and safety data sheets. General information on the handling and disposal of carcinogens has been provided in response to inquiries. This contract enables the NCI to provide compounds for pertinent experiments in chemical carcinogenesis which could not be carried out otherwise. Carcinogenesis research has been greatly stimulated by the availability of authentic reference standards and/or substrates. This can be attested to by the volume of published accounts of research citing the NCI Chemical Carcinogen and Radiochemical Repositories (IITRI and MRI) as the source of materials.

On April 1, 1983 the Chemical Research Resources program introduced a user's fee, or payback system, for samples distributed under the program. A price structure was developed which includes cost centers for the chemical cost, the handling/-packaging cost and the shipping cost. Because of the great expense involved in developing a synthesis route for a new chemical, the NCI will still be significantly involved in the support of these contract efforts. The repository contractors (IITRI and MRI) will bill the requestors and deduct the net income from their operating costs. The NCI then covers the balance of each month's operating

cost to the contractors. The amount billed under the payback system, between April 1, 1983 and June 1, 1984, by MRI was \$34,195 and \$71,195 by IITRI.

Retinoid derivatives which are to be tested by contractors in the Biological and Chemical Prevention component of this Branch are synthesized under a resource contract with the Southern Research Institute (7). The retinoids are synthesized in quantities ranging from several grams up to kilogram amounts so that contractors can assess the chemopreventive activity of the compounds in inhibition or suppression of cancer development in animal models of carcinogenesis. The toxicity of the compounds in rodents is also assessed. Compounds are provided at very high purity having been characterized by infrared and U.V. spectrophotometry, mass spectrometry, proton and  $^{13}\text{C}$ -magnetic resonance spectrometry, melting point, elemental analysis, thin layer and high pressure liquid chromatography. During this period, an additional two kilograms of 13-cis-N-ethylretinamide have been synthesized for continuing evaluation of its anticarcinogenic efficacy against bladder carcinogenesis, and four other multistep syntheses of arotenoid compounds (originally synthesized at SRI International by Dr. M. Dawson) were completed in quantities allowing for initial toxicological evaluation. Work on several other retinoids, for initial toxicity studies also involving long synthesis routes, is currently in progress.

At SRI International (3) radiolabeled retinoids are synthesized for use as tracers in metabolic studies, for pharmacokinetic investigations and for mechanistic studies. This involves the synthesis of small quantities of several different retinoids, the choice of which is dictated by the needs and interests of the Biological and Chemoprevention program of the Branch and the research community. The contractor currently offers an inventory of 16 compounds which are provided to requestors on a payback basis. Recipients of these compounds have included grantees, contractors, NCI staff, and other investigators conducting chemoprevention, nutrition, or other basic research in the retinoid area. During the past year four new compounds were synthesized and tritium labeled: N-ethyl-13-cis-(11- $^3\text{H}$ )-retinamide, N-ethyl-all-trans-(11- $^3\text{H}$ )-retinamide, stilbenoid- $^3\text{H}$ -(THTMNPB- $^3\text{H}$ ) and trans-(11,11'- $^3\text{H}_2$ )-beta-carotene. During the past year, 23 requests for labeled retinoids were filled. The total billing for chemicals shipped by this contractor between April 1983 and June 1984 was \$9,000.

At IIT Research Institute (6) bioassays of new retinoids are performed in the hamster tracheal organ culture system. Evaluation in this system has been a standard bioassay for many years for potential efficacy of a retinoid in chemoprevention of carcinogenesis. This differentiation assay measures the capacity of a retinoid derivative to reverse keratinizing squamous metaplasia in retinoid-deficient hamster trachea to the normal mucociliary epithelium. This biological assay can be precisely quantified, is reproducible and quite sensitive at physiological levels of retinoids (all-trans-retinoic acid employed as an internal standard in each bioassay has an  $\text{ED}_{50}$  for reversal of keratinization of approximately  $1 \times 10^{-11}\text{M}$ ). This project provides an initial evaluation of retinoids provided to the program by contractors, grantees, independent investigators and, occasionally, commercial sources.

RESEARCH RESOURCES  
CONTRACTS ACTIVE DURING FY84

<u>Investigator/Institution/Contract No</u>	<u>Title</u>
1. BODINE, Richard S Midwest Research Institute N01-CP-41001 (formerly N01-CP-05719)	Synthesis of Selected Chemical Carcinogen Standards
2. HECHT, Stephen S American Health Foundation N01-CP-15747	Synthesis of Derivatives of Poly- nuclear Aromatic Hydrocarbons
3. RHEE, Sung W SRI International N01-CP-05601	Synthesis of Radiolabeled Retinoids for Metabolic and Pharmacologic Studies
4. KEITH, James N IIT Research Institute N01-CP-05612	Chemical Carcinogen Standard Reference Repository
5. REIST, Elmer J SRI International N01-CP-41028 (formerly N01-CP-05614)	Synthesis of Selected Chemical Carcinogens
6. SCHIFF, Leonard J IIT Research Institute N01-CP-31012	Bioassay of Retinoid Activity by Tracheal Organ Culture System
7. SHEALY, Y Fulmer Southern Research Institute N01-CP-26009	Synthesis of Kilogram Amounts of Retinoids for Chemoprevention and Toxicity Studies
8. WILEY, James C Midwest Research Institute N01-CP-05613	Synthesis of Derivatives of Poly- nuclear Aromatic Hydrocarbons





















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